

# The Pathway of Membrane Fusion Catalyzed by Influenza Hemagglutinin: Restriction of Lipids, Hemifusion, and Lipidic Fusion Pore Formation

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**Abstract.** The mechanism of bilayer unification in biological fusion is unclear. We reversibly arrested hemagglutinin (HA)-mediated cell–cell fusion right before fusion pore opening. A low-pH conformation of HA was required to form this intermediate and to ensure fusion beyond it. We present evidence indicating that outer monolayers of the fusing membranes were merged and continuous in this intermediate, but HA restricted lipid mixing. Depending on the surface density of HA and

the membrane lipid composition, this restricted hemifusion intermediate either transformed into a fusion pore or expanded into an unrestricted hemifusion, without pores but with unrestricted lipid mixing. Our results suggest that restriction of lipid flux by a ring of activated HA is necessary for successful fusion, during which a lipidic fusion pore develops in a local and transient hemifusion diaphragm.

LIKE many other enveloped viruses, the influenza virus uses membrane fusion to inject its genome into host cells (Hernandez et al., 1996). Influenza virus binds to the sialic acid residues of cell surface receptors and enters these cells by an endocytic pathway. The low-pH environment in the endosome triggers a sequence of changes in the conformation of the influenza virus hemagglutinin (HA),<sup>1</sup> which leads to fusion. Fusion mediated by influenza HA is the best characterized example of biological fusion (White, 1996). Remarkable progress has been achieved recently in understanding the nature of these conformational changes, which include the insertion of the NH<sub>2</sub>-terminal peptide sequence of HA (“fusogenic peptide”) into membranes (Tsurudome et al., 1992; Weber et al., 1994) and extension and folding of the triple-stranded  $\alpha$ -helical coiled coil of HA (Carr and Kim, 1993, 1994; Bullough et al., 1994; White, 1996; Weissenhorn et al., 1997). Three to six HA trimers cooperate in fusion (Blumenthal et al., 1996; Danieli et al., 1996), substantiating

the picture in many models that a ring of HA transmembrane helices surrounds the initial fusion pore (for review see Kemble et al., 1994). Baculovirus gp64 also forms a cooperative ring of approximately six trimers in fusion pore opening (Plonsky and Zimmerberg, 1996), so ring formation by fusion proteins may be general.

Investigators have focused on the fusion pore, an intermediate that creates ionic continuity between the two previously separated aqueous spaces, to understand the mechanism of membrane fusion (Zimmerberg et al., 1987, 1994; Spruce et al., 1989, 1991; Tse et al., 1993; Melikyan et al., 1995*a,b*). A critical issue for HA-mediated fusion is which occurs first: a proteinaceous pore, whose expansion leads to lipid merger (Lindau and Almers, 1995), or a lipidic connection (hemifusion), which then breaks to form a pore (Zimmerberg et al., 1993; Kemble et al., 1994; Melikyan et al., 1995*b*, 1997). Proteinaceous pores are suggested by the small size and abrupt opening of the fusion pore, analogous to gap junctions. The strongest evidence for a proteinaceous fusion pore is obtained by measuring lipid dye flux simultaneously with fusion pore conductance: initially, small fusion pores join the cytoplasm of fusing cells but restrict the transfer of lipid dyes between fusing membranes (Tse et al., 1993; Zimmerberg et al., 1994; Blumenthal et al., 1996).

On the other hand, several studies suggest that hemifusion occurs during HA-mediated fusion. First, there is a lipid-sensitive stage of fusion, occurring after the low pH-dependent conformational change in HA but before pore

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1. *Abbreviations used in this paper:* CF, 6-carboxyfluorescein; FIF, frozen intermediate of fusion; GPI, glycosylphosphatidylinositol; GPI-HA, HA ectodomain linked to GPI; HA, influenza virus hemagglutinin; LPC, lysophosphatidylcholine; LPC-S, LPC-arrested fusion stage; NBD-taurine, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) taurine; OA, oleic acid; R18, octadecyl rhodamine B; RBC, red blood cells.

formation. Lipids like lysophosphatidylcholine (LPC), which increase the energy barrier for phospholipid bilayer hemifusion, reversibly arrest, while lipids like oleic acid (OA), which decrease this energy barrier, augment the rate and extent of HA-mediated fusion (Chernomordik et al., 1997). This lipid-sensitive, LPC-arrested stage (LPC-S) is common to biological and phospholipid bilayer fusion (Chernomordik et al., 1993). Second, part of the HA molecule is capable of merging outer membrane monolayers without merging inner membrane monolayers. A mutant of HA (GPI-HA) whose HA ectodomain is anchored in membranes by a glycosylphosphatidylinositol, rather than by the transmembrane domain present in wild type HA (Kemble et al., 1994; Melikyan et al., 1995b), mediates "unrestricted hemifusion" (lipid dye redistribution without fusion pores), indicating that the ectodomain of HA has hemifusion activity. Unrestricted hemifusion also occurs for a truncated fusion protein of paramyxovirus SV5, so hemifusion may be a general phenotype of the ectodomain of fusion proteins (Bagai and Lamb, 1996).

That wild-type HA may also be capable of causing unrestricted hemifusion was suggested by experiments where lipid dye flux without aqueous dye flux was observed at room temperature (Melikyan et al., 1997). Lacking electrophysiological experiments, Melikyan et al. (1997) termed this state "stunted fusion," rather than hemifusion, since the smallest fusion pores do not allow transfer of aqueous fluorescent dyes but can still be detected as ion flux by electrophysiological recording (Zimmerberg et al., 1994).

To resolve the apparent conflict between data supporting proteinaceous pores and data supporting lipidic intermediates, it was conjectured that the HA transmembrane domain may constrain lipid motion in the initial fusion pore (Kemble et al., 1994). We report here data showing the restriction of lipid flux even before fusion pore formation, in an intermediate that we call "restricted hemifusion." This restricted hemifusion state is after the lipid-sensitive stage but before either lipid mixing or fusion pores. Our findings establish the role of wild-type HA in promoting hemifusion, restricting dye flux, and transforming the restricted hemifusion into a lipidic fusion pore. Together, these data provide substantial evidence for the idea that HA-mediated fusion proceeds via lipidic intermediates that develop within a ringlike complex of HA trimers in low-pH conformation.

## Materials and Methods

### Preparation of Cells

HAb2 cells expressing A/Japan/305/57 HA (Doxsey et al., 1985), HA300a cells expressing X:31 HA, and BHA-PI cells expressing the GPI-anchored ectodomain of X:31 HA (Kemble et al., 1993) were grown as described. Human red blood cells (RBC), freshly isolated from whole blood, were labeled with fluorescent lipids: PKH26 (Sigma Chemical Co., St. Louis, MO) or octadecyl rhodamine B (R18; Molecular Probes, Eugene, OR) as in Chernomordik et al. (1997). Under these conditions, R18 incorporated into RBC membranes to self-quenching concentration. In some experiments, to label RBC by either DiI<sub>16</sub>, DiI<sub>18</sub> and DiI<sub>12</sub> (Molecular Probes), or lissamine rhodamine B diacyl phosphatidylethanolamine (Avanti Polar Lipids, Birmingham, AL), we rapidly added 10  $\mu$ l of dye solution in methanol (1 mg/ml) to 10 ml of an RBC suspension (1% hematocrit) in PBS. Unbound dye was removed by washing RBC with complete medium followed by four washings with PBS.

To label RBC with the fluorescent water-soluble dye carboxyfluorescein (CF), we used mild hypotonic lysis followed by resealing as described (Ellens et al., 1989; Melikyan et al., 1995a,b). *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD)-taurine was loaded into RBC as described (Morris et al., 1993). To double label RBC with CF and PKH26, membranes were first loaded by CF, and then, after washing out the unbound CF, the loaded RBC ghosts were labeled by PKH26.

### Fusion Experiments

HAb2 cells were treated by 5  $\mu$ g/ml trypsin (Fluka, Buchs, Switzerland) for 10 min at room temperature to cleave HA0 into its fusion-competent HA1-S-S-HA2 form. For HA300a and BHA-PI cells, trypsin (5  $\mu$ g/ml) was supplemented with neuraminidase (0.2 mg/ml; Sigma Chemical Co.) to improve binding of RBC. The enzymes were applied together for 10 min at room temperature. To terminate the reaction, HA-expressing cells were washed twice with complete medium containing 10% fetal serum. After two washings with PBS, cells were incubated for 10 min with a 1 ml suspension of RBC (0.05% hematocrit). HA-expressing cells with zero to two bound RBC per cell were washed three times with PBS to remove unbound RBC and then used. When measuring RBC binding to cells, several areas of the dish were selected. We screened at least 200 cells to find the average number of RBC bound to each HA-expressing cell.

Fusion was triggered by incubation of cells with PBS titrated by citrate to acidic pH. After low-pH treatment, acidic solution was replaced by PBS. Fusion extent was assayed by fluorescence microscopy more than 20 min after low-pH application as the ratio of dye-redistributed bound RBC to the total number of the bound RBC. Longer incubations (up to 2 h) did not increase the extent of fusion. We performed the fluorescence microscopy for lipid and content mixing either in a cold room at 4°C or at room temperature, as required.

For spectrofluorometric measurements (SLM-Aminco, Urbana, IL), excitation and emission wavelengths were 550 and 590 nm for R18, and 473 and 515 nm for NBD-taurine. The standard fusion assay was performed as in Chernomordik et al. (1997). Suspensions of HA-expressing cells with bound RBC in PBS were placed into a thermostated fluorescence cuvette and stirred with a Teflon-coated flea. Citric acid was injected into the cuvette to lower pH to 4.9. The increase in fluorescence was normalized to that at infinite dilution of the probe by lysing cells with 0.06% SDS. Spectrofluorometry was also used to evaluate LPC incorporation into RBC membranes at different temperature from the decrease in R18 quenching caused by adding exogenous lipid to HAb2 cells with bound R18-labeled RBC (Chernomordik et al., 1997). To induce swelling of cells by applying hypotonic medium (osmotic shock), HA-expressing cells with bound RBC were placed into PBS diluted by H<sub>2</sub>O (1:3) as in Melikyan et al. (1995b).

Each set of experiments for each graph presented here was repeated on at least three occasions with similar results. Presented data were averaged from the same set of experiments.

### Application of Exogenous Lipids and Enzymatic Treatments

Stock solutions of lauroyl LPC (Avanti Polar Lipids) and OA (Sigma Chemical Co.) were freshly prepared as a 0.5% (wt/wt) aqueous dispersion and 25 mM ethanolic solution, respectively. In spectrofluorometry experiments, LPC was added directly to the cell suspension by rapid injection of a few microliters of the stock solution into the cuvette under continuous stirring. In fluorescence microscopy experiments, the cell medium bathing the plastic or glass attached HA-expressing cells with bound RBC was replaced by 0.5 ml of medium supplemented with LPC or OA 5 min before low-pH application to the cell medium. Low-pH medium (used to trigger fusion) and "normal"-pH medium (used to terminate the low-pH treatment) were supplemented with the same concentration of lipid.

In some experiments, RBC membranes were loaded with lauroyl LPC along with CF by the same mild hemolysis protocol as the one used for loading RBC with CF. RBC were lysed in the solution containing 280  $\mu$ M of LPC and 130 mM of CF. To remove lauroyl LPC from the outer membrane monolayers, cells were washed by PBS with no LPC. To extract OA, we washed cells by PBS supplemented by 10 mg/ml of fatty acid-free BSA.

In the experiments presented in Fig. 4 A, HAb2 cells with bound RBC were treated by neuraminidase or proteinase K (0.1 mg/ml, 20 min, if not stated otherwise), or both enzymes together in PBS before or after low-pH application. Reactions were terminated by washing cells twice with complete medium. BSA and enzymes were from Sigma Chemical Co.

## Electrophysiological Recordings

To prepare HAb2 cells for patch clamp experiments, we followed the protocol described above. After binding RBC labeled with PKH26 and, in some experiments, with both PKH26 and CF to HAb2 cells, we washed out unbound ghosts and then lifted HAb2-RBC aggregates using PBS supplemented with EGTA/EDTA (both 0.5 mg/ml). Cells were diluted in 4°C external solution: 155 mM *N*-methyl-glucamine glutamate, 5 mM MgCl<sub>2</sub>, 2 mM Cs-Hepes, pH 7.4 (Tse et al., 1993). HAb2-RBC complexes were kept on ice until the experiment (1–3 h). Electrophysiological recordings were made using glass micropipettes (Microcaps, 75 μl, Drummond Sci., Broomall, PA) coated with Sylgard (World Precision Instruments, Inc., Sarasota, FL). Patch pipettes with resistances of 1–3 MΩ contained (in mM): 155 Cs-glutamate, 5 MgCl<sub>2</sub>, 5 BAPTA, 10 Cs-Hepes, pH 7.4 (Tse et al., 1993). Capacitance measurements were performed using an in-house software lock-in amplifier (“Browse,” program available upon request) to simultaneously apply 250, 500, and 750 Hz sine waves (Melikyan et al., 1995a) superimposed over a –29-mV holding potential. The sine waves were applied for 4 ms, and then circuit parameters were recalculated during the next 12 ms. Pore conductance was calculated from these parameters as described previously (Melikyan et al., 1995a; Plonsky and Zimmerberg, 1996). The time resolution was 16 ms, yielding a 50–100 pS background noise level in the whole-cell mode.

We patched partially attached cells with bound RBC. After establishing tight contact between the tip of the pipette and the cell membrane (“gigaseal”), a short pulse of suction was applied to the pipette solution to break the patch of membrane beneath the pipette and to achieve whole-cell configuration: a conductive pathway between the cell interior and the pipette. Then cells were pulled off the glass coverslip and held in suspension. A micropipette containing pH 4.9 medium (external solution with 20 mM succinic acid replacing 2 mM Hepes) was positioned ~40 μm from the cell, and a 100-s pulse of positive pressure from a pneumatic picopump (model PV830; World Precision Instruments) forced a flow of low-pH solution around the patched cell.

In the experiments on the frozen intermediate of fusion, cells were patched at 3 ± 2°C. After the end of the electrical recording, cells were warmed to 33°C. No electrical measurements were performed on cells during warming since access resistance lowered abruptly. For the fusion experiments at 33 and 22°C, cells were patched at 10 ± 2°C, lifted, warmed to the required temperature, and then treated with low pH. Because of limitations of the microscope stage, we could not reproducibly control temperature above 33°C. In control fluorescence microscopy experiments on HAb2 cells, no significant difference was found between the extents of complete fusion and unrestricted hemifusion at 33 vs. 37°C. During electrical recording, we followed PKH26 and CF transfer from RBC to the patched

HAb2 cell using an inverted microscope with a water-immersion objective (PlanApo 60/1.2; Nikon Inc., Melville, NY) and an intensified camera (model VE1000SIT; Dage-MTI, Inc., Michigan City, IN) connected to a VCR. In the experiments at 3°C (Fig. 3 C), to reliably maintain the temperature of the microscope stage we had to replace the water-immersion objective with a dry objective (MPlan60/0.7; Nikon, Inc.). Because of the limited light gathering capability of the dry objective (due to lower N.A.), we were not able to achieve the same image quality at 3°C as at higher temperatures. PKH26 redistribution was measured as previously described (Zimmerberg et al., 1994). Briefly, recorded images were first digitized, and then all pixel values in an area of interest surrounding the HAb2 cell were integrated to measure unidirectional dye flux.

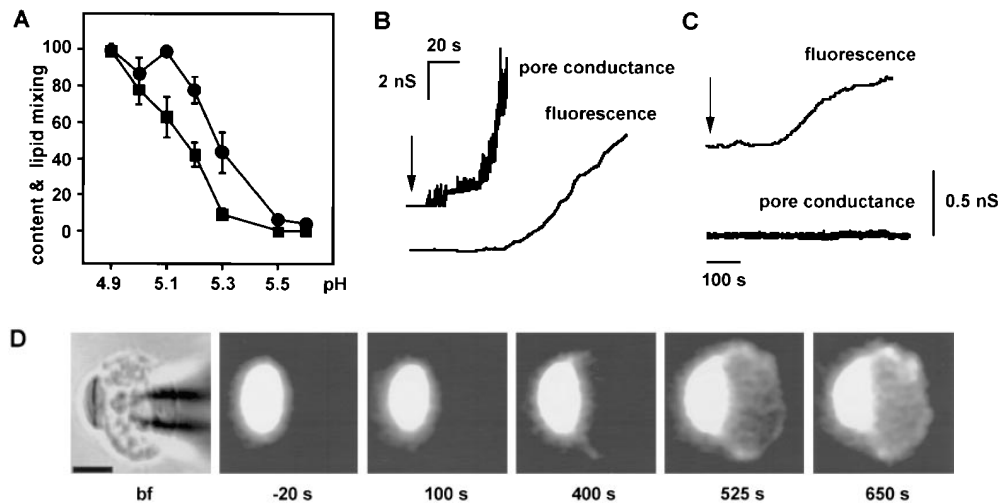
## Results

### Unrestricted Hemifusion Mediated by HA Side-steps the Fusion Pathway

**HA Can Cause Unrestricted Hemifusion.** To test for hemifusion as an intermediate of fusion, we attempted to impair fusion by using suboptimal conditions. When fusion of HA-expressing cells (HAb2 cells) with RBC was triggered by a 10-min application of pH 4.9 medium at 37°C (optimal conditions), transfer of both lipid (Fig. 1 A, circles) and aqueous (squares) dyes from RBC to HAb2 cells was uniformly observed (Fig. 1 A, pH 4.9). At less acidic pH values, a higher percentage of HAb2 cells stained with lipid but not with aqueous dye (Fig. 1 A, pH 5.1–5.3).

We then tested for the presence of fusion pores that were permeable to ions but impeded the passage of aqueous dyes (Zimmerberg et al., 1994; Blumenthal et al., 1996) by electrophysiological measurements. As observed previously (Tse et al., 1993; Zimmerberg et al., 1994), pore opening occurred before lipid dye transfer in experiments where pH 4.9 medium was used to trigger cell fusion (Fig. 1 B). Fusion pores usually enlarged sufficiently to give a conductance of over 10 nS within 2 to 80 s after pore opening (*n* = 9).

By contrast, the spectrum of response was significantly different at pH 5.3 (Table I, second column). In 7 out of 17



**Figure 1.** HA causes unrestricted hemifusion at suboptimal pH. (A) Fusion of HAb2 cells with bound RBC was triggered by a 10-min application of pH 4.9 medium at 37°C. The extent of fusion was assayed by fluorescence microscopy at neutral pH 20 min after the end of low-pH application as lipid dye, PKH26 (circles), and aqueous dye, CF, (squares) redistribution. (B) Pore conductance and PKH26 flux (fluorescence) in a HAb2/RBC pair treated by pH 4.9 at 33°C. Arrow indicates start of pH pulse. At these “optimal” conditions, pores opened

before transfer of membrane dye. (C and D) Unrestricted hemifusion of HAb2 and RBC at pH 5.3. (C) Increase in the integrated PKH26 fluorescence of the HAb2 membrane is not accompanied by any appearance of fusion pore conductance. Arrow indicates start of pH pulse. (D) Bright field (bf) and fluorescent images for the same HAb2/RBC as in (C) pair at different moments of time, 0 time at start of pH pulse. Patch pipette is seen on the right; a PKH26-labeled RBC is seen on left. This lipid dye is seen to redistribute, although no fusion pores are detected (C). Bar, 10 μm.

Table I. Frequency of Hemifusion and Fusion Pores

	HA	HA	HA, low trypsin*	HA
pH	4.9	5.3	4.9	4.9
Temperature, °C	33	33	33	22
Trypsin*	normal	normal	low	normal
No. of trials	14	17	11	21
Unrestricted hemifusion	0	7	3	4
Nonexpanded pores <sup>‡</sup>	3	6	2	10
Complete fusion	9	0	1	2
No response	2	4	5	5

\*Normal treatment: 5  $\mu$ g/ml, 10 min at room temperature; low trypsin treatment: 1  $\mu$ g/ml, 2 min at room temperature.

<sup>‡</sup>Nonexpanded pores include transient ones and pores that expanded slowly and had conductances less than 3 nS 420 s after low-pH application.

trials, we recorded unrestricted hemifusion, i.e., notable lipid dye redistribution, but no pore conductance (Fig. 1, C and D). The level of resolution in these electrophysiological experiments (50–100 pS) would be sufficient to detect fusion pores even smaller than any reported to date for any viral fusion. When fusion pores were detected (in 6 out of 17 trials), they either expanded very slowly or flickered, i.e., opened and closed reversibly for a short time and then closed completely while lipid dye transfer continued.

We next experimentally manipulated the number of HA molecules that could undergo low pH-dependent confor-

mational change to see if this was associated with hemifusion. We took advantage of the fact that the HA precursor HA0 has to be cleaved into its fusion-competent HA1-S-S-HA2 form by trypsin before low-pH application (White, 1996). Shorter trypsin treatment led to fewer fusion-competent HA molecules (Clague et al., 1991) and yielded more HAb2 cells that were stained only by lipid-soluble but not by aqueous dyes at pH 4.9 (Fig. 2 A). In simultaneous admittance and fluorescence measurements, lipid dye transferred without any detectable fusion pore formation, evidence of unrestricted hemifusion, in 3 out of 11 trials (Table I, *third column*, Fig. 2, B and C). Thus, lowering the surface density of fusion-competent HA yields unrestricted hemifusion.

Lowering the temperature is another approach to impair fusion at the optimal pH (Wharton et al., 1986; Morris et al., 1989; Melikyan et al., 1997). At 22°C, the majority of fusion pores failed to enlarge or enlarged slowly, attaining conductances of 0.29 to 3 nS at 200 s after opening,  $n = 12$  (example: Fig. 2 D). In 4 out of 21 trials (Table I, *fourth column*), lipid dye exchange was detected in the absence of fusion pore opening. This unrestricted hemifusion may be explained by a significantly lower mobility of HA molecules at 22°C than at 33°C (Junankar and Cherry, 1986; Gutman et al., 1993).

All together, that wild-type HA can cause unrestricted

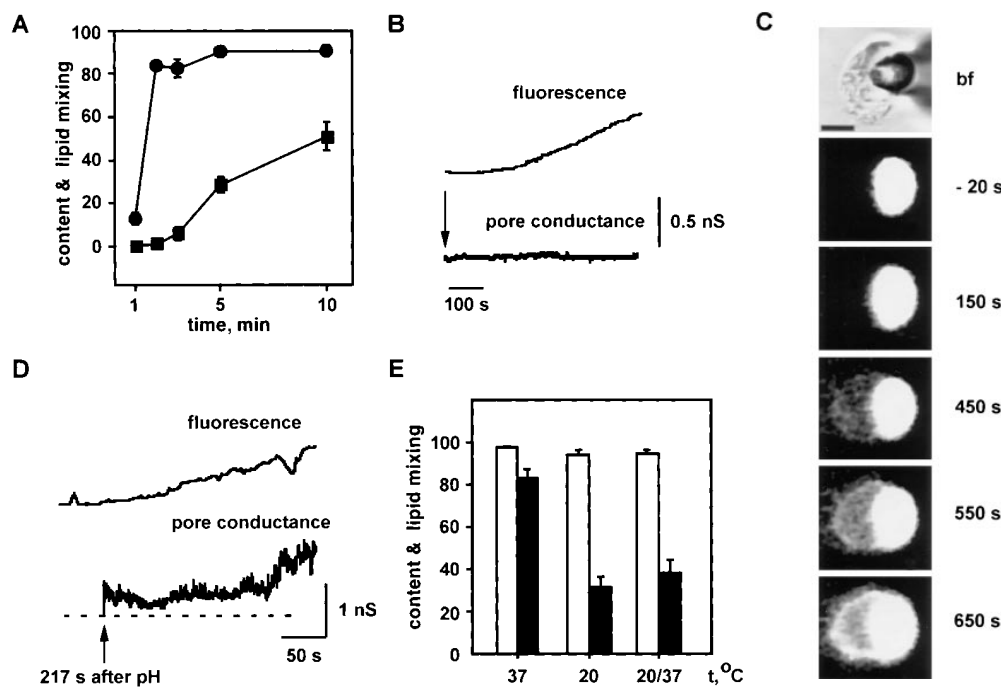


Figure 2. Decreasing either the number of trypsin-activated HA or the temperature results in unrestricted hemifusion. (A) The surface density of fusion-competent HA molecules was varied by altering the duration of trypsin treatment (1  $\mu$ g/ml, room temperature) of HAb2 cells, which cleaves inactive HA0 into HA1-S-S-HA2 form. Fusion of HAb2 cells with bound RBC was triggered by pH 4.9 medium (10 min, 37°C). Extent of fusion was assayed by fluorescence microscopy at neutral pH 20 min after the end of low-pH application as PKH26 (*circles*) and CF (*squares*) redistribution. (B and C) Unrestricted hemifusion of RBC to HAb2 cells mildly treated by trypsin (1  $\mu$ g/ml, 2 min, room temperature) was triggered by a pH 4.9 pulse applied

plied at 33°C. (B) The fusion pore conductance and the integrated fluorescence of HAb2 membrane for the cell pair in C, arrow at pH pulse. (C) The same cell pair as in B, bright field (*bf*) and fluorescent images of the HAb2/RBC cell pair at the times indicated. 0 time was at the beginning of the pH pulse. Patch pipette is seen on the right, overlying a PKH26-labeled RBC. This lipid dye is seen to redistribute, although no fusion pores were detected (see B). (D) Nonexpanding pores. When HA is triggered by suboptimal conditions, pores can form that are either transient or fail to expand. Here we triggered with pH 4.9 at 22°C. The pore opened (*arrow*) 217 s after low-pH application. Nonexpanding pores like this were seen in all conditions (Table I). (E) Unrestricted hemifusion is not an intermediate to complete fusion. Fusion of HAb2 cells with PKH26- and CF-labeled RBC membranes was triggered by a 10-min application of pH 4.9 medium at 37 or 20°C. After low-pH application at 20°C, cells were kept in neutral medium for 10 min more at 20 or 37°C, and then fusion extents were assayed by fluorescence microscopy as redistribution of PKH26 and CF (*open and closed bars*, respectively). Bar, 10  $\mu$ m.

hemifusion (lipid dye flux without any fusion pore opening) was documented in 14 electrophysiological experiments performed under different suboptimal conditions.

**Establishing the Unrestricted Hemifusion State Precludes Formation of a Fusion Pore.** When HA caused unrestricted hemifusion, we never saw an expanding fusion pore after the onset of lipid mixing, although we waited more than 650 s after low-pH application. This is much longer than the lag time for pore formation under any of the conditions we tried ( $68 \pm 39$  s [SD,  $n = 5$ ] at pH 5.3;  $71 \pm 61$  s [SD,  $n = 3$ ] for short trypsin treatment, and  $158 \pm 62$  s [SD,  $n = 12$ ] at 22°C).

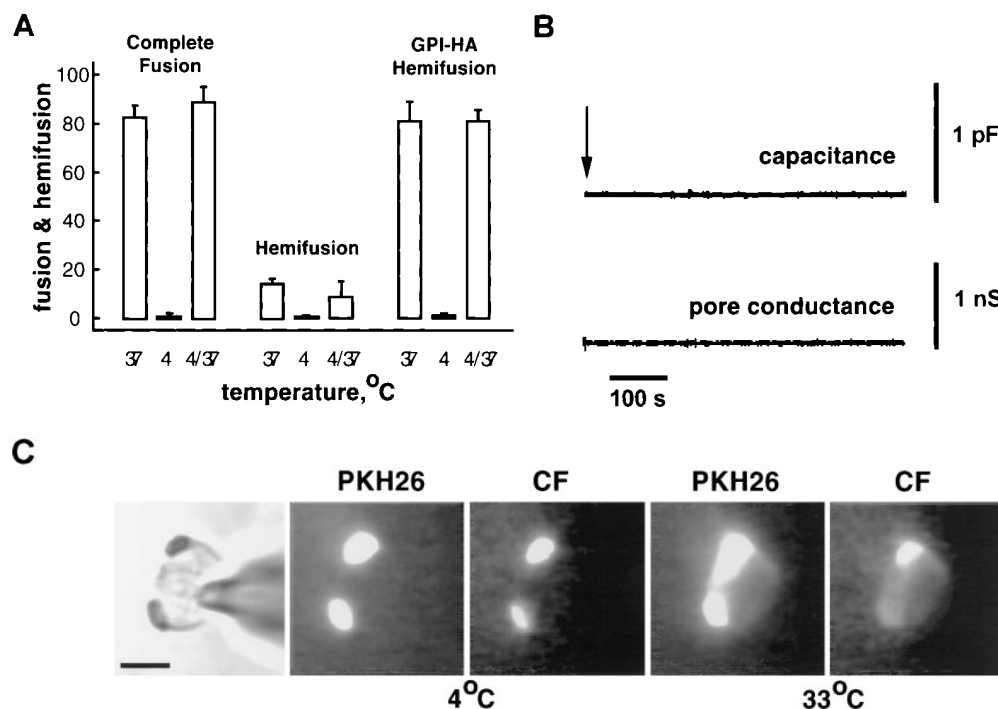
To further test whether unrestricted hemifusion can proceed to fusion, we returned populations of cells from 20 to 37°C after 10 min of incubation at low pH (Fig. 2 E). No increase in the number of cell pairs with transfer of aqueous dye was observed. This effect was not due to a reversal of HA conformation because the same result occurs in the continued presence of pH 4.9 (data not shown). Also, it is not likely that this was due to inactivation of HA because the Japan strain of HA inactivates very slowly (Puri et al., 1990; Gutman et al., 1993; Korte et al., 1997). Longer incubations (up to 2 h) were not sufficient to transform unrestricted hemifusion into complete fusion. Similar results (i.e., Fig. 2 E) were also obtained for X:31 HA-mediated fusion of RBC to HA300a cells (data not shown). However, fast inactivation of X:31 HA (Korte et al., 1997) complicates interpretation.

Our finding that no fusion pores open and dilate after the onset of lipid mixing suggests that unrestricted hemifusion is a branch off the normal fusion pathway.

### Frozen Intermediate of Fusion

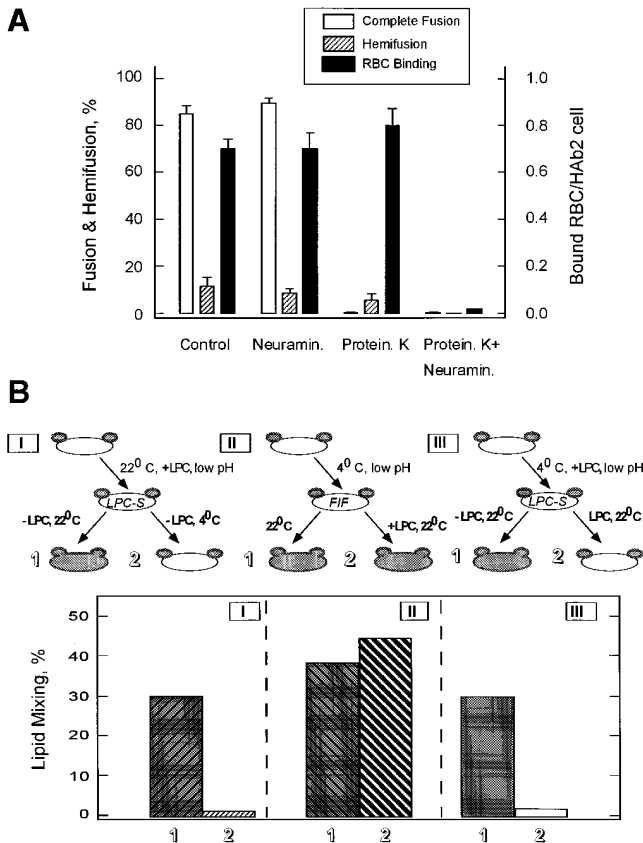
**Both Fusion and Unrestricted Hemifusion Are Reversibly Arrested at 4°C.** The fusion of influenza virus to RBC and to liposomes is slowed (Stegmann et al., 1990; Schoch et al., 1992; Tsurudome et al., 1992; Ramalho-Santos et al., 1993) or even reversibly arrested (Schoch et al., 1992) at low temperatures. To trap fusion intermediates preceding unrestricted hemifusion, we lowered temperature to 4°C.

At 4°C, HAb2 cells bound to doubly labeled RBC were exposed to pH 4.9 for 10 min and then returned to neutral pH for 1 h, still at 4°C. Neither the lipid dye nor the aqueous probe redistributed from RBC to HAb2 cells at this fusion stage, which we call the “frozen intermediate of fusion” (FIF) (Fig. 3 A). Warming to 37°C, still at neutral pH, led to fast redistribution of both lipid- and water-soluble dyes. Hereafter, HAb2-RBC pairs showing both lipid and aqueous dye mixing are presented as complete fusion (fusion in figure axes), and cell pairs where HAb2 cells were stained with lipid but not aqueous dye are presented as unrestricted hemifusion (hemifusion in figure axes). FIF was observed only at temperatures at and below 6°C. 4°C reversibly arrested complete fusion and unrestricted hemifusion mediated by wild-type HA of Japan strain of influ-



**Figure 3.** Frozen intermediate of fusion precedes both lipid mixing and fusion pore opening. (A) Complete fusion and unrestricted hemifusion are both reversibly arrested at 4°C. HA-expressing HAb2 cells or GPI-HA-expressing BHA-PI cells with bound PKH26- and CF-labeled RBC were triggered to fuse at pH 4.9, 10 min, 4°C and then incubated in neutral medium for an additional hour at 4°C. Then, still at neutral pH, the temperature was raised to 37°C. Complete fusion (cell pairs with both PKH26 and CF transferred from RBC to HAb2 cells) and hemifusion (cell pairs with PKH26 but not CF transferred) were assayed just before warming cells, still at 4°C, or 10 min after warming. In controls, fusion was triggered at pH 4.9, 10 min, 37°C. The extent of

fusion was measured at neutral pH 10 min after the end of low-pH application. Bars are mean  $\pm$  SE,  $n > 3$ . (B) FIF precedes fusion pore formation. No detectable changes in cell capacitance and fusion pore conductance and thus no fusion pore opening was observed when low pH was applied to a HAb2 cell with two bound RBC at 4°C. Arrow at pH pulse. (C) Bright field and fluorescence microscopy images at different stages of the same experiment. Bright field image was taken before acid medium application. The patch pipette is seen on the right; two RBC stained with both PKH26 and CF are seen attached to the left side of the HAb2 cell. Videomages of PKH26 and CF fluorescence of these cells were taken at 4°C at the onset of the pH pulse and then 10 min after the end of the electrical recording shown in B, when cells were warmed up to 33°C (e). Bar, 10  $\mu$ m.



**Figure 4.** Frozen intermediate of fusion is subsequent to a low pH-triggered change in HA conformation (A) and the LPC-arrested stage (B). (A) FIF is subsequent to the change in fusion sensitivity to neuraminidase and proteinase K upon low pH refolding of HA. Fusion of HAb2 cells with bound RBC was triggered by a 10-min pH 4.9 pulse applied at 4°C. After 45 min at neutral pH at 4°C, cells were warmed to 37°C. During the incubation at neutral pH, 4°C, cells were treated by proteinase K (0.1 mg/ml, 45 min), neuraminidase (0.2 mg/ml, 45 min), or both enzymes simultaneously. In controls, no enzymes were applied. Complete fusion (cell pairs with both PKH26 and CF transferred from RBC to HAb2 cells) and hemifusion (cell pairs with PKH26 but not CF transferred) were assayed 10 min after warming to 37°C using fluorescence microscopy. The binding of RBC to HAb2 cells (*righthand y-axis*) is presented as the ratio of the number of bound RBC to the number of HAb2 cells at 4°C. Each bar is mean  $\pm$  SE,  $n > 3$ . In the control experiments, we verified that incubation of cells with proteinase K (0.1 mg/ml, 45 min, 4°C) before low-pH application affects neither binding nor complete fusion and unrestricted hemifusion (see also Chernomordik et al., 1997). (B) FIF is subsequent to LPC-arrested stage. Fusion of HAb2 cells (depicted as *large ellipses* in the illustration of the experimental protocol and result) with bound R18-labeled RBC (*two small, shaded ellipses*) was triggered by a 10-min pulse of pH 5.0 medium, followed by reneutralization. Final fusion extent was assayed at neutral pH by spectrofluorometry as R18 dequenching. (I) Fusion of cells released from LPC-S was arrested at 4°C. Cells were treated by low pH at 22°C in the presence of 140  $\mu$ M lauroyl LPC to establish the LPC-arrested stage. 10 min after the end of the pH pulse, cells were placed into a cuvette containing LPC-free solution at either 4 or 22°C. (II) LPC did not block fusion of cells released from FIF. At the end of the pH pulse at 4°C, LPC was added to cells to 180  $\mu$ M final concentration, still at 4°C. 10 min later, cells were transferred into a cuvette, and fusion was assayed at 22°C in the presence or absence of LPC. We used

enza (HAB2 cells; Fig. 3 A) and by wild-type HA of the X:31 strain (HA300a cells, not shown). 4°C also blocked the unrestricted hemifusion mediated by GPI-HA (BHA-PI cells; Fig. 3 A). The lack of fluorescent probe redistribution between membranes at FIF was independent of dye chemistry, judged from the experiments on complete fusion and hemifusion of HAb2 cells using lipid dyes DiIC<sub>18</sub>, DiIC<sub>16</sub>, DiIC<sub>12</sub>, PKH26, and rhodamine phosphatidylethanolamine and aqueous dyes 6-carboxyfluorescein and NBD-taurine.

The absence of fusion pores at FIF suggested by the fluorescence microscopy experiments (Fig. 3 A) was verified by electrophysiology. There was never any opening of fusion pores in 11 experiments where the low-pH pulse was applied at 4°C, judged by pore conductance measurements (Fig. 3 B). However, we observed both lipid and aqueous dye redistribution 1–6 min after warming FIF cells (7/11 trials; Fig. 3 C).

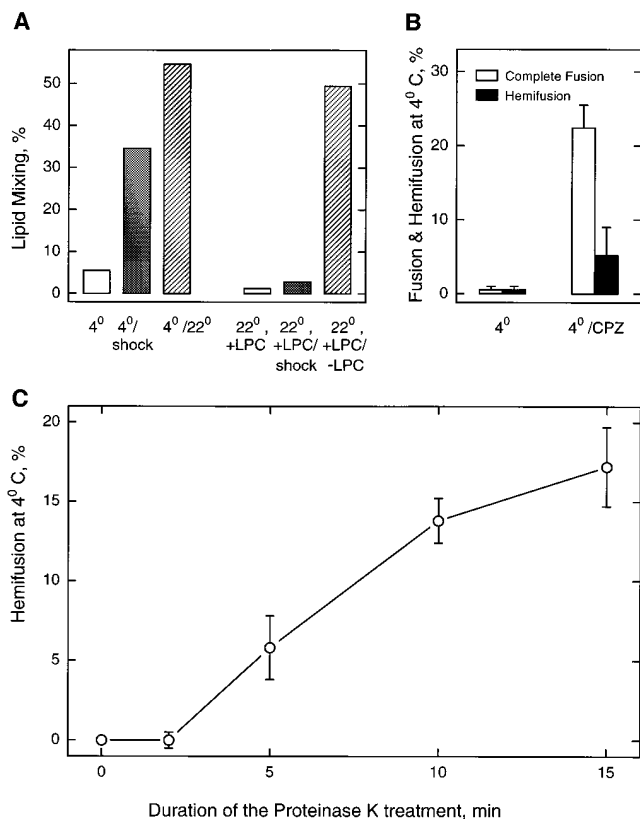
**FIF Occurs after Low pH-Dependent Conformational Changes in HA.** We tested whether the low pH-dependent conformational change in HA precedes the 4°C block to fusion. HA refolding changes fusion sensitivity to specific glycolytic and proteolytic enzymes (Doms et al., 1985; van Meer et al., 1985). Neuraminidase treatment of HAb2 cells with bound RBC (0.2 mg/ml, 45 min, 4°C) before low-pH application released >90% of bound RBC (data not shown), presumably by cleaving the sialic acid residues at RBC that are required for HA1-receptor binding. FIF, however, was insensitive to the same neuraminidase treatment (Fig. 4 A). Cells that were committed to fuse but arrested at 4°C were incubated with neuraminidase at neutral pH. Both the binding of cells and their fusion upon warming were unaffected by neuraminidase treatment.

In contrast, proteinase K, which cleaves a low-pH conformation of HA but not the neutral one (Doms et al., 1985), inhibited fusion when applied to FIF (Fig. 4 A). RBC binding to HAb2 cells in the FIF state was not susceptible to proteinase K applied alone, but proteinase K in combination with neuraminidase released most of the bound RBC.

These results suggest that in our experiments, complete fusion is blocked at 4°C at a stage when the fusion peptides of HA are already inserted into the RBC membrane and activated HA is needed to proceed beyond FIF. HA1-sialic acid binding at this fusion stage is complemented by insertion of HA fusion peptides into the RBC membrane.

**FIF Occurs after a Lipid-sensitive Stage.** Thus 4°C, as LPC (Chernomordik et al., 1997), reversibly arrested fusion after low pH-dependent refolding of HA but before lipid mixing and fusion pore formation. We tested whether FIF came before or after the LPC-arrested stage, LPC-S. The protocols of these experiments are illustrated in the diagrams in Fig. 4 B. To establish LPC-S, cells were triggered to fuse by low-pH medium at 22°C in the presence of lauroyl LPC. Then, already at neutral pH, cells were cooled

higher concentrations of LPC at 4°C than at 22°C to achieve the same incorporation of LPC. (III) LPC block prevented the system from reaching FIF. Fusion was triggered at 4°C in the presence of 180  $\mu$ M lauroyl LPC. 10 min after the end of the low-pH pulse, cells were transferred into a cuvette, and fusion was assayed at 22°C either in the presence or absence of LPC.



**Figure 5.** The stage arrested at 4°C is restricted hemifusion between fusing membranes. (A) Application of hypotonic osmotic shock at FIF but not at LPC-S results in lipid mixing. HAB2 cells with bound R18-labeled RBC were triggered to fuse by pH 5.0, 10 min at 4°C (to establish FIF) or at 22°C with 140  $\mu$ M lauroyl LPC (to establish LPC-S). Final fusion extent was assayed at neutral pH by spectrofluorometry at 4°C for FIF or with LPC for LPC-S. Application of 4°C, low-tonicity medium to FIF resulted in significant R18 dequenching (more than 60% of that observed in PBS upon warming cells to 22°C). In contrast, almost no R18 dequenching was observed when low-tonicity medium containing 140  $\mu$ M lauroyl LPC was applied to cells at the LPC-arrested stage (less than 6% of that observed upon washing out LPC by PBS). (B) Chlorpromazine treatment transforms FIF into complete fusion. HAB2 cells with bound, PKH26- and CF-labeled RBC were triggered to fuse at pH 4.9, 10 min, 4°C, incubated at neutral pH, 4°C for 5 min, and then exposed to 0.5 mM CPZ for 2 min, neutral pH, still at 4°C. Complete fusion (cell pairs with both PKH26 and CF transferred from RBC to HAB2 cells) and hemifusion (cell pairs with PKH26 but not CF transferred) were assayed with fluorescence microscopy, still at 4°C. In controls, where cells were treated by pH 4.9, 10 min, 4°C, incubated at neutral pH, 4°C for 10 min, and then warmed up to 37°C, fusion and hemifusion extents assayed 10 min after warming to 37°C were 81 and 17%, respectively. Each bar is mean  $\pm$  SE,  $n > 3$ . (C) Lipid mixing at FIF is promoted by mild treatment with proteinase K. HAB2 cells with bound, doubly labeled RBC were treated at pH 4.9, 10 min, 4°C, incubated for 20 min at neutral pH at 4°C, and then treated with proteinase K (0.05 mg/ml; 4°C) for different times. 1 h after the end of low-pH application, fusion was assayed with fluorescence microscopy, still at 4°C. While no transfer of CF was detected for any of these conditions, proteinase K treatment resulted in a significant percentage of hemifusion events (cell pairs with PKH26 transferred). Each point is mean  $\pm$  SE,  $n > 3$ . Warming the cells at this point yields unrestricted hemifusion (not shown, see Fig. 6 C). Because the proteinase K applica-

tion (0.05 mg/ml, 15 min, 4°C or 0.1 mg/ml, 45 min, 4°C) to HAB2 cells with bound RBC before low-pH pulse (pH 4.9, 10 min, 4°C) did not result in any measurable transfer of membrane or aqueous dyes at FIF (not shown), the facilitation of the lipid flow at FIF by mild proteinase K treatment is caused by cleaving HA in its low-pH conformation rather than by some nonspecific effects of this enzymatic treatment.

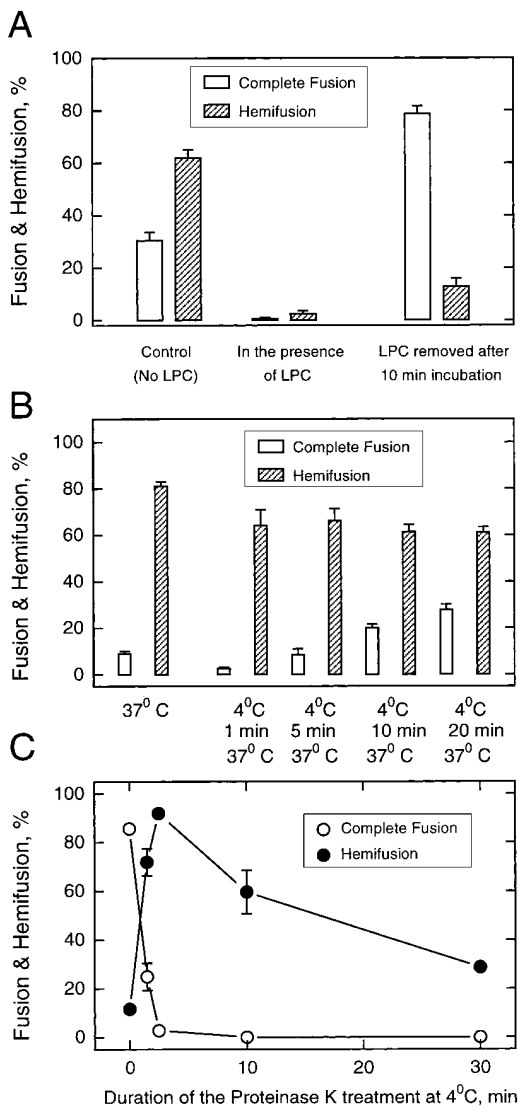
to 4°C in the presence of lauroyl LPC, and then, still at 4°C, LPC was washed out (Fig. 4, B, I). No lipid mixing was observed, indicating that 4°C arrests fusion (or at least lipid dye redistribution) after the system is released from LPC-S. We also treated cells in FIF at 4°C with an inhibiting concentration of LPC (Fig. 4, B, II). In the presence of LPC, warming to 22°C resulted in fast lipid mixing, indicating that completion of fusion from FIF is insensitive to LPC. In another experiment, low pH was applied at 4°C in the presence of LPC (Fig. 4, B, III), and then the temperature was raised to 22°C. Lipid mixing was inhibited until removal of LPC. Thus, fusion triggered at 4°C did pass through LPC-S, and membranes were accessible for the lipid at the fusion site at 4°C. We verified LPC incorporation into membranes and subsequent release of LPC upon washing at 4°C by measuring the membrane concentration of LPC (data not shown). Our experimental approach was based on the fact that fluorescence of the self-quenched R18 in RBC membranes increases upon lowering the membrane concentration of R18 by adding exogenous lipids (Chernomordik et al., 1997).

The results of all these experiments indicated that FIF is downstream of the LPC-arrested intermediate. However we cannot exclude the possibility that the local properties of the membranes in the fusion sites at FIF somehow hinder LPC incorporation in these sites, eliminating the inhibiting effect of this lipid on membrane fusion. In the next section, we present data on the structure of FIF, which confirmed that FIF follows LPC-S, and that can explain why FIF is no longer sensitive to LPC.

#### *FIF Is a Restricted Hemifusion That Precedes Fusion Pore Formation*

At this point, we can divide the set of hypotheses for the structure of FIF into two groups. Either the membranes are topologically distinct, bound together by HA, but with no continuity of the outer lipid monolayers, or the membranes do have continuity of the outer monolayers, but lipid mixing is restricted. Three different experimental approaches were used to distinguish between these two possibilities.

First, hemifusion can be tested with osmotic shock. Osmotic shock should pull a preexistent hemifusion into complete fusion, as it does in the transformation of GPI-HA-induced unrestricted hemifusion into full fusion (Melikyan et al., 1995b). Indeed, application of 4°C, low tonicity medium to cells in FIF resulted in significant lipid mixing, indicating membrane fusion (Fig. 5 A). In contrast, osmotic shock did not cause fusion of cells at the LPC-S. Interestingly, HA is needed not only to proceed beyond FIF upon raising temperature (see Fig. 4 A) but also to stabilize this fusion intermediate. When low tonicity medium



**Figure 6.** Redirecting cells committed to unrestricted hemifusion toward complete fusion and vice versa. (A and B) HAB2 cells were redirected from unrestricted hemifusion to complete fusion by preventing premature fusion with LPC (A) or with 4°C (B). Each bar is the mean  $\pm$  SE,  $n > 3$ . (A) The percentage of cells committed to complete fusion versus unrestricted hemifusion increased if the onset of actual membrane merger was delayed by applying an LPC block. Fusion was triggered by pH 4.9, 1 min, 22°C without LPC or with 170  $\mu$ M lauroyl LPC. After a 10-min incubation at neutral pH, LPC was removed by washing cells with LPC-free PBS. Fusion was assayed at neutral pH 20 min after the end of low-pH application. (B) The percentage of cells committed to complete fusion increased with time at 4°C. Fusion was triggered by pH 5.3 medium, 10 min, 4°C. Cells were incubated at neutral pH at 4°C for different time intervals and finally warmed up to 37°C, still at neutral pH. Fusion was assayed 10 min after warming to 37°C. In controls, fusion was triggered by pH 5.3 medium, 10 min, 37°C. Fusion extents were measured at neutral pH 20 min after the end of low-pH application. (C) Cells committed to complete fusion but blocked at FIF could still be redirected to unrestricted hemifusion by cleaving some activated HA molecules. Fusion was triggered by pH 4.9 medium, 10 min, 4°C. Cells were incubated in neutral pH medium still at 4°C, 30 min and then warmed to 37°C. 20 min later fusion was assayed. In the time interval between the end of the low-pH application and warming, cells were treated with proteinase K (0.1 mg/ml, 4°C) for different

was applied to cells in FIF pretreated at 4°C with 0.1 mg/ml proteinase K for 45 min (harsh treatment as in Fig. 4 A), no lipid mixing occurred (data not shown).

Second, short-term treatment of cells with the cationic amphiphath chlorpromazine (CPZ) transforms GPI-HA-mediated unrestricted hemifusion and HA-mediated “stunted fusion” into complete fusion, presumably because of preferential partition of CPZ into cytoplasmic membrane monolayers, where it promotes pore formation in hemifusion diaphragms (Melikyan et al., 1997). If there is a hemifusion diaphragm in FIF, CPZ treatment should transform FIF into complete fusion. It did (Fig. 5 B). No dye redistribution was observed for HAB2 cell-RBC pairs at LPC-S and for cell pairs not exposed to acidic pH or exposed to acidic pH but without prior trypsinization to cleave HA0 into fusion-competent form (data not shown).

Third, if FIF is really hemifusion restricted by HA trimers, then we should be able to remove this restriction to lipid redistribution by creating breaks in the molecular fence of HA trimers (see Fig. 5 C). We could (Fig. 5 C). Partial cleaving of low-pH forms of HA was attained by very mild treatment with proteinase K at 4°C. Harsher proteinase K treatment at FIF completely inhibited fusion (Fig. 4 A). Mild proteinase K treatment led to a statistically significant increase in lipid dye redistribution between cells in FIF (still at 4°C) in eight out of nine independent experiments (Fig. 5 C). The percentage of cell pairs with lipid flux was from 7 to 23% after mild proteinase K treatment vs. 0–1% without proteinase K applied. Lipid mixing was not accompanied by any transfer of the aqueous dye. Identical mild proteinase K treatment at 4°C also resulted in some membrane dye redistribution between RBC and BHA-PI cells at FIF (data not shown). The simplest explanation for these results is that both HA-mediated complete fusion and GPI-HA-mediated unrestricted hemifusion proceed through the stage of restricted hemifusion arrested at 4°C.

### Switching Fusion from Unrestricted Hemifusion towards Fusion Pore Formation

Fusion is mediated by multiple HA molecules acting in a cooperative manner (Danieli et al., 1996; Blumenthal et al., 1996). If interactions between a number of low pH-activated HA molecules are required for complete fusion, and premature development of unrestricted hemifusion prevents pore formation, then we should be able to compensate for low protein density and mobility by allowing activated HA molecules to assemble over a longer period of time. Indeed, in experiments when fusion was blocked by LPC and then, 10 min later, LPC was removed (to allow fusion or unrestricted hemifusion to proceed), the percentage of cells demonstrating complete fusion was significantly higher than in the control experiments with no lipids added (Fig. 6 A). In similar experiments, cells committed to unrestricted hemifusion by a pulse of pH 5.3, 4°C, were then held at neutral pH at 4°C before warming to 37°C

times. Complete fusion (cell pairs with both PKH26 and CF transferred from RBC to HAB2 cells) and hemifusion (cell pairs with PKH26 but not CF transferred) were assayed with fluorescence microscopy. Each point is mean  $\pm$  SE,  $n > 3$ .



(Fig. 6 B). Complete fusion increased as the time at neutral pH at 4°C increased. These data indicate that cells in the unrestricted hemifusion pathway can be gradually redirected to complete fusion.

Conversely, cells committed to complete fusion can be redirected to unrestricted hemifusion by treating them with proteinase K, which only cleaves low-pH conformations of HA trimers. In the experiments presented in Fig. 6 C, HAb2 cells with bound RBC were incubated in low-pH medium at 4°C. Then cells were returned to neutral pH medium and treated with proteinase K, still at 4°C. We found that proteinase K treatment inhibited complete fusion faster than unrestricted hemifusion. Decreasing the surface density of activated HA molecules at FIF transformed some precomplete fusion intermediates into pre-unrestricted hemifusion intermediates, redirecting cells from complete fusion to unrestricted hemifusion. Note that the increase in unrestricted hemifusion at shorter proteinase K treatments precisely corresponds to a decrease in complete fusion (Fig. 6 C), indicating that such treatment did not promote lipid mixing. At longer incubations with enzyme, we observed almost no content mixing and a gradual decrease in the lipid mixing, and, thus, inhibition of unrestricted hemifusion.

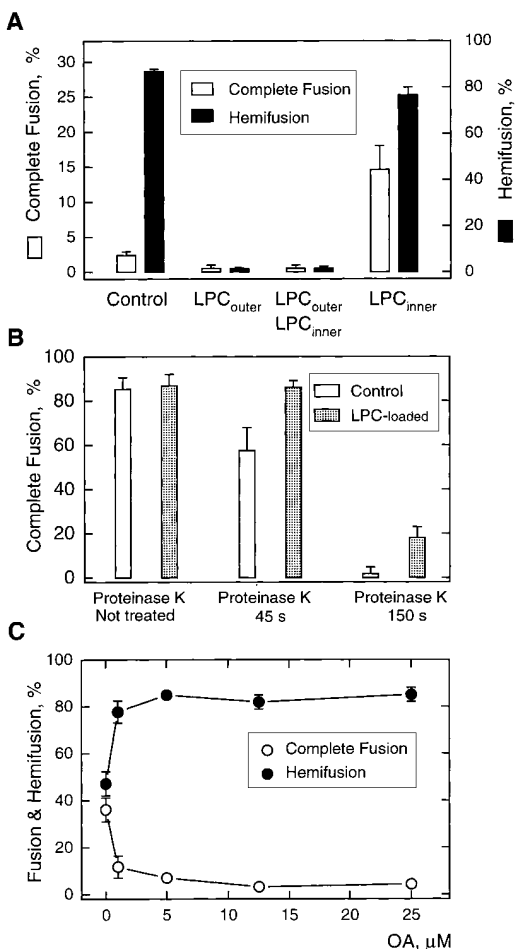
#### Fusion Pore Formation Depends on the Lipid Composition of the Inner Membrane Monolayers

If lipids are present in the edge of a fusion pore, pore formation should be sensitive to the membranes' lipid composition. Furthermore, if such a lipidic pore opens in a hemifusion diaphragm, pore formation should be sensitive to the composition of the inner (cytoplasmic) membrane monolayers that form this hemifusion diaphragm.

Indeed, loading LPC inside RBC, so that it was added to the inner monolayer of RBC membrane, promoted pore development. In these experiments, we chose conditions (pH 5.1) that gave a low percentage of complete fusion to start with. When LPC-loaded RBC were the target cells, membrane dye redistribution did not differ from controls lacking LPC. However, transfer of aqueous dye, and thus the percentage of complete fusion, were significantly higher (approximately fivefold) than in controls (Fig. 7 A). The LPC effect was not related to colloid-osmotic swelling of RBC (Bierbaum et al., 1979) because adding an impermeable sugar (20 mM stachyose) to the external medium just before low-pH application did not affect these results. Fusion required that the RBC loaded with LPC in the inner monolayers undergo treatment at acidic pH and that the HAb2 cells were treated with trypsin (data not shown).

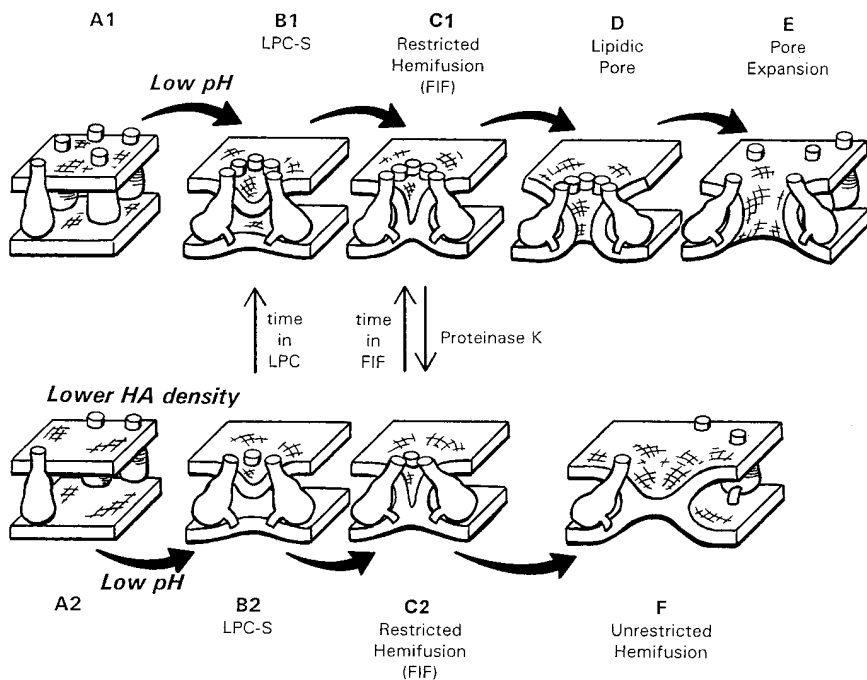
LPC in the inner monolayer of the RBC membrane did not interfere with inhibition of lipid mixing by LPC added to the contacting (outer) monolayers of the membranes: when LPC was added to the medium to partition into outer membrane monolayers of HAb2 cells and LPC-loaded RBC, almost no lipid mixing was detected (Fig. 7 A). This argues against LPC-induced buckling of RBC membranes due to asymmetrical incorporation as a mechanism of LPC inhibition.

Interestingly, the presence of LPC in the inner monolayer of the RBC membrane was not sufficient to cause the transition from FIF to complete fusion while still at



**Figure 7.** Promotion and inhibition of complete fusion by altering membrane lipid composition. (A) LPC in the inner monolayer of RBC membrane promotes complete fusion. Fusion was triggered at 22°C, pH 5.1, 10 min. No exogenous lipid was added in the control experiments. Lauroyl LPC was either added to the medium to incorporate into outer membrane monolayers (170 μM), loaded into RBC to partition into inner membrane monolayer, or both loaded into RBC and added to the outer membrane monolayers (170 μM). (B) Loading LPC into RBC ghosts decreases the number of activated HA molecules required for fusion pore formation and expansion, downstream of FIF. Fusion of HAb2 cells with either RBC loaded with LPC or control RBC was triggered at 4°C, pH 4.9, 10 min. Then cells were incubated in neutral pH medium for 10 min, treated by proteinase K (0.1 mg/ml, still at 4°C) for 45 s, 150 s, or not treated at all. After 20 min more at neutral pH, 4°C, the temperature was raised to 22°C. (C) Complete fusion is inhibited by OA. Fusion was triggered at 22°C, pH 4.9, 10 min in the medium containing different concentrations of OA. In A–C, complete fusion (cell pairs with both PKH26 and CF transferred from RBC to HAb2 cells) and hemifusion (cell pairs with PKH26 but not CF transferred) were assayed with fluorescence microscopy after 20 min of incubation at neutral pH. Each point is mean ± SE,  $n > 3$ .

4°C (data not shown), suggesting that destabilization of the hemifusion diaphragm by LPC appears to be less profound than that by CPZ (see Fig. 5 B). This can be explained by the fact that LPC was added only to the inner monolayer of the RBC membrane and CPZ was appar-



**Figure 8.** Pathway for the fusion of HA-expressing membrane with RBC membrane. In the schematic diagram of the fusion site, the top membrane is a section of an HA-expressing cell, transmembrane domain of HA pointing up. The bottom membrane is a section of a target cell membrane (e.g., RBC), fusion peptide pointing down. *A1*, *B1*, *C1*, *D*, and *E* illustrate the pathway of complete fusion. Acidification causes a conformational change in HA from its initial state (*A1*), leading to insertion of the HA fusion peptide into the target membrane. (*B1*) The fusion intermediate after assembly of refolded HA but before membrane merger, which is the LPC-arrested state (LPC-S). (*C1*) Restricted hemifusion intermediate within a fence of activated HA, which restricts lipid diffusion between the two membranes: the stage arrested at 4°C (FIF). (*D*) Formation of a fusion pore, which can close and open again within the fence (flicker). (*E*) Expansion of the fusion pore beyond the fence to complete fusion. The lower set of intermediates (*A2–F*) illustrates the unrestricted hemifusion pathway

for decreased surface density of activated HA near the fusion site. (*A2*) Initial state. (*B2*) LPC-arrested state. (*C2*) Restricted hemifusion intermediate arrested at 4°C (FIF). Note that the fence of activated HA assembled around fusion site, which is formed here by a lower number of proteins than the one in *C1*, still restricts lipid transfer between the two membranes. (*F*) The extension of the hemifusion intermediate precedes the assembly of a proper HA complex and yields an unrestricted hemifusion state that allows dye mixing but is incapable of supporting fusion pore development. Blocking the premature merger of membranes and development of unrestricted hemifusion with either LPC (at *B2*) or 4°C (at *C2*) allows activated HA molecules to assemble over a longer period of time and thus redirects the cells from the unrestricted hemifusion pathway to the complete fusion pathway. In contrast, cleaving of some of the activated HA molecules at *C1* with proteinase K redirects cells from the complete fusion pathway to the unrestricted hemifusion pathway.

ently present in both membrane monolayers forming the hemifusion diaphragm (Melikyan et al., 1997). While LPC was not capable of transforming FIF into complete fusion at 4°C, loading of LPC into RBC promoted the HA-dependent completion of fusion upon raising the temperature. In the experiments where cells were briefly treated by proteinase K at FIF to partially shift cells committed to complete fusion into cells committed to hemifusion by cleaving part of the activated HA molecules (see Fig. 6 C), the percentage of complete fusion events was significantly higher for RBC loaded with LPC than for control RBC (Fig. 7 B). Thus, altering the lipid composition of these monolayers to make them more permissive for fusion pore formation decreased the number of the activated HA molecules required to convert hemifusion into complete fusion.

In contrast to LPC, OA stabilizes the hemifusion state. Unlike LPC it rapidly equilibrates between outer and inner monolayers (Broring et al., 1989; Kamp et al., 1995; Kleinfeld et al., 1997). By triggering fusion with more acidic pH media (pH 4.9), we minimized the OA-induced promotion of lipid mixing (Chernomordik et al., 1997) and focused on the effects of OA on aqueous content mixing. Adding OA inhibited fusion pore development (Fig. 7 C). OA worked only within the first few minutes after low-pH application, before the development of unrestricted hemifusion that precludes pore formation (see above). Adding BSA to extract OA from membranes within the first 3 min after the beginning of low-pH application increased the percentage of complete fusion to that observed in control

experiments with no OA added (data not shown). When BSA was added 10 min after low-pH application, unrestricted hemifusion was already irreversible, and OA removal did not promote complete fusion.

## Discussion

To arrest and isolate short-lived intermediates of HA-mediated fusion, we slowed fusion progression by decreasing the numbers of activated HA, lowering temperature, and altering the membrane lipid composition to that nonpermissive for fusion. We found the sequential formation of a hemifusion intermediate and a lipidic pore in the fusion pathway (Fig. 8, *A1–E*). Our results indicate that fusion proceeds through a transient stage of restricted hemifusion (Fig. 8 *C1*) that is subsequent to both conformational changes in HA upon its low pH-induced activation and to the LPC-arrested stage (Fig. 8 *B1*) but precedes fusion pore opening and lipid mixing (Fig. 8, *D* and *E*). Because this restricted hemifusion intermediate can be reversibly arrested by 4°C, we refer to this intermediate as a frozen intermediate of fusion. In addition to this restricted hemifusion, wild-type HA is also capable of producing unrestricted hemifusion, i.e., mixing the lipids of fusing membranes without forming a pore that would allow ions through (Fig. 8 *F*). Unrestricted hemifusion occurred as the density or mobility of active HA trimers was reduced. Rather than being a transient intermediate of fusion, this unrestricted hemifusion proved to be a stable alternative state that can

occur instead of an expanding fusion pore. Altering either the number of activated hemagglutinin available at the fusion site or the lipid composition of inner membrane monolayers leads to switching fusion sites between fusion pore development and unrestricted hemifusion. These findings indicate that the initial fusion pore involves lipids (Fig. 8 D) and develops within the restricted hemifusion diaphragm only when the number of activated HA forming the complex around the fusion site is sufficiently high, suggesting a functional role for the aggregation of HA trimers in fusion pore formation.

### **Unrestricted Hemifusion**

Unrestricted hemifusion is the final state of fusion mediated by engineered, lipid-anchored HA molecules (GPI-HA), suggesting hemifusion can also be caused by wild-type HA (Kemble et al., 1994; Melikyan et al., 1995a,b, 1997). This was contested by the alternative suggestion that wild-type HA can not cause hemifusion. In this view, the engineered HA lacking the pore-forming transmembrane domain causes hemifusion, and wild-type HA forms proteinaceous pores without hemifusion (Lindau and Almers, 1995). The recent finding that under suboptimal conditions wild-type HA promotes lipid mixing without mixing of water-soluble probes (stunted fusion; Melikyan et al., 1997), while consistent with the establishment of a hemifusion state, did not prove it because pores in HA-mediated fusion are often too small to be detected by assays less sensitive than electrophysiological measurements (Tse et al., 1993; Zimmerberg et al., 1994). Here we present the electrophysiological proof that wild-type HA causes unrestricted hemifusion. We found that stunted fusion events similar to those reported by Melikyan et al. (1997) can correspond not only to unrestricted hemifusion but also to nonexpanding, often transient fusion pores that are too small to allow measurable transfer of water-soluble dyes. Thus, any presumption of hemifusion in dye mixing experiments needs to be verified electrophysiologically.

It is possible that the two different phenotypes for fusion (complete fusion and unrestricted hemifusion) are mediated by two different conformations of HA. For example, pH 5.3 may activate a few of the monomers of a trimer, and mild trypsin treatment may create trimers with only one or two cleaved monomers. However, the structure of the fusion-competent conformation of HA of the Japan strain of influenza virus appears to be the same for pH 5.0 vs. pH 5.4 and for 37°C vs. 20°C (Korte et al., 1997). The presence of unrestricted hemifusion in each of our three experimental approaches and the finding that unrestricted hemifusion occurs when the conformational change in HA is triggered under “optimal” conditions (pH 4.9, 33–37°C) (Fig. 2, A–C) argue against the possible role of “nonphysiological” alterations of HA conformation. If the same low-pH trimer conformation of HA mediates both unrestricted hemifusion and complete fusion, it is the supramolecular complex of these active trimers that dictates the functional outcome.

Additional evidence that unrestricted hemifusion is caused by the same conformations of HA as complete fusion came from our experiments on shifting the destiny of cells from unrestricted hemifusion to complete fusion merely

by increasing the duration of HA interaction. HA activation was presumably complete within 1 min (White and Wilson, 1987; Stegmann et al., 1990; Krumbiegel et al., 1994; Korte et al., 1997) before the beginning of the interval of redirection from unrestricted hemifusion to complete fusion. This suggests that during this interval, activated HA still have sufficient mobility to assemble into functional multiprotein complexes. In contrast, cells committed to complete fusion were redirected to unrestricted hemifusion by cleaving a portion of the HA molecules already activated on the cell surface. These results indicate that the opening of a fusion pore requires more HA molecules than unrestricted hemifusion. Biochemical characterization of fusion complexes at different stages is clearly needed.

A crucial question is whether unrestricted hemifusion is a stage in wild type HA-mediated fusion. The answer is no. There is no productive fusion pore opening after the onset of lipid mixing. Thus, unrestricted hemifusion is an inactivated, side branch in the fusion pathway. Interestingly, a recent publication of Nussler et al. (1997) suggests that GPI-HA-mediated unrestricted hemifusion can be less stable against transition into complete fusion than that mediated by wild-type HA. While for wild-type HA we did not observe any change in unrestricted hemifusion extent from 20 min to 2 h after low-pH pulse, for GPI-HA, the extent of unrestricted hemifusion decreased from 10 to 0% for 24 and 40 min after pH application with a corresponding increase in complete fusion (Nussler et al., 1997). At present, it is difficult to draw any conclusion from this difference. In our work, the extent of unrestricted hemifusion was evaluated as a difference between lipid mixing and content mixing. Nussler et al. (1997) measured the difference between the mixing of membrane dyes present in the outer and inner membrane monolayers. In addition, these authors used a more intensive trypsin treatment of HA-expressing cells than the one used in our work (50 vs. 5 µg/ml). This can be important because intensive trypsin treatments often increase membrane permeability, which may disturb colloid osmotic equilibrium causing slow swelling of cells and a building up of membrane tension.

In the next section we will discuss a type of hemifusion where, in contrast to unrestricted hemifusion, membrane continuity is apparently not accompanied by measurable lipid mixing. The lifetime of this intermediate, which appears to be a transient intermediate in the pathway of complete fusion, dramatically increased at 4°C.

### **The Frozen Intermediate of Fusion Is Restricted Hemifusion**

Temperatures as low as 4°C inhibit various fusion reactions (Kielian and Cohn, 1980; Hart et al., 1996). In particular, the fusion of influenza virus to RBC and to liposomes is slowed (Stegmann et al., 1990; Ramalho-Santos et al., 1993; Tsurudome et al., 1992) or even reversibly arrested (Schoch et al., 1992). Brief exposure of RBC complexes with influenza virus (X:31 strain but not PR/8 strain) to low pH at 4°C results in establishment of a long-lived “fusion-committed” state that develops into fusion only after raising the temperature (Schoch et al., 1992; Pak et al., 1994).

We report here the FIF for fusion of RBC with cells ex-

pressing HA of either X:31 or Japan strains of influenza virus. Our data confirm earlier findings that 4°C blocks fusion at a stage after a low pH-dependent conformational change in HA and apparently after the insertion of the fusion peptide into the RBC membrane (Stegmann et al., 1990; Schoch et al., 1992; Tsurudome et al., 1992). We established that FIF follow the LPC-S and precedes both lipid mixing and fusion pore opening. 4°C similarly inhibited complete fusion and unrestricted hemifusion mediated either by wild-type HA or by GPI-HA.

The lack of lipid mixing at FIF can occur in the absence of lipidic continuity between fusing membranes at this stage or if lipid flow through such connections is somehow hindered. While lateral diffusion of lipids in biological membranes is relatively slow at low temperatures, e.g.,  $0.7\text{--}3.5 \times 10^{-9}$  cm<sup>2</sup>/s at 5°C vs.  $0.2\text{--}1 \times 10^{-8}$  cm<sup>2</sup>/s at 37°C (Jacobson et al., 1981; van Meer et al., 1985), it is not slow enough to account for our results. At 37°C, the onset of lipid dye PKH26 redistribution was maximal within a minute, while at 4°C, the temperature at which FIF occurs, no lipid dye transfer between cells was detected for 24 h (data not shown). If even the whole influenza particle, presumably bound to a number of receptors on the cell surface, can move more than 4 μm in 4 h at 4°C (Anderson et al., 1992), one would expect measurable lipid flow between connected fusing membranes if there would be no barrier for lipid transfer through the connection. In fact, such lipid flux at 4°C was observed when cells in FIF were mildly treated by proteinase K (see Fig. 5 C).

Our results indicate that there is a local lipidic connection between the outer monolayers of fusing membranes in FIF: (a) Lipid mixing at FIF, but not at LPC-S, can be promoted by applying osmotic shock (Fig. 5 A), suggesting that low temperature blocks fusion at a stage when membranes are connected. (b) CPZ, known to selectively break the hemifusion diaphragm (Melikyan et al., 1997), can convert FIF but not LPC-S into complete fusion (Fig. 5 B). (c) Partial cleaving of low-pH forms of HA by very mild treatment with proteinase K of cell pairs in FIF resulted in membrane dye redistribution between some fusing cells still at 4°C (Fig. 5 C). This increase in our assay for fusion, after proteolysis of the protein that catalyzes fusion, may seem paradoxical. However, this finding is entirely consistent with the hypothesis that 4°C reversibly arrests fusion by stabilizing local hemifusion sites surrounded by HA aggregates, which restrict lipid diffusion. Proteolytic treatment just facilitated lipid flow through this preexisting connection and allowed its detection.

The results of the osmotic shock, CPZ, and proteinase K experiments all indicate that FIF is restricted hemifusion. This conclusion substantiates our finding that FIF is downstream of LPC-S. LPC presumably inhibits merger of outer monolayers (Chernomordik et al., 1997), and at FIF outer monolayers are already merged. Could FIF correspond to some very special and tight proteinaceous connection between membranes that develops before actual merger of membranes? If so, (a) the transition from this "tight contact" state to either opening of a fusion pore or unrestricted hemifusion would have to be independent of the lipid composition of the contacting monolayers of the membranes (not responsive to LPC); (b) osmotic shock and CPZ treatment must destabilize this tight contact state

and transform it into complete fusion; and (c) mild proteinase K treatment must transform it into unrestricted hemifusion. The existence of another fusion intermediate combining all these properties seems improbable to us. FIF, then, is most likely identical to the restricted hemifusion intermediate originally postulated to explain the lack of lipid flux in initial fusion pores at physiological temperatures.

### *Lipidic Fusion Pore*

The effects of the lipid composition of membranes on fusion pore are consistent with the hypothesis that as fusion of purely lipid bilayers (Kozlov and Markin, 1983; Chernomordik et al., 1987, 1995a,b; Siegel, 1993; Lee and Lentz, 1997), HA-mediated fusion involves two bent lipid-involving fusion intermediates: a localized hemifusion between contacting membrane monolayers—a stalk—and a lipidic pore, which have opposite net curvatures and are formed by different monolayers of fusing membranes. The dependence on the lipids in the inner membrane monolayers for HA-mediated hemifusion is another piece of evidence that complete fusion proceeds through lipidic rather than proteinaceous intermediates. For proteinaceous pores, these monolayers would only connect after fusion pore widening. Most recently we have shown flickering fusion pores in fusion of protein-free lipid bilayers (Chanturiya et al., 1997).

As mentioned above, fusion pore development is promoted by CPZ added to membranes at a state of unrestricted hemifusion (Melikyan et al., 1997). Because CPZ preferentially partitions into inner membrane monolayers and, like LPC, promotes the micelle-like curvature of the lipid monolayer required in the edge of a lipidic pore, this elegant finding is consistent with the hypothesis that HA forms lipidic pores in a hemifusion diaphragm (Melikyan et al., 1997). However, because CPZ was added to the membranes at a state of unrestricted hemifusion, which, as we report here, is actually a branch off the normal fusion pathway, CPZ may have destabilized hemifusion diaphragms through pores that are different from those in HA-mediated fusion. Indeed, we found that CPZ-induced transformation of unrestricted hemifusion into complete fusion is HA independent. This promotion was not affected by cleaving activated HA with rather harsh proteinase K treatment (0.2 mg/ml, 20 min, room temperature) of hemifused BHA-PI cells and RBC before CPZ application (data not shown). Similar results were obtained for HAb2 cells in the unrestricted hemifusion state. In contradistinction, lipid-sensitive pores that develop in restricted hemifusion diaphragms were HA dependent (Fig. 4 A). In our experiments, the lipid composition of inner membrane monolayers was altered before low-pH application, and thus added lipids were present and acting during the earliest fusion stages. OA, which should increase the energy barrier for lipidic pore formation, did inhibit HA-mediated pore formation, switching some of the fusion sites from fusion pore development to unrestricted hemifusion.

### *Lipid Bilayer Fusion within the Ringlike Complex of Activated HA Trimers*

Our data support a model of fusion (Fig. 8, A1–E) wherein, after their conformational changes, HA trimers activated

by low pH assemble into a ringlike complex (Bentz et al., 1990; Zimmerberg et al., 1991; Danieli et al., 1996; Blumenthal et al., 1996) in a manner similar to that proposed in the scaffold model for exocytosis (Monck and Fernandez, 1992). Fusion starts with a local merger of the outer membrane monolayers that can be prevented by adding LPC. The ring of HA molecules enclosing this lipidic connection restricts the flow of lipids between fusing membranes and, thus, prevents expansion of the hemifusion intermediate beyond this proteinaceous "fence." This fence would keep tension focused within the fusion site to facilitate the transformation of this restricted hemifusion into an expanding fusion pore. In its early state, the lipidic fusion pore is still surrounded by this assembly of HA (Fig. 8 D) since lipid mixing remains restricted for small initial pores (Tse et al., 1993; Zimmerberg et al., 1994). If the number of activated HA available at the fusion site is inadequate to form a sufficiently large and tight ringlike complex (less fusion-competent HA, higher pH, or lower mobility of HA) or if the interactions between HA molecules in the complex are weaker (as in the case of GPI-HA [Kemle et al., 1993]), the system shifts to the alternative pathway (Fig. 8, A2–F). In this case, the growing hemifusion intermediate can break the HA molecular fence and expand into unrestricted hemifusion, a branch off the normal fusion pathway (Fig. 8 F). The cells can be redirected from unrestricted hemifusion to complete fusion by allowing more time for HA assembly, or from complete fusion to unrestricted hemifusion by decreasing the number of activated HA at the fusion site.

Since the characteristics of the fusion pore and the lipid dependence of fusion appear to be common to all biological fusion events (Monck and Fernandez, 1992; Chernomordik et al., 1995b; White, 1995), the mechanism of protein-driven membrane rearrangements described here for HA-mediated fusion may be universal. In all systems, dissecting the membrane fusion pathway into a series of distinct, arrested intermediates may facilitate the identification of specific conformation(s) of the proteins that form and stabilize these fusion intermediates and assemble into multiprotein complexes around the fusion site. In the future, this knowledge of the general mechanism of membrane fusion could be used to discover agents that inhibit or facilitate specific fusion events.

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