Membrane Perturbation and Fusion Pore Formation in Influenza Hemagglutinin-mediated Membrane Fusion

A NEW MODEL FOR FUSION*

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Low pH-induced fusion mediated by the hemagglutinin (HA) of influenza virus involves conformational changes in the protein that lead to the insertion of a “fusion peptide” domain of this protein into the target membrane and is thought to perturb the membrane, triggering fusion. By using whole virus, purified HA, or HA ectodomains, we found that shortly after insertion, pores of less than 26 Å in diameter were formed in liposomal membranes. As measured by a novel assay, these pores stay open, or continue to close and open, for minutes to hours and persist after pH neutralization. With virus and purified HA, larger pores, allowing the leakage of dextrans, were seen at times well after insertion. For virus, dextran leakage was simultaneous with lipid mixing and the formation of “fusion pores,” allowing the transfer of dextrans from the liposomal to the viral interior or vice versa. Pores did not form in the viral membrane in the absence of a target membrane. Based on these data, we propose a new model for fusion, in which HA initially forms a proteinaceous pore in the target, but not in the viral membrane, before a lipidic hemifusion intermediate is formed.

Influenza virus enters its host cells by endocytosis, followed by fusion between the endosomal and the viral membrane. Fusion is mediated by the trimeric integral membrane protein hemagglutinin (HA)† (for reviews see Refs. 1 and 2) and is triggered by the low endosomal pH, which induces a conformational change in the protein. Each monomer of HA consists of two disulfide-linked subunits, the smaller one, HA2, is membrane-anchored at the C terminus. The N terminus of this subunit consists of a hydrophilic stretch of amino acids known as the “fusion peptide,” which is buried in the stem of the HA trimer at neutral pH (3). The conformational change at low pH moves this peptide to the outside of the protein (4), and studies of liposome-virus fusion have shown that the peptide then enters the hydrophobic interior of the target membrane for fusion (5, 6). Insertion could perturb the target membrane locally, providing a starting point for fusion. We and others have found that virus (7) or a single purified trimer of the ectodomain of HA (8), prepared by bromelain digestion of HA (BHA), produce a pore in target membranes at low pH, allowing the leakage of water-soluble molecules across these membranes. Whereas the conformational change and insertion of the fusion peptide are clearly required for fusion (9, 10), little is known of the mechanism whereby the protein achieves the merger of the lipid bilayers. The formation of either lipidic or proteinaceous intermediate structures has been proposed to precede membrane merger. Lipidic intermediates could be “stalks” of fused outer membrane leaflets, formed after a focal perturbation of the target membrane bilayer by the inserted viral fusion peptides (11, 12). This “hemifusion” intermediate would then expand laterally, be followed by breakthrough and merger of the inner leaflets at this point, leading to complete fusion. Although several observations are compatible with this theory (13–15), it follows that lipids would line the first aqueous connection between the viral and the target membrane interior, the so-called “fusion pore.” However, electrophysiological measurements have shown that fusion pores form before lipid mixing can be detected (16). Thus, alternatively, it was proposed that the fusion pores may be proteinaceous (17) and would expand gradually, incorporating lipids as they open. More recent data suggest, however, that a lipidic connection between the membranes may form before fusion pore formation after all but that extensive lipid mixing would be hindered by the presence of HA in the membrane, thus supporting the first theory while explaining the data that gave rise to the second theory (18).

Here we show that BHA induces the formation of small pores that stay open, or open and close, for a long time but do not allow the passage of dextrans (mass 3 kDa). Viral HA or purified intact HAs initially create small pores also, but much larger pores are formed later. Large pores also formed in reconstituted viral membranes during fusion with liposomes but not in the absence of target membranes. By taking into account the available evidence for lipidic intermediates containing a hemifusion diaphragm in the literature, we propose that although lipidic hemifusion intermediates are involved in influenza virus-induced fusion, the perturbation of the target membrane at the site of fusion initially leads to formation of a large pore in the target membrane, and we discuss the role of this pore in initiating fusion.

EXPERIMENTAL PROCEDURES

Liposomes—Multilamellar vesicles were produced by resuspension of dry lipid films of egg phosphatidylcholine, egg phosphatidylethanolamine (both from Avanti Polar Lipids, Birmingham, AL), gangliosides (type III from bovine brain, estimated molecular weight 1,500 g/mol, from Sigma) at a molar ratio of 6:3:1 in buffer A (NaCl 145 mM, HEPES...
2.5 mM EDTA 1 mM, pH 7.4). This suspension was frozen and thawed five times, and large unilamellar vesicles were made from the multilamellar vesicles by extrusion through 0.1- or 0.4-μm defined pore polycarbonate filters (Nuclepore, Pleasanton, CA) (19). Remaining multilamellar liposomes were removed by centrifugation. Phospholipid phosphorus was determined according to Bradford (30) using the Bio-Rad dye-binding assay (Bio-Rad), using bovine serum albumin as a standard.

Leakage Measurements—Calcein was encapsulated into liposomes by hydrating the lipid film in buffer containing 75 mM calcein, 85 mM NaCl, 2.5 mM HEPES, 1 mM EDTA, pH 7.4. After extrusion as described above, free dye was removed by molecular sieve chromatography on Sephadex G-75 with buffer A. Tetramethylrhodamines coupled to dextrans with a molecular mass of 3,000 or 10,000 Da (TMRD-3,000 or TMRD-10,000) were entrapped into liposomes by hydrating a lipid film in buffer A containing 20 mg/ml of the dyes. After liposome extrusion as described above, through a 0.4-μm filter for maximum entrapment efficiency, free dye was removed by molecular sieve chromatography on Sephadex G-75 for TMRD-3,000 or G-200 for TMRD-10,000 using buffer A. Relief of self-quenching due to dilution upon leakage or fusion was measured by monitoring calcein fluorescence at 515 nm, with excitation at 495 nm, and TMRD fluorescence at 580 nm, with excitation at 530 nm. Residual fluorescence intensity was set to zero and the intensity at 495 nm, and TMRD fluorescence at 580 nm, with excitation at 530 nm. These measurements were carried out in buffer containing 135 mM NaCl, 15 mM sodium citrate, 10 mM MES, 5 mM HEPES, 1 mM EDTA at pH 5.1 or 7.4, or 0 or 37 °C (buffer B). Fluorescence data were normalized by setting the initial fluorescence intensity of TMRD or calcein-loaded liposomes to the initial fluorescence of zero dye entrapped fluorophores, obtained after lysis of the liposomes with Triton X-100 (0.5% v/v, from Sigma), to 100. The lag time before the onset of leakage was defined as the time between the addition of BHA or virus and the intercept of the tangent to the inflection point of the leakage curve with the time axis (21), as proposed by Bentz (22).

Fusion Measurements—Fusion between virus and labeled liposomes was measured with a resonance energy transfer assay (23). Labeled liposomes, prepared as described above, form a lipid film containing 0.6 mol % each of N-lissamine rhodamine B sulfonyl-phosphatidylethanolamine (N-Rh-PE) and N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (N-ND-BPE). Fusion was measured in buffer B at excitation and emission wavelengths of 465 and 530 nm, respectively, with a 515-nm long pass filter placed between cuvette and emission monochromator (24) on a Photon Technologies International (South Brunswick, NJ) fluorometer with continuous stirring in a thermostated cuvette holder. For calibration of the fluorescence scale, the initial residual fluorescence intensity was set to zero and the intensity at infinite probe solution 100%. The latter value was obtained after lysis of the liposomes with Triton X-100 (0.5% v/v) with correction for the quenching of symmetric liposomes by about 20%.

Asymmetric Liposome Preparation—Asymmetrically labeled liposomes were prepared by adding 20 μl of a freshly made solution of 1 mM sodium dithionite, 1 mM Tris, pH 10, to 1 μmol of a unilamellar, N-NBD-PE and N-Rh-PE containing liposome preparation in 1.4 ml of buffer B at excitation and emission wavelengths of 465 and 530 nm, respectively, with a 515-nm long pass filter placed between cuvette and emission monochromator (24) on a Photon Technologies International (South Brunswick, NJ) fluorometer with continuous stirring in a thermostated cuvette holder. For calibration of the fluorescence scale, the initial residual fluorescence intensity was set to zero and the intensity at infinite probe solution 100%. The latter value was obtained after lysis of the liposomes with Triton X-100 (0.5% v/v) with correction for the quenching of symmetric liposomes by about 20%.

BHA Preparation—BHA was prepared as described by Brand and Skehel (28) with minor modifications as specified by Harter et al. (29). Briefly, virus was pelleted by centrifugation, resuspended in 1 ml of buffer containing 100 mM Tris, 1 mM EDTA, 50 μM β-mercaptoethanol, pH 8.0, and digested with 10 mg/ml bromelain (Calbiochem) at 37 °C for 20 h. Subsequently, virus was removed by centrifugation, and BHA was purified from the supernatant by molecular sieve chromatography on Sephadex G-75 in buffer A. As assessed by SDS-polyacrylamide gel electrophoresis the protein was more than 95% pure. Protein concentrations were determined according to Bradford (30) using the Bio-Rad protein assay (Bio-Rad), using bovine serum albumin as a standard.

Preparation of HA Rosettes—An aliquot of virus was pelleted by ultracentrifugation and solubilized in 30 mM β-α-toctyl glucoside (Roche Molecular Biochemicals) in buffer A for 20 min at 0 °C. The viral nucleocapsid and matrix protein were removed by centrifugation at 100,000 × g for 35 min at 4 °C. The supernatant containing the solubilized viral membrane was then passed over an affinity chromatography column containing Ricinus communis lectin coupled to Sepharose beads (Sigma), and the column was washed with 5 volumes of buffer A containing 30 mM β-α-toctyl glucoside to remove the viral lipids. HA was eluted with 0.2 M D(+)-galactose, 30 mM β-α-toctyl glucoside in buffer A; the fractions containing HA were pooled, and the detergent was removed by dialysis against 1000 volumes of buffer A for 24 h at 4 °C, with 3 changes at 10 h. HA was then purified according to Bradford (30), and the purity of the preparation was checked by SDS-polyacrylamide gel electrophoresis. No proteins other than HA were detected on gel.

Virosome Preparation—Virosomes were prepared as described by Stegmann et al. (31), with minor modifications. Briefly, an aliquot of virus was pelleted by ultracentrifugation, solubilized in octaethylene glycol monododecyl ether (C12E8, Fluka) 50 mM in buffer A. The viral nucleocapsid and matrix protein were then removed by ultracentrifugation at 100,000 × g for 35 min at 4 °C. The supernatant containing the solubilized viral membrane was then either added to a dry lipid film containing N-ND-BPE and N-Rh-PE (final concentration in the reconstructed membrane 0.8 mol % each) or mixed with TMRD-10,000. To remove the detergent, 100 μl of the mixtures was added to Bio-Beads SM-2 (30 mg) and shaken at 2500 rpm for 1 h at room temperature, after which the supernatant was added to 15 mg of Bio-Beads SM-2 and shaken at 2500 rpm for 10 min at room temperature. The virosomes were then purified by molecular sieve chromatography on Sephadex G-200 in buffer A to eliminate remaining detergent and to separate the virosome-entrapped from free TMRD-10,000.

RESULTS

Pores Formed by BHA Are Small and Are Open or Continue to Close and Open for Minutes to Hours—We have previously shown that the purified ectodomain of HA, BHA, produces pores in large unilamellar liposomal membranes at low pH, causing low molecular weight water-soluble molecules encapsulated in the liposomes to leak out (8). To estimate the size of these pores, liposomes were produced containing self-quenching concentrations of calcein (623 Da) or tetramethylrhodamine coupled to 3,000- or 10,000-Da dextrans (TMRD-3,000 or TMRD-10,000, respectively). Although BHA readily induced the leakage of calcein, relief of self-quenching due to the leakage of neither dextran was found at pH 5.1, 0 °C (at this pH, BHA is not stable at 37 °C (8)). Therefore, the pores formed by BHA are between 13 Å, the diameter of calcein, and 26 Å, the diameter of TMRD-3,000 (32, 33).

To characterize the lifetime of these pores, we developed an assay that measured the amount of time for which the interior of the liposomes remained accessible to membrane-impermeant molecules. For this purpose, asymmetric liposomes were produced from liposomes symmetrically labeled with the phospholipid analogues N-ND-BPE and N-Rh-PE, by dithionite reduction of the NBD moiety present on the outer leaflet of the membrane at pH 10, as described under “Experimental Procedures.” Dithionite, a strongly negatively charged molecule at the pH of the experiment, cannot pass membranes and thus reduces only the N-ND-BPE present in the outer leaflet to a non-fluorescent product (34). Accordingly, dithionite reduced the N-ND-BPE fluorescence in lipid bilayers by about 50%. At this point, further additions of dithionite no longer affected the fluorescence, indicating the reduction of all of the outer leaflet N-ND-BPE. The liposomes were then purified from the dithionite and incubated with BHA at pH 5.1, 0 °C, after which the pH was adjusted to 9.0 and dithionite added. Entry of dithionite into the liposomes via the HA-induced pores resulted in a decrease in fluorescence due to dithionite reduction of the inner leaflet N-ND-BPE (Fig. 1A). Reduction of N-ND-BPE fluorescence could also result from transmembrane movement of N-ND-BPE, but neither HA-induced membrane fusion (15) nor HA rosette-induced pore formation (see above) gave rise to such transmembrane movement, as tested with phospholipase D from Streptomyces species. This enzyme is a membrane-impermeant molecule that efficiently removes the fluorescent head group of N-ND-BPE from the
lipid, at 37 °C; the head group is not fluorescent in an aqueous environment (15). Since the enzyme is not active at 0 °C, we could not directly test this for BHA. Furthermore, the effect of dithionite reduction of N-NBD-PE no longer increased at times beyond 1 h following the addition of BHA (Fig. 1B, see below), arguing against the possibility that dithionite reduced fluorescent lipid translocated to the outside leaflet of the liposomes by BHA, rather than acting on N-NBD-PE of the inside leaflet. No reduction of fluorescence was seen when BHA and liposomes were incubated at pH 7.4 before addition of dithionite at pH 5.1. If inactivated BHA was added to liposomes at pH 5.1 (Fig. 1A).

The pores formed by BHA appeared remarkably stable; if dithionite was added up to 15 min after increasing the pH to 9.0, it still entered the liposomes (not shown). The accessibility of the liposomes as a function of time after addition of BHA was quantitated by measuring the initial rate of the N-NBD-PE reduction. Efficient dithionite entry could still be detected 2 h after the addition of BHA to liposomes (Fig. 1, A and B); in fact, the initial rate of N-NBD-PE reduction was maximal an hour after addition of BHA (Fig. 1B). It is not likely that at 1 h after addition, BHA present in the solution still forms new pores in the membranes, given that BHA which is not liposome-associated becomes inactivated with a half-time of 10 min (8). Moreover, fewer new pores were formed at 60 min after addition of BHA, although at this point, calcein-induced leakage from liposomes was less than 60% (8). Because BHA-induced calcein leakage is an all or none process (8), 40% of the calcein-filled liposomes were therefore still available after 1 h. Therefore, membrane-bound BHA is also much less active at this point, and yet 2 h after incubation, dithionite still reduced N-NBD-PE. Together, the above data indicate that the small pores formed by BHA either remain open a long time or, alternatively, they continue to open and close.

Viral HA Initially Forms Small Pores and the Onset of Fusion Coincides with the Formation of Larger Pores—Shangguan et al. (7) have already demonstrated the leakage of fluorescent dextrans across a liposomal membrane during fusion with influenza virus (strain A/PR/8/34, H1N1 serotype) at pH 5.1, 37 °C, and we confirm their results for the X-31 strain (H3N2 serotype, Fig. 2A). At this temperature, fusion is preceded by a short (1–2-s) lag phase, which cannot be resolved by stirrer mixing techniques (35). At 0 °C, however, fusion is slowed down and preceded by a lag phase of several minutes following the low pH-induced conformational change (35) (Fig. 2B). This at time
point large pores were formed at 0 °C, the leakage of TMRD-3,000 (Fig. 2B) or TMRD-10,000 (not shown) across the liposomal membrane was determined. An increase in fluorescence was observed using either large probe after a lag phase of about 10 min. If fusion of virus with N-NBD-PE and N-Rh-PE containing liposomes was measured under the same circumstances, lipid mixing started after a lag of about 10 min also (Fig. 2B). Therefore, kinetically, the dequequenching of large molecules coincided precisely with the onset of fusion as measured by lipid mixing.

The increase in fluorescence observed for the dextrans could be due either to transfer of dextrans from the liposomal to the viral interior through fusion pores during fusion or to leakage. To determine the amount of leakage versus fusion (Fig. 3), virus was mixed with TMRD-10,000 containing liposomes at a 3:1 virus to liposome ratio at pH 5.1, 37 °C. In a parallel experiment, fusion of virus with liposomes containing N-NBD-PE and N-Rh-PE containing liposomes was measured under the same conditions. Under these circumstances, the increase in fluorescence using TMRD-10,000 containing liposomes was approximately 80%, and 92% of the liposomes fused with the virus as measured by lipid mixing. The lipid mixing-based data somewhat overestimate fusion, as 100% fusion was defined as the calculated fluorescence increase for complete mixing of the viral and liposomal phospholipids; since viruses also contain cholesterol and integral membrane proteins, the actual dilution of the probes upon fusion is slightly higher. We then separated free TMRD-10,000 from dextran entrapped in liposomes and fusion products by gel filtration on a Sephadex G-200 column (Fig. 3). 43% of the dextran was found to elute with the void volume of the column, whereas 53% was in the second peak, indicating that it had leaked from the liposomes. Upon addition of a detergent to these fractions, it was found that the dextran included in the void volume was partially quenched, retaining two-thirds of the original quenching, and thus present in membranes (Fig. 3). Given that 92% of the liposomes participated in fusion, about 40% of the void volume fluorescence was thus from tetramethylrhodamine dextran present in fusion products, and 3% was present in unfused liposomes. Therefore, the signal observed in Fig. 2B is due to transfer of dextrans from liposomes to virus by fusion as well as from leakage. By using calcein as a probe, we did not find residual quenched calcein in fusion products, indicating that small pores permeate the membranes of fusion products. More extensive dextran leakage was reported by Shangguan et al. (7) for the A/PR/8/34 strain.

Leakage could occur across the liposomal membrane or through pre-existing defects in the viral membrane, allowing the dextrans to leak across this membrane after virus-liposome fusion. However, the viral membranes used did not appear to be damaged, as precisely the same kinetics and extent of leakage were observed using freshly prepared virus that had never been frozen, thawed, or pelleted (not shown). Furthermore, although some spontaneous leakage of dextrans entrapped in reconstituted viral membranes was observed at pH 5.1, the kinetics of this leakage were different (see below). Taken together, these data indicate that dextrans start to flow through pores at the onset of membrane fusion; these pores appear to be of two types, “fusion” pores between the viral membrane and the liposomal membrane, and “leakage” pores formed by HA in the liposomal membrane.

HA Rosettes Give Rise to Large Pores in Liposomal Membranes in the Absence of Fusion—In contrast to viral HA, BHA does not contain the membrane anchor, and the local concentration of BHA on the liposomal membrane is much lower than that of HA presented by a viral membrane. To determine if these factors are important for large pore formation, we solubilized viral membranes with octyl glucoside, and we purified HA by lectin affinity chromatography as described under “Experimental Procedures.” The octyl glucoside was then removed by dialysis. The aggregation of the hydrophobic transmembrane domain of HA during dialysis leads to the formation of “HA rosettes,” multimers of 6–8 HA trimers (36) with the membrane anchors at the core of the complex and HA1 on the outside. In contrast to BHA, the rosettes are stable at 37 °C. Rosettes do not induce fusion (9). Leakage of both calcein and dextrans across liposomal membranes was found to occur at 37 and 0 °C (Fig. 4, A and B). HA rosettes induced complete leakage of calcein at 37 °C (Fig. 4A, curve a) after a short lag, whereas leakage of TMRD-3,000 and -10,000 was observed after a longer lag (Fig. 4A, curves b and c). The final level of leakage decreased with increasing probe size. At 0 °C, essentially complete leakage of calcein and somewhat less of TMRD-3,000 occurred after a lag of about 10 min, whereas leakage of TMRD-10,000 started after a slightly longer lag and was much less extensive (Fig. 4B). In conclusion, with multimeric, membrane anchor-containing preparations of HA, we do see large pore formation.

Large Fusion Pores Form in the Viral Membrane during Fusion—The above data indicate that the formation of large pores in the liposomal membrane mediated by HA was in itself independent of the presence of a viral membrane. However, leakage pore formation coincided kinetically with what appeared to be fusion pore formation, allowing the passage of dextrans from the liposomal to the viral interior (Fig. 3), suggesting that large leakage pores and fusion pores are related structures. However, in contrast to leakage pores, fusion pores would necessarily also permeate the viral membrane. To determine whether large pores would also form in the viral membrane during fusion, viral membranes were solubilized with C12E6 (octaethylene glycol dodecyl ether), purified, and reconstituted in the presence of TMRD-10,000 by detergent removal as described under “Experimental Procedures.” If these reconstituted viral membranes (“virosomes”) were incubated in the presence of target membranes at pH 5.1, 0 °C (Fig. 5, curve a), leakage of dextrans across the viral membrane was observed after a lag phase of about 10 min, or about the same lag that was found to precede fusion between virosomes and liposomes as measured by lipid mixing under the same conditions (Fig. 5, a).
In this paper, we show that a variety of HA preparations (BHA, HA rosettes, and virus) all induce pores of less than 26 Å in diameter in liposomal membranes at low pH. These pores are long lasting and begin to open shortly after the low pH-induced conformational change in HA. HA rosettes and virus also induced the formation of much larger pores (which we call leakage pores here to distinguish them clearly from all other pores), allowing the leakage of fluorescent dextrans across the liposomal membrane, at times well after the conformational change. For virus and reconstituted viral membranes, the opening of these large leakage pores was simultaneous with the onset of membrane fusion as measured by lipid mixing (Figs. 2B and 5), and with the formation of fusion pores aqueous connections allowing the passage of dextrans from the liposomal to the viral interior (Fig. 2B) and vice versa (Fig. 5). Since influenza-induced membrane fusion is characterized by a very typical sigmoidal time course, the onset of fusion being preceded by a lag phase of several minutes at 0 °C, and since molecules of the same size pass through fusion and leakage pores at the same time, these observations suggest that fusion pores and leakage pores are related structures.

Formulation of the target membrane half of the structure was independent of the presence of a viral membrane, but leakage pores were not formed in viral membranes at low pH in the absence of target membranes. As neutralization of virus-liposome complexes during the lag phase before the onset of fusion arrested lipid mixing (35), but not leakage pore formation (Fig. 6), it is clear that formation of leakage pores alone does not suffice to induce fusion. Therefore, these data suggest that fusion pores may start out as leakage pores in the target membrane, with the viral membrane still separating the viral and liposomal interior (Fig. 7).

**Formation of Small and Large Leakage Pores**—The small pores that are formed shortly after the conformational change...
are clearly the result of insertion of the fusion peptide into the target membrane, perturbing the bilayer; with virus >50% of the calcein leaks out before fusion even starts (Fig. 2B). The structure of this pore is not clear. Given that they have a defined size and prolonged lifetimes, it is difficult to imagine that the pores are lipid defects, given the flexibility and fast diffusion of lipids. Also, as we have argued before, these are not classical “barrel stave” pores (8). Other types of peptide-induced pores have a lifetime of milliseconds (37), or open and close continuously for a long time (38). Although our measurements do not allow us to distinguish between pores that stay open or those that close and open for a long time, and flickering fusion pores induced by influenza hemagglutinin have been described (39), the small pores that we see are not fusion pores. Therefore, the small pores that we observe must be of a novel type, unlike a lipid defect but also different from the known pores induced by peptides.

With virus, the development of small pores was followed by that of large (leakage and fusion) pores (small and large pores are clearly resolved at 0 °C, Fig. 2B). Pore enlargement does not lead to lysis of the membranes, as witnessed by the intact resonance energy transfer between lipidic probes and the quenching of dextrans in fusion products (Fig. 3). The increase in fluorescence was more limited with dextrans than with calcein. Whereas some of this difference may be due to dextran remaining quenched in fusion products (Fig. 3), the data obtained with rosettes seem to suggest that large pores developed in fewer liposomes than small pores (Fig. 4). Rosettes do not induce fusion pores and give rise to slower and less extensive leakage of TMRD-10,000 than TMRD-3,000 leakage. TMRD-3,000 leakage is again slower and less extensive than calcein leakage (Fig. 4). Given a random distribution of HA trimers over the liposomes, fewer liposomes will have a large number of bound HAs than a lower amount of bound HA. Therefore, these data suggest that more HA trimers were required to form a pore allowing the passage of TMRD-10,000 than a pore allowing the passage of TMRD-3,000, and we know that 1 trimer suffices to allow the passage of calcein (8). With virus, multiple trimers are presented to the target membrane together (on the viral membrane), so the difference between calcein and dextrans is much smaller. Thus, most likely, pores increase in size gradually by recruiting additional trimers. Such behavior has been described for HA-induced fusion pore formation, and large pores are thought to be formed following complex formation by multiple HA trimers (17, 18, 40, 41), again suggesting that fusion and leakage pores are related structures. A new element contributed by our observations is that since large leakage pores would be formed in a similar way, the target membrane half of the fusion pore may initially be a leakage pore (Fig. 7c).

BHA does not give rise to large pore formation. These data appear to suggest that the transmembrane anchor of HA, lacking in BHA, plays a role in this process. Cells expressing HA mutants that are anchored by a phosphatidylinositol (GPI-HA) do not induce fusion as measured by the transfer of aqueous dyes from one cell to the other (14), but the mutants do induce lipid mixing of outer membrane leaflets (hemifusion). Therefore, the anchor probably does not have a structural role in organizing the HA complex giving rise to large pore formation. We suggest that it may instead be the higher local concentration of HA in rosettes, viral membranes, or cellular membranes containing GPI-anchored HA that facilitates large pore formation. Under the circumstances of the experiments shown in this paper, we have found that 25 BHA trimers bind per liposome (results not shown). At pH 5.1, 0 °C, 5% of the added BHA is active in pore formation (8). Of course, this interpretation implies that GPI-HA should induce large pore formation at low pH; we have tried this experiment but find that, in our hands, not only GPI-HA but also wild type-HA, at the densities at which they are expressed on the surface of cells, induce no significant fusion or hemifusion with liposomes.

**Proteinaceous Versus Lipidic Fusion Pores**—Our data would be quite compatible with the numerous models proposing that fusion is the result of complex formation by multiple HA molecules in a ring-like structure (35, 40, 42–44), assuming that the small pores correspond to the action of individual HA trimers before complex formation (Fig. 7a) and that the large pores are caused by the complex. Two completely different pathways have been proposed to lead from HA complex formation to fusion as follows: complexes could either be proteinaceous fusion pores, whose lateral expansion would break the ring and lead to lipid merger (reviewed in (17)), or they could induce the formation of a lipidic connection between the two membranes, which would then break through to form a fusion pore lined by lipids (11, 18, 35).

All the data reported in this paper are compatible with a proteinaceous nature of the fusion pore. Evidence for proteinaceous nature of the fusion pore...
aceous pores was provided by experiments showing that an aqueous connection forms between cells expressing HA and other cells at low pH, before lipid merger can be detected (16, 18, 41, 45). However, strong experimental evidence for the formation of lipidic intermediates in hemagglutinin-mediated fusion is also available (14, 15, 18, 46). This evidence is mostly interpreted in terms of one of the versions of the stalk theory for fusion (11, 47). In theory, an unspecified initial membrane perturbation at the site of fusion first leads to fusion of the outer leaflets of the two membranes. In one version, lateral expansion of this stalk connecting the membranes gives rise to a special bilayer, the “hemifusion diaphragm,” and breakthrough of this diaphragm would finally lead to fusion of the inner leaflets also (48). No leakage would be expected to occur at any stage during fusion involving stalk intermediates.

The more recent observations of Chernomordik et al. (18) appeared to reconcile the proteinaceous and the lipidic fusion pathways. Following an earlier suggestion (14), they showed that limited outer leaflet fusion occurs before fusion pore formation but that the lipids cannot spread beyond the area of this initial defect, because of steric constraints imposed by the presence of the complex of HA molecules. Expansion of the complex would allow lipid mixing. These data are compatible with both the observed aqueous fusion pore opening before the onset of extensive lipid mixing and the existence of lipidic intermediates (16, 18, 41, 45).

We think that there is sufficient evidence for the involvement of lipidic intermediates, involving a hemifusion diaphragm, in fusion, but neither the stalk theory nor this recent model sufficiently take into account the leakage of large molecules during fusion as observed by us and others (7). We therefore propose a different model for fusion (Fig. 7). Initially, small stable pores resulting from insertion of the fusion peptide form anywhere in the target membrane (Fig. 7a). HA complex formation, involving a pH-independent, viral membrane-independent interaction between HA trimers, then leads to the formation of larger leakage pores in the membrane (Fig. 7b). This process takes several minutes at low temperature. With HA present in the horizontal position as shown in Fig. 7, consistent with recent models of fusogenic HA structure and alternative models (35, 49), as well as indications that HA may tilt with respect to the membrane normal during fusion (50, 51), viral fusion intermediates (16, 18, 41, 45).

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We think that there is sufficient evidence for the involvement of lipidic intermediates, involving a hemifusion diaphragm, in fusion, but neither the stalk theory nor this recent model sufficiently take into account the leakage of large molecules during fusion as observed by us and others (7). We therefore propose a different model for fusion (Fig. 7). Initially, small stable pores resulting from insertion of the fusion peptide form anywhere in the target membrane (Fig. 7a). HA complex formation, involving a pH-independent, viral membrane-independent interaction between HA trimers, then leads to the formation of larger leakage pores in the membrane (Fig. 7b). This process takes several minutes at low temperature. With HA present in the horizontal position as shown in Fig. 7, consistent with recent models of fusogenic HA structure and alternative models (35, 49), as well as indications that HA may tilt with respect to the membrane normal during fusion (50, 51), viral fusion intermediates (16, 18, 41, 45).
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Hemagglutinin-mediated Membrane Fusion: A NEW MODEL FOR FUSION
Pierre Bonnafous and Toon Stegmann

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