The HA2 Subunit of Influenza Hemagglutinin Inserts into the Target Membrane Prior to Fusion*

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The interaction between influenza virus and target membrane lipids during membrane fusion was studied with hydrophobic photoactivatable probes. Two probes, the newly synthesized bisphospholipid diphosphatidylethanolamine trfluoromethyl diazirine and the phospholipid analogue 1-palmitoyl-2(11-[4-[3-(trifluoromethyl)diazirinyl]phenyl]-[2-3H]-undecanoyl)-sn-glycero-3-phosphocholine (Harter, C., Bächli, T., Semenza, G., and Brunner, J. (1988) Biochemistry 27, 1856-1864), were used. Both labeled the HA2 subunit of the virus at low pH. By measuring virus-liposome interactions at 0 °C, it could be demonstrated that HA2 was inserted into the target membrane prior to fusion. As we have recently demonstrated, at this temperature, exposure of the fusion peptide of HA2 takes place within 15 s after acidification, but fusion does not start for 4 min (Stegmann, T., White, J. M., and Helenius, A. (1990) EMBO J. 9, 4231-4241). HA2 was labeled at least 2 min before fusion. No labeling of the HA1 subunit was seen. These data indicate that fusion is triggered by a direct interaction of the HA2 subunit of a kinetic intermediate form of HA with the lipids of the target membrane. Most likely, it is the fusion peptide of HA2 that is inserted into the target membrane. Just before fusion, HA is thus an integral membrane protein in both membranes. In contrast, the bromelain-derived ectodomain of HA was labeled by 1-palmitoyl-2(11-[4-[3-(trifluoromethyl)diazirinyl]phenyl]-[2-3H]-undecanoyl)-sn-glycero-3-phosphocholine at low pH but not by diphosphatidylethanolamine trfluoromethyl [3H]phenyl diazirine. This indicates that insertion of the fusion peptide of the bromelain-derived ectodomain of HA into a membrane differs from that of viral HA during fusion.

Membrane fusion plays a pivotal role in the entry of enveloped viruses into their host cells. Fusion is mediated by viral envelope glycoproteins (For recent reviews see Marsh and Helenius, 1989; Stegmann et al., 1989a; Wilschut and Hoekstra, 1990; White, 1990). Of these, the hemagglutinin of influenza virus (HA)1 is the best-characterized. It is a homotrimeric integral membrane protein. Each monomer consists of two disulfide-bonded polypeptides, HA1 and HA2. As we have have recently shown (Stegmann et al., 1990), the low pH-induced fusion is mediated by HA and is triggered by a transient intermediate conformation of the protein. In this intermediate, the hydrophobic N termini of the HA2 polypeptides, the so-called “fusion peptides,” are exposed, while the trimers remain largely intact. In the x-ray structure of the neutral pH form, these peptides are buried in the interface between the monomers of the trimer (Wilson et al., 1981).

While the models proposed for HA-mediated membrane fusion differ in several respects, it is generally assumed that a direct interaction between HA and the lipids of the target membrane is involved. The most convincing evidence for such an interaction was provided by Brunner and co-workers (Harter et al., 1988, 1989). They incorporated a hydrophobic photoactivatable reagent, PTPC (a diazirine coupled to the acyl chain of a synthetic phospholipid), in the bilayer of liposomes. Since photolysis of the probe results in covalent modification of whatever is present in the membrane, they could demonstrate that the fusion peptides of bromelain-solubilized ectodomains of HA (BHA) insert into the outer leaflet of the liposomal bilayers. Their results suggested, moreover, that the peptides adopted an α-helical configuration (Brunner, 1989). Recently, these results were extended to intact influenza virus fusing with liposomes containing PTPC (Brunner et al., 1991).

We have further investigated the role of HA2 in triggering fusion. As was recently shown, studying fusion at reduced temperatures allows analysis of the component steps in the fusion reaction (Stegmann et al., 1990). In this paper we demonstrate that, at pH 5.1 and 0 °C, HA2 is labeled both by PTPC and by the newly synthesized hydrophobic photoactivatable probe DIPETPD present in liposomal target membrane bilayers at least 2 min before fusion. The implications of these findings for the membrane fusion mechanism are discussed.

1 The abbreviations used are: HA, hemagglutinin of influenza virus; PTPC, 1-palmitoyl-2(11-[4-[3-(trifluoromethyl)diazirinyl]phenyl]-[2-3H]undecanoyl)-sn-glycero-3-phosphocholine; BHA, bromelain-derived ectodomain of HA; DIPETPD, diphosphatidylethanolamine trifluoromethyl [3H]phenyl diazirine; N-Rh-PE, N-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; N-NBD-PE, N-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PC, phosphatidyicholine; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polycrylamide gel electrophoresis.
MATERIALS AND METHODS

**Photoactivatable Reagents**—The synthesis and properties of ²H-labeled DIPETPD will be described in detail elsewhere. ²Synthesis of an analogue of DIPETPD lacking the photoactivatable group was reported by Delfino et al. (1987). The structure of DIPETPD is shown in Fig. 1. Specific radioactivity of the DIPETPD preparation used was 3.5 Ci/mmol. [²H]PTPC (22.2 Ci/mmol) synthesized according to Harter et al. (1988) was the generous gift of Dr. J. Brummer. Both reagents were stored in the dark in toluene/ethanol 1:1 at -20 °C.

Liposomes—N-(Lissamine rhodamine B sulfonyl)-phosphatidyethanolamine (N-Rh-PE) and DND-7-nitro-2,1,3-benzoazadiol-4-yllphosphatidylethanolamine (N-Rh-PE), egg phosphatidylethanolamine (PE), and egg phosphatidylcholine (PC) from Avanti Polar Lipids (Birmingham, AL) were used without further purification. Bovine brain gangliosides (type III), HEPEs, 2,4,6-trinitrobenzenesulfonic acid (TNBS), and Sepharose-coupled Ricinus communis agglutinin were purchased from Sigma, and MES and Tris-HCl were purchased from Fisher, Bromelain and the detergent C12E8 were purchased from Calbiochem, scintillation fluid (Opti-fluor) from Packard (Drowners Grove, IL).

**Virus, BHA, and Reconstituted Viral Membranes**—The X-31 recombinant strain of influenza virus was propagated from plaque-purified virus (plaque C-22; Doms et al., 1986) in the alveolar cavity of embryonated eggs, purified, handled, and stored as described before (Steigmann et al., 1985). To measure the concentration of virus, viral phospholipid was extracted according to Folch et al. (1957) and the phosphate fraction of the lipids was determined according to Bottcher et al. (1961).

BHA was produced by digestion of virus (1 mg/ml) with bromelain (10 mg/ml) in the presence of β-mercaptoethanol (50 mM) for 24 h at 37 °C (Brand and Skehel, 1972). The BHA was then purified by affinity chromatography on Sepharose-immobilized R. communis agglutinin. Reconstituted viral membranes were produced essentially as in Steigmann et al. (1987), except that photoactivatable probes, dissolved in C12E8, were added directly to the solubilized membranes in C12E8. The procedure was carried out under conditions of low illumination.

**Liposomes**—Large unilamellar liposomes were prepared by repeated low pressure extrusion of multilamellar liposomes (Mayer et al., 1986) through defined pore polycarbonate filters of decreasing pore size (0.4, 0.2, and 0.1 μm in diameter). An extruder with a 10-ml barrel from Lipex Biomembranes (Vancouver, Canada) was used. Multilamellar liposomes were produced by resuspending thin films of lipids, prepared by exhaustive evaporation of solvent (toluene/ethanol 1:1) in vacuo, into aqueous buffers. The suspensions were then frozen and thawed three to five times before extrusion. After extrusion, residual multilamellar liposomes were removed by pelleting at 16,000 × g for 20 min. For liposomes containing DIPETPD, the temperature during resuspension of the lipids and the subsequent extrusion was kept at 60 °C. Liposomes were used within 2 days of their production.

DIPETPD-containing liposomes were stored at room temperature; kept at 60 °C. Liposomes were used within 2 days of their production.

**Fusion Experiments**—To measure fusion by a resonance energy transfer based assay, liposomes were produced containing 0.6 mol% each of N-NBD-PE and N-Rh-PE (Struck et al., 1981). Measurements were performed under continuous stirring, in a thermostatted cuvette holder containing 2 ml of 135 mM NaCl, 15 mM sodium citrate, 10 mM MES, 5 mM HEPEs set to various pH values by HCl or NaOH. The increase in N-NBD-PE fluorescence emission, resulting from dilution of the fluorophores into the viral membrane upon fusion, was recorded continuously at excitation and emission wavelengths of 465 and 530 nm, respectively. A 515-nm long-pass filter was placed between cuvette and emission monochromator (Steigmann et al., 1985). An SLM-8000 fluorometer was used for all measurements. The fluorescence scale was calibrated by setting the initial fluorescence of the liposomes to zero and the fluorescence at infinite probe dilution to 100%.

**RESULTS**

**Properties of Liposomes Containing PTPC or DIPETPD**—Large unilamellar liposomes were used as target membranes for fusion. They were composed of natural zwitterionic phospholipids (PC and PE) with added gangliosides. The gangliosides served as receptors for the viral HA, allowing virus-liposome complexes to form at neutral pH (Steigmann et al., 1986, 1988). To produce liposomes containing PTPC or DIPETPD, trace amounts of the lipid were added to stock solutions in toluene/ethanol (1:1) to PC/PE/ganglioside mixtures in the same solvent. After evaporation of solvents, multilamellar liposomes were produced from the dry lipid films by resuspension in an aqueous buffer. They were made unilamellar by repeated extrusion through polycarbonate filters (see "Materials and Methods"). The whole procedure was performed under conditions of low illumination to avoid photolysis of the probes. For DIPETPD-containing liposomes the temperature during resuspension of the lipids and extrusion was maintained at 60 °C. Negative stain electron microscopy (not shown) confirmed that unilamellar liposomes with a diameter of about 0.1 μm were formed. We found that up to 20% by weight of a nonradioactive analogue of DIPETPD could be incorporated into liposomal bilayers without affecting stability or tightness.

As a bisphospholipid, DIPETPD (Fig. 1) could potentially span the membrane of the liposomes from the outer leaflet of the bilayer to the inner leaflet and thus confine the photoactivatable group to the center of the bilayer. Alternatively, it could be present in a U-shaped configuration either in the outer or the inner leaflet of the bilayer with both headgroups present on one side of the bilayer. To determine the fraction...
of DIPETPD in these different configurations the membrane impermeant reactant TNBS was used, which reacts with the ethanolamine headgroup of the probe. When present in a liposome, transmembrane conformers will be labeled only on one end, the external-facing U-shaped molecules will be labeled on both ends, and the interior-facing ones will not be labeled. Liposomes were reacted with TNBS at pH 8.0 and the formation of a reaction product was determined spectrophotometrically. After a level was reached, the remaining unreacted TNBS was quenched with lysine, and lipids were extracted and separated by thin-layer chromatography. It was found that 50% of the probe was in a membrane-spanning configuration, and that 25% resided in each of the monolayers of the bilayer in a U-shape. The method and the results will be described in detail elsewhere.2

Next, we investigated the ability of DIPETPD-containing liposomes to serve as targets for fusion with influenza virus. The fusion assay was based on resonance energy transfer between two fluorescent phospholipid analogues (N-NBD-PE and N-Rh-PE) when they are both present in the target liposomes (Struck et al., 1981). Fusion of a liposomal with a viral membrane resulted in dilution of the fluorescent lipids. This led to decreased energy transfer and an increase in energy donor (N-NBD-PE) fluorescence emission (Stegmann et al., 1986). Trace amounts of the fluorescent probes (0.6 mol% each) and DIPETPD were incorporated into liposomes. Virus was injected into a thermostatted fluorometer cuvette containing the liposomes in buffers at different pH and the change in fluorescence recorded.

A low-pH-dependent increase in fluorescence was observed both at 37 and at 0 °C (Fig. 2, A and B). To confirm that this was caused by fusion and not by other modes of lipid transfer between membranes, virus alone was incubated at pH 5.1, 37 °C and then added to liposomes at pH 5.1. We have previously shown that after such treatment the virus will still bind to the ganglioside-containing liposomes but is unable to fuse (Stegmann et al., 1986). The lack of fluorescence increase in this control and the lack of an increase at neutral pH (Fig. 2A) indicated that the increase in fluorescence measured at pH 5.1 represents bona fide membrane fusion. At 37 °C, fusion started immediately after addition of virus to liposomes, leveling off in about 2 min at a fluorescence increase of 20%. At 0 °C, fusion was preceded by a lag phase of about 4 min, after which the fluorescence increase slowed, reaching a level of about 25% after 2 h. The results showed that the characteristics of fusion of influenza virus with liposomes containing DIPETPD at either temperature were essentially identical to those with liposomes lacking the probes (Stegmann et al., 1990).

The HA2 Subunit of Intact Virus Is Labeled by Both Probes—Next, we investigated whether the two probes could be used to detect fusion of influenza virus strain X-31 with liposomes. Virus was incubated with liposomes at pH 5.1, 37 °C. Immediately after lowering the pH, samples were photolyzed. Fusion and photolysis were allowed to continue for 15 min, after which the samples were neutralized. In control experiments, samples were either kept at neutral pH during photolysis or not photolyzed during fusion at low pH. To determine whether covalent association of DIPETPD and PTPC with protein had taken place, samples were extensively delipidized by repeated chloroform/methanol precipitation of the protein before electrophoresis.

As shown in Fig. 3, HA2 was labeled by both probes at low pH. Since HA2 runs at the same position as does the viral matrix (M) protein, nonreducing gels were run to substantiate the identification of the labeled protein as HA2. No labeling was found in M (not shown). A second band, migrating just below the viral nucleoprotein, was labeled to some extent (1-4% of that found in HA2). Although invisible in the Coomassie-stained gel, this band could perhaps represent the viral neuraminidase, whose membrane anchor is present in the membrane of the fusion product. For either probe, the total amount of protein-associated label was around 0.03% of the input radioactivity (as determined by immunoprecipitation of the viral proteins with a polyclonal antibody against influenza virus). With PTPC, no labeling was seen at neutral pH, or in

**FIG. 2. Fusion of influenza virus with liposomes containing DIPETPD.** In A: a, pH 5.1, 37 °C; b, pH 5.1, 37 °C after a preincubation of the virus alone at pH 5, 37 °C for 2 min; c, pH 7.4, 37 °C. In B: pH 5.1, 0 °C. Liposome to virus ratio was 1:1.5 (12.5 μM of membrane phospholipid phosphorous products total). Liposomes consisted of egg PC:egg PE:bovine brain gangliosides:DIPETPD (12:6:2:1), N-NBD-PE, and N-Rh-PE (0.6 mol% each). Fluorescence was measured as described under "Materials and Methods."
nonphotolyzed controls after fusion. In similar controls with DIPETPD, a small amount (about 3% of the protein-associated radiolabel) was found associated with HA2. These data indicate that both probes were able to label HA2 during or after fusion at 37 °C.

The Membrane Anchor of HA2 Can Be Labeled by PTPC and DIPETPD—The radioactivity found associated with HA2 at low pH could arise from labeling of the fusion peptide, the membrane anchor of HA2, or both. To investigate whether the probes used in the current study could label the membrane anchor of HA2, influenza virus membranes were solubilized with the detergent C12E8 and added to PTPC or DIPETPD in an aqueous buffer containing C12E8, after which the membranes were reconstituted according to Stegmann et al. (1987). After photolysis, 90–98% of the label was found associated with HA2 (Fig. 4), and the rest in HA1. No radioactivity was detected in the region of the gel where the neuraminidase massie-stained M protein, which runs as a very narrow band at about the same position as HA2, acts as a filter during autoradiography for these very low quantities of radioactivity. HA did not appear as a doublet in nonreducing gels, and no labeling of M was found on those gels (not shown). Traces of radioactivity were found associated with HA2 at pH 7.4. Extensive delipidization by repeated chloroform/methanol precipitation of the samples did not remove this radioactivity (not shown). No labeling was seen in samples that were fused but not photolyzed. Other faintly visible labeled bands were similar to those seen after fusion at 37 °C (Fig. 3). The total protein-associated radioactivity found after 1 h of fusion was about 0.01% of the added radioactivity.

With DIPETPD, some labeling of HA2 was seen even at pH 7.4 or in the nonphotolyzed control (Fig. 5B). Extensive delipidization by repeated chloroform/methanol precipitation of the protein removed about 95% of the input radioactivity, but did not lower the labeling in these controls appreciably (not shown). The radioactivity found associated with HA2

![Fig. 4. Autoradiograph of products formed from reconstituted viral membranes containing PTPC or DIPETPD.](image)

![Fig. 5. HA2 was labeled before fusion at 0 °C. Autoradiograph of products formed after incubation of liposomes containing PTPC or DIPETPD with virus at 0 °C. A, PTPC; B, DIPETPD.](image)
bands were similar to those seen at 37 °C. The total protein-obtained during the 2-min interval following 1 h was subtracted. Intensities are expressed relative to the labeling was determined and integrated over the surface area of the band. The amount of radioactivity in the controls. After the onset of fusion, HA2-associated radioactivity increased. Other faintly visible bands were similar to those seen at 37 °C. The total protein- obtained during the 2-min interval following 1 h was subtracted. Intensities are expressed relative to the labeling was determined and integrated over the surface area of the band. The amount of radioactivity in the controls. After the onset of fusion, HA2-associated radioactivity increased. Other faintly visible bands were similar to those seen at 37 °C. The total protein- obtained during the 2-min interval following 1 h was subtracted. Intensities are expressed relative to the labeling was determined and integrated over the surface area of the band. The amount of radioactivity in the controls. After the onset of fusion, HA2-associated radioactivity increased. Other faintly visible bands were similar to those seen at 37 °C. The total protein-

Importantly, only a slight increase in labeling was found during the lag phase.

Taken together, these results indicate that HA2 entered the hydrophobic interior of the target membrane before fusion took place. No significant additional insertion occurred during the later part of the lag phase. HA1 did not come into contact with the hydrophobic interior of the bilayer during fusion.

BHA2 Is Labeled by Interaction with PTPC— but Not DIPETPD-containing Liposomes at Low pH—To determine whether the newly synthesized probe DIPETPD incorporated into liposomal membranes was able to label the fusion peptide of HA at low pH as does PTPC, the experiments of Brunner and coworkers (Harter et al., 1988) with the bromelain-solubilized ectodomain of HA (BHA) were repeated with DIPETPD. PTPC-containing liposomes were used as a control. BHA was incubated with liposomes at pH 5.1, 37 °C. Immediately after lowering the pH, samples were photolyzed for 15 min and then neutralized and prepared for SDS-PAGE. Control experiments were carried out at neutral pH. The results are shown in Fig. 6. As expected, BHA2 was labeled by PTPC at low pH. Radioactivity was also found associated with a band migrating at the position of HA1 at low pH (approximately 30% of BHA2 labeling by densitometry). It has been reported by Harter et al. (1989) that at least part of this band is not HA1, but a dimer of BHA2 which comigrates with HA1 under these conditions. Only a small amount of labeling was seen at neutral pH (Fig. 6) and no labeling was found in nonphotolyzed controls (not shown). By comparison, Harter et al. (1988), working with the BHA of strain A/PR/8/34 found less labeling at neutral pH but a somewhat higher incorporation of label into BHA2 relative to HA1 at low pH. Strain differences or a difference in protein/lipid ratio (Harter et al., 1988) might account for these discrepancies.

With DIPETPD, very little labeling was observed at low or neutral pH (Fig. 6). Our interpretation of these results is as follows. The results of Brunner (1989) indicate that the fusion peptides of BHA are inserted as shallow α-helices into the outer leaflet of the liposomal bilayer. They are accessible to the photoactivatable group of PTPC, which probably requires transient movement of the diazirine toward the headgroup or transient dipping of the BHA helix further into the bilayer. The lack of reactivity with DIPETPD may suggest that the photoactivatable group of this probe, whether present in a transmembrane or a U-shaped configuration, is deeper within the bilayer and therefore not within reach of the inserted fusion peptide of BHA2. Even in the U-shaped configuration, the diazirine on DIPETPD is likely to be much more restricted near the middle of the bilayer than that on PTPC, because it is covalently attached to two fatty acid chains (cf. Fig. 1). Therefore, in all likelihood, DIPETPD does not label the fusion peptide of BHA because of its more defined location as compared to PTPC.

**DISCUSSION**

**HA2 Is Inserted into the Target Membrane Bilayer before Fusion**—In this study, it was demonstrated with two different photoactivatable probes that the HA2 subunit of influenza virus, strain X-31, inserts into a liposomal bilayer prior to fusion of the virus with liposomal membranes. We were able to measure this because at pH 5.1, 0 °C fusion is preceded by a 4-min lag phase (cf. Fig. 2B; Steggmann et al., 1990). As reported previously, the fusion peptide is already exposed within 15 s after acidification at this temperature and pH, and concomitantly virus interacts hydrophobically with target membranes. Harter et al. (1988; 1989) have demonstrated that, with the bromelain-solubilized ectodomain of HA (BHA), PTPC labels only the fusion peptide of the BHA2 subunit at low pH. In the intact virus at 0 °C, the fusion peptide of HA2 is the only hydrophobic moiety known to be exposed early in the lag phase by the limited conformational change taking Place at low pH (Stegmann et al., 1990). Therefore, it is most likely that the observed labeling of HA2 during the lag phase (Fig. 5, A and B) is in the fusion peptide of HA2. A more direct way of identifying the fusion peptide would have been to sequence peptides of HA2, but we were unable to do so because of the low level of radioactive labeling obtained.

BHA was found to interact somewhat differently with target

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**TABLE I**

**Quantitation of the label associated with HA2 before and during fusion at 0 °C.**

| Interval | Radioactivity |
|----------|---------------|
|          | DIPETPD | PTPC |
| min      |         |      |
| 0–2      | 14      | 27   |
| 2–4      | 17      | 30   |
| 4–6      | 36      | 36   |
| 15–17    | 60      | 70   |
| 60–62    | 100     | 100  |

**FIG. 6. Labeling of BHA by PTPC but not by DIPETPD.** Autoradiograph of SDS-PAGE separation of products obtained after incubation of liposomes containing PTPC or DIPETPD with BHA at 37 °C. Liposomes (50 μM of membrane phospholipid phosphorous) were incubated with BHA (4 μg/ml) in a small volume at neutral pH, 0 °C for 15 min. Aliquots of the mixture were transferred to a large volume of buffer at 37 °C and, at the pH indicated, immediately photolyzed for 15 min. Samples were then neutralized, precipitated by the addition of trichloroacetic acid (10%), washed twice with acetone, dissolved in sample buffer, and subjected to SDS-PAGE using a Tris/tricine buffer system (10% T/3% C gels) (Schägger and von Jagow, 1987). The noncovalently bound lipid (L) ran behind the dye front (Rf approximately 0.8).
membranes than the the HA of intact virus. While labeled by PTPC as reported by Harter et al. (1988) it was not labeled by DIPETPD. The fusion peptide of BHA2 probably inserts into the bilayer as a shallow α-helix parallel to the plane of the membrane (Brunner, 1989). Such an orientation could put it out of reach of the photoactivatable group of DIPETPD which may be more deeply buried in the bilayer than that of PTPC. In contrast, the fusion peptides of viral HA could be inserted in a more perpendicular orientation, explaining their labeling with DIPETPD. Other differences between BHA and the viral HA were also observed. HA1 from BHA but not viral HA1 was labeled at low pH by PTPC. This probably indicates that hydrophobic domains not normally exposed on HA1 are accessible after removal of the membrane anchor and exposure of the fusion peptide. This may in part be due to the aberrant trimer dissociation which is observed with BHA but not with intact HA (Doms and Helenius, 1986). Moreover, both subunits of BHA were also labeled by PTPC to a significant extent at neutral pH, indicating that some hydrophobic moieties are exposed even before the pH is lowered.

In a recent study, Brunner et al. (1991), working with intact virus, strain A/PR/8/34, found weak labeling of HA2 after incubation at pH 5, 0 °C with PTPC-containing liposomes. Although the virus bound to zwitterionic liposomes, fusion was not detected at this temperature. Unfortunately, little is known about the kinetics of fusion and conformational change for this strain. It is possible that strain differences resulted in the different threshold temperature for fusion. Importantly, significant labeling was found during the first part (10–15 s) of a biphasic labeling pattern, probably indicative of a lag phase, at 23 °C (Brunner et al., 1991).

Properties of DIPETPD—The results presented in this study demonstrate the usefulness of the newly synthesized hydrophobic photoactivatable reagent DIPETPD (Fig. 1). The molecule was designed to provide enhanced geometric resolution compared to existing hydrophobic photoactivatable reagents such as the phospholipid-like PTPC. In our hands, 50% of DIPETPD was present in a membrane-spanning configuration, which would most likely restrict the photoactivatable diazirine of DIPETPD to the middle of the membrane bilayer. However, this remains to be demonstrated directly. Despite their differences in structure, PTPC and DIPETPD labeled HA with similar efficiency.

Implicit in our interpretation of the results is the notion that the labeling seen with DIPETPD at 0 °C in the absence of photolysis or at neutral pH, can be subtracted from the labeling obtained at low pH. Labeling at neutral pH could be due to a population of fusion peptides that is already exposed at neutral pH. However, very little labeling was seen at 37 °C or with PTPC, indicating that such a population is probably small. Labeling without photolysis (Fig. 5B) can only occur if the association between protein and lipid is noncovalent or if a side reaction takes place. As the association withstood repeated washing with chloroform/methanol or acetone, boiling in the presence of SDS and separation on a gel it is probably covalent. Therefore, it was assumed that the labeling was due to a nonspecific side reaction. Considering the small percentage of specific labeling obtained with these probes, side reactions involving as little as 0.001% of label are readily problematic. We do not know why it is so much more extensive at 0 °C than at 37 °C. The amount depended somewhat on the age of the preparation and on the solvent in which DIPETPD was stored.

The Mechanism of Membrane Fusion—Several models for the role of HA in fusion have been presented. In one model the fusion peptide of HA2 is not inserted into either the viral or the target membrane, but it serves as a hydrophobic surface on the HA molecules which enables the flow of lipids during fusion (White, 1990). Our observation that HA2 was labeled by both probes before any lipid mixing takes place would appear to contradict this. In a second model, the fusion peptide of HA2 is thought to interact with the viral membrane,
or with other fusion peptides (Ruigrok et al., 1988). While we cannot exclude that this occurs for some of the fusion peptides, we found clear evidence for an interaction between HA2 and the target membrane.

We have recently proposed a model for membrane fusion in which there is a limited conformational change in HA at low pH (Stegmann et al., 1990). The model is summarized in Fig. 7. After the conformational change (Fig. 7B), the tops of the trimers remain intact, while exposed fusion peptides insert into the target membrane (Fig. 7C). Considering the distance between the target membrane and the fusion peptide, it was necessary to assume that the proteins are able to bend sideways in order for the peptide insertion to take place, as schematically indicated in Fig. 7C. The present results are consistent with this hypothesis. They indicate that HA2 is an integral membrane protein in both the viral membrane and the target membrane just before fusion.

We hypothesized further that the lag phase which precedes fusion at 0 °C could represent the time needed for multiple spikes to form a fusion complex, for the lipids to be destabilized, or for further insertion of fusion peptides to reach a critical concentration that would trigger fusion (Stegmann et al., 1990). We found that the amount of labeled HA2 did not increase significantly during the lag phase (Table I). This indicates that little additional insertion of HA2 occurred during the later part of the lag phase. Thus, the insertion of HA2 molecules into the target membrane occurs almost immediately upon acidification. The lag phase then presumably represents the time needed to rearrange the bound trimers to assemble to a functional fusion complex (Fig. 7D). The initial association could, for instance, involve attachment of spikes at the focal site of interaction in a random orientation. For the final fusion complex, we have hypothesized the formation of a rosette-like structure involving several HA trimers. After complex formation, merger of the membranes, the final event in membrane fusion (Fig. 7E), takes place.

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