Fusion Activity of Influenza Virus PR8/34 Correlates with a Temperature-Induced Conformational Change within the Hemagglutinin Ectodomain Detected by Photochemical Labeling†

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ABSTRACT: Fusion of influenza viruses with membranes is catalyzed by the viral spike protein hemagglutinin (HA). Under mildly acidic conditions (~pH 5) this protein undergoes a conformational change that triggers the exposure of the "fusion peptide", the hydrophobic N-terminal segment of the HA2 polypeptide chain. Insertion of this segment into the target membrane (or viral membrane?) is likely to represent a key step along the fusion pathway, but the details are far from being clear. The photoreactive phospholipid 1-palmitoyl-2-[11-[4-[3-(trifluoromethyl)diazirinyl]phenyl][2-^{3}H]undecanoyl]-*sn*-glycero-3-phosphocholine ([^{3}H]PTPC/11), inserted into the bilayer of large unilamellar vesicles (LUVs), allowed us to investigate both the interaction of viruses with the vesicles under "prefusion" conditions (pH 5; 0 °C) and the fusion process itself occurring at elevated temperatures (>15–20 °C) only. Despite the observed binding of viruses to LUVs at pH 5 and 0 °C, labeling of HA2 was very weak (<0.002% of the radioactivity originally present). In contrast, fusion could be readily monitored by the covalent labeling of that polypeptide chain. We have studied also the effect of temperature on the acid-induced (pH 5) interaction of bromelain-solubilized HA (BHA) with vesicles. Labeling of the BHA2 polypeptide chain was found to show a remarkable correlation with the temperature dependence of the fusion activity of whole viruses. A temperature-induced structural change appears to be critical for both the interaction of BHA with membranes and the expression of fusion activity of intact viruses.

Influenza viruses enter cells by receptor-mediated endocytosis followed by fusion of the viral envelope with the membrane of the endosome (Matlin et al., 1981; Bergelson et al., 1982; White et al., 1983; Yoshimura & Ohnishi, 1984; Stegmann et al., 1989a). The factor responsible for fusion is hemagglutinin (HA),[1] the major spike protein of the viral membrane. HA is a homotrimer, each subunit of which consists of two polypeptide chains, HA1 and HA2, linked together through a single disulfide bond (Wiley et al., 1977; Lazarowitz & Choppin, 1975). The membrane fusion activity of HA is triggered by an acid-induced irreversible conformational change (Skehel et al., 1982), whereby the hydrophobic N-terminal region of the HA2 subunit, the "fusion peptide", is exposed. The high degree of sequence conservation among fusion peptides of hemagglutinins originating from viruses of different subtypes and strains (Skehel & Waterfield, 1975; Skehel et al., 1982; White et al., 1983) and the fact that site-specific mutations within this region abolish, or severely affect, the fusion activity of hemagglutinin (Gething et al., 1986) suggest a crucial functional role for this peptide.

Many aspects of the acid-induced conformational change of hemagglutinin have been studied with BHA, the bromelain-solubilized ectodomain of HA. BHA undergoes a similar conformational change around pH 5, whereby it acquires amphiphilic properties as exhibited by its ability to bind to liposomes and nonionic detergents (Skehel et al., 1982; Doms et al., 1985). In order to characterize in more detail the interaction of BHA with liposomes, we applied the technique of hydrophobic photolabeling (Brunner, 1981, 1989a). By using three membrane-restricted carbene-generating reagents, we could demonstrate that the interaction with the lipid bilayer is mediated solely by the fusion peptide (Harter et al., 1988, 1989). Moreover, several molecular details regarding this interaction could be derived. Thus, (i) the segment mediating this interaction is about 21 residues long, (ii) in all likelihood, it adopts a helical structure upon binding to membranes, and (iii) the peptide does not cross the entire bilayer (Brunner, 1989b).

In this paper, we now apply hydrophobic photolabeling to analyze the acid-induced fusion of influenza virus with a target membrane. The main question we asked is whether fusion is triggered by a mechanism that involves the insertion of the (acid-exposed) fusion peptide into the target membrane. The occurrence of such an initial (hydrophobic) interaction is suggested by the finding that at pH 5 and 0 °C, conditions under which fusion is slow or absent, liposomes not containing a receptor do bind to viruses (Stegmann et al., 1987). The

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[1] Abbreviations: HA, HA1, and HA2, influenza virus hemagglutinin and the two polypeptides derived therefrom; BHA, bromelain-solubilized ectodomain of HA consisting of HA1 and BHA2 polypeptides; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; [^{3}H]PTPC/11, 1-palmitoyl-2-[11-[4-[3-(trifluoromethyl)diazirinyl]phenyl][2-^{3}H]undecanoyl]-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; PS, 1,2-diacyl-*sn*-glycero-3-phosphoserine; *N*-Rh-PE, *N*-(Lissamine rhodamine B sulfonyl)phosphatidylethanolamine; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; TLC, thin-layer chromatography; Tris, Tris(hydroxymethyl)-aminomethane; Tricine, *N*-[Tris(hydroxymethyl)methyl]glycine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RET, resonance energy transfer.

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apparent hydrophobic character of the virus–liposome interaction further supports the view that the fusion peptide is involved. Hydrophobic photolabeling with a reagent introduced into and confined to the target bilayer should be ideally suited to test this model. Indeed, using a similar experimental approach, Novick et al. (1988) have recently been able to demonstrate that Sendai virus glycoproteins penetrate the target membrane during the early stage of fusion.

Materials and Methods

Chemicals. [^{125}I]TID and [^{3}H]PTPC/11 were synthesized according to protocols described earlier (Brunner & Semenza, 1981; Harter et al., 1988). The specific radioactivities were 20 and 22.2 Ci/mmol, respectively. The radiochemical purity of [^{3}H]PTPC/11 was checked by TLC and was found to be >98%. Egg lecithin (grade I) was from Lipid Products (South Nutfield, England). Disialoganglioside (G_{D1a}) was from Supelco Inc. (Bellefonte, PA). POPC, POPE, and bovine brain phosphatidylserine (PS) as well as *N*-Rh-PE and *N*-NBD-PE were from Avanti Polar Lipids, Inc. (Birmingham, AL). Prior to use, the fluorescent probes were purified by TLC as recommended by Struck et al. (1981).

Viruses and Hemagglutinin. Influenza virus PR8/34 (strain A/PR/8/34 Mount Sinai) was propagated in the allantoic cavity of embryonated eggs and purified as described before (Gerhad, 1976). Bromelain-solubilized hemagglutinin (BHA) was produced by digestion of purified PR8/34 viruses with bromelain (Sigma) as described by Brand and Skehel (1972) with minor modifications as specified by Harter et al. (1988).

Preparation of LUVs and of SUVs. The mixture of phospholipids (<1 μmol) containing [^{3}H]PTPC/11 was dried at 10^{-2} mmHg for 1 h at room temperature. The dried lipid was then dispersed by vortexing in 1.0 mL of fusion buffer (130 mM NaCl, 15 mM sodium citrate, 10 mM MES, and 5 mM HEPES, pH 7.2). After five freeze–thaw cycles, the liposomes were sized by extrusion through two stacked polycarbonate filters with a pore size of 0.2 μm (Hope et al., 1985; Mayer et al., 1986). The extrusion was repeated four more times, and any remaining larger or multilamellar liposomes were removed by centrifugation (Szoka & Papahadjopoulos, 1978). Small unilamellar vesicles (SUVs) were prepared by sonication of LUVs using a bath sonifier (Laboratory Supplies Co., Hicksville, NY; Model T-80-80-1RS).

Fusion Measurements. In the resonance energy transfer fusion assay, we proceeded as described by Stegmann et al. (1987) except that the final volume was 2.0 mL and that an SPF-500 fluorometer (SLM/Aminco, Urbana, IL) was used. For calibration of the fluorescence scale, the initial residual fluorescence of the liposomes was set to zero and the fluorescence at infinite probe dilution (determined by the addition of 0.50% Triton X-100 to the liposomes) to 100%.

Photolabeling Procedures. For photolabeling experiments, the light source and general equipment described earlier (Brunner & Semenza, 1981) were used. [^{125}I]TID labeling of the viruses was carried out in a Pyrex tube following standard protocols. For photolabeling with [^{3}H]PTPC/11, 0.2 mL of the mixture containing the viruses and liposomes was incubated in an Eppendorf tube under the conditions specified. The tubes were immersed in a double-walled Pyrex vessel, thermostated with circulating water, and irradiated for 10 s. It was found that under these conditions >80% of the diazirine is photolyzed and that the temperature of the sample did not increase during the irradiation. Special care was taken that within a series of an experiment each sample was photolyzed under exactly the same conditions. Following photolysis, the (photolabeled) proteins were then precipitated by the addition of 3 volumes of chloroform/methanol (1:2 v/v). After 1 h at room temperature, the precipitate was sedimented in a Sorvall HB-4 swinging bucket rotor for 10 min at 7000 rpm. The supernatant was carefully removed and discarded and the sediment dried in a Speed-Vac evaporator.

SDS–Polyacrylamide Gel Electrophoresis. For separation of photolabeled virus proteins a Tris/Tricine buffered system according to Schägger and Jagow (1987) was employed [separating gel 135 × 90 × 1 mm (16 × 6),[2] spacer gel 25 mm (10 × 3);[2] stacking gel 20 mm (4 × 3)[2]]. The protein bands were visualized by staining with Coomassie Blue. For quantitation of the radioactivity bound to the HA2 polypeptide chain, the corresponding band was excised and cut in 2-mm slices; each slice was treated with 0.6 mL of NCS tissue solubilizer (Amersham International plc, Amersham, U.K.) for 2 h at 50 °C and then subjected to scintillation measurements.

Analytical Methods. Viral phospholipids were extracted according to Folch et al. (1957). For analysis of phospholipids, the lower organic phase was evaporated, and the residue was either subjected to phosphate determination (Chen et al., 1956) or dissolved in a small volume of chloroform/methanol for subsequent TLC analysis of the phospholipids. Silica gel plates (Merck) were used and were developed in the solvent system chloroform/methanol/acetic acid/water (25:15:4:2 v/v/v/v). The individual phospholipids were visualized by exposure to iodine and were identified on the basis of their R_f values (comparison with reference samples). Protein was determined according to a modified Lowry procedure (Peterson, 1977).

Results

Lipid/Protein Ratio and Lipid Composition of Influenza Virus PR8/34. All experiments described were performed with viruses obtained from two preparations. The lipid/protein ratio (micromoles of phospholipid per milligram of protein) of the viruses was found to be 0.31 (batch I) and 0.25 (batch II). The membrane of the virus consists of the following (major) components: sphingomyelin (16%), phosphatidylcholine (27%), phosphatidylserine (19%), and phosphatidylethanolamine (38%).

[^{125}I]TID Labeling of Influenza Virus PR/8/34. When samples of whole viruses PR8/34, or of lipid-extracted virus proteins, were subjected to SDS–PAGE under reducing conditions, a pattern of four major bands is obtained (Figure 1). Since the experimental approach used in this study relies on our ability to determine the covalent incorporation of radioactivity (specific labeling) into the HA2 polypeptide chain, we have first studied the relative extents of labeling of the viral proteins by [^{125}I]TID, a hydrophobic reagent freely diffusible within the membrane. Labeling was carried out on native viruses suspended in fusion buffer, pH 7.3, at 0 °C by using the photolysis equipment and labeling protocol described earlier (Brunner & Semenza, 1981). As shown in Figure 1, the HA2 subunit of hemagglutinin was by far the most heavily labeled protein. It was calculated that approximately 0.6% of the [^{125}I]TID radioactivity originally added to the viruses became bound covalently to this polypeptide. A minor fraction (approximately 0.05%) was associated with an unidentified 18-kDa component (slices 29 and 30), and very little label coelectrophoresed with HA1 and nucleoprotein (slices 12–15). Since neither of the latter is an integral protein, it is likely that

[2] According to Hjertén (1962), the first figure in parentheses indicates the total concentration of monomers [percent (w/v)], and the second is that of *N,N'*-methylenebisacrylamide as a percentage of the monomer concentration.

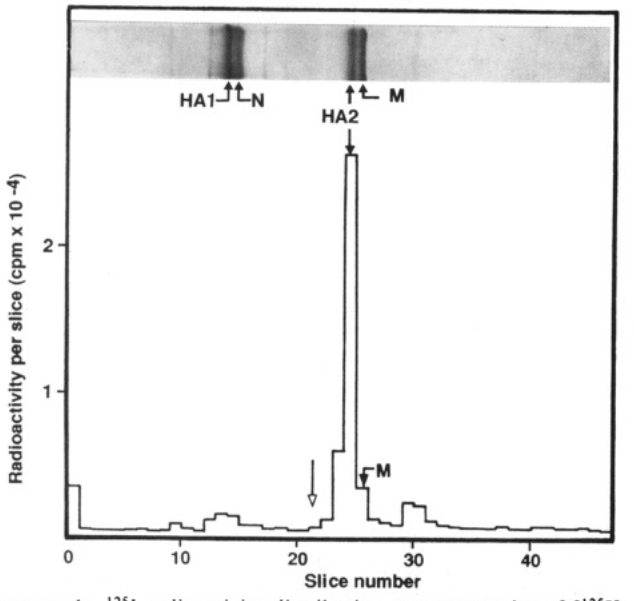

FIGURE 1: ^{125}I radioactivity distribution among proteins of [^{125}I]-TID-labeled influenza virus PR8/34. Viruses (150 μg of protein in 1 mL of fusion buffer, pH 7.3) and 25 μCi of [^{125}I]TID (added as a solution in 2 μL of ethanol) were incubated for 15 min at 0 °C and then subjected to photolysis for 30 s (Brunner & Semenza, 1981). Labeled viruses (30 μg of protein in 0.2 mL) were subjected to lipid extraction by chloroform/methanol (1:2 v/v). The precipitated protein was collected by centrifugation and subjected to SDS–polyacrylamide gel electrophoresis using the Tris/Tricine system. The gel was overrun to achieve better separation of the matrix protein (M) and the HA2 polypeptide as well as to remove residual labeled phospholipids that migrated slightly behind the tracking dye front. After electrophoresis, the polyacrylamide gel was stained with Coomassie Blue (photograph on top) and subsequently cut into 2-mm slices. Each slice was subjected to γ-counting. The open arrow denotes the position of the 26.6-kDa molecular weight standard protein.

the radioactivity in these slices is due to labeled neuraminidase, a minor integral protein, and/or some dimeric HA2 which, as noted previously (Harter et al., 1988), has a mobility similar to that of HA1. It was of particular interest to note that the matrix protein M, which migrates slightly ahead of HA2, was labeled very weakly, or not at all (slices 26 and 27). Therefore, although it is possible that the HA2 band, when excised from the gel, is not totally free of matrix protein, such contamination would not represent a problem for the present analysis. We have also established that the recoveries of HA2 of individual samples within a series of an experiment were virtually identical.

Temperature Dependence of Fusion Activity of Influenza Virus PR8/34. The acid-induced fusion of virus PR8/34 with LUVs (egg PC/G_{D1a}) shows a pronounced temperature dependence (Figure 2). Under standard assay conditions (phospholipid of viruses and LUVs at 5 μM each) the lower threshold temperature (no detectable fusion) was found to be around 15 °C. At much higher vesicle and virus concentrations [80 μM each as used in photolabeling experiments (see below)] this temperature appears to be shifted toward a somewhat lower value. Thus, some fusion (corresponding to 1.5% of that measured at 37 °C) was detectable even at 13.5 °C.

Regardless of whether fusion was triggered when LUVs were prebound to viruses or when the viruses were added to the acidified (pH 5) LUVs, there was no detectable delay at the onset of fusion. However, with our procedure of performing the assay (pipetting of a small volume of buffer into a 2-mL solution in a stirred cuvette) it was not possible to monitor fluorescence changes within the first 5–10 s.

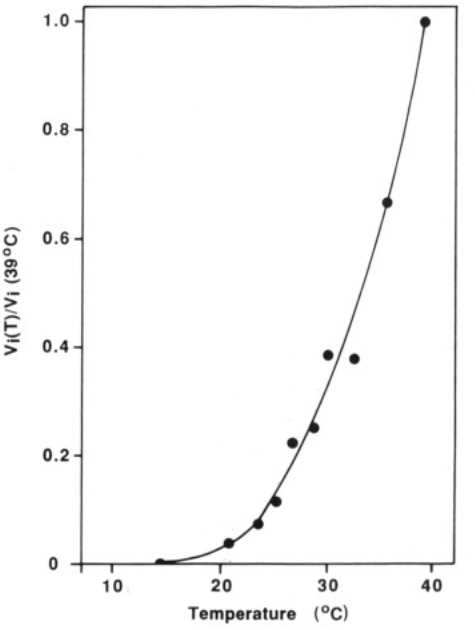

FIGURE 2: Effect of temperature on the fusion of influenza virus PR8/34 with LUVs prepared from egg PC/G_{D1a} (molar ratio 20/1) and each 0.6 mol % of *N*-NBD-PE and *N*-Rh-PE. Fusion between the virus and vesicles was measured by the RET assay after equilibration of the viruses and the LUVs in fusion buffer (2 mL), pH 7.4, at the desired temperature. Fusion was initiated by the addition of 50 μL of 1 M HCl. The initial fusion rate [$V_i(T)$] was determined and normalized to the initial rate obtained at 39 °C [V_i(39 °C)]. The ratio was plotted as a function of temperature.

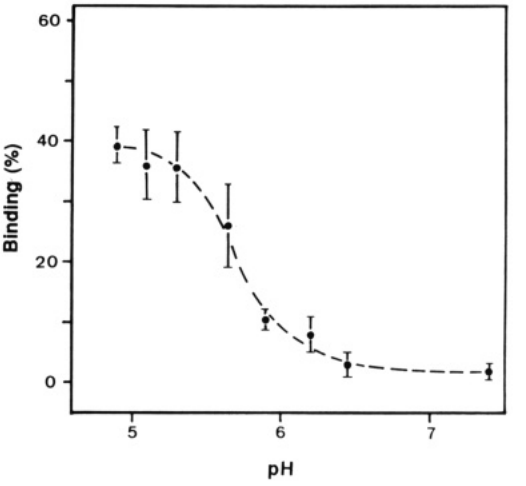

FIGURE 3: Binding of liposomes to influenza viruses PR8/34 as a function of pH. SUVs (sonicated liposomes) prepared from egg PC and a trace of [^3H]PTPC/11 were incubated with viruses in fusion buffer adjusted to the desired pH for 15 min at 0 °C (concentration of viruses and that of the liposomes corresponded to 10 μM phospholipid each). The mixture was centrifuged at 0 °C for 15 min at 12000*g*. Binding was calculated as the percentage of the total radioactivity that was pelleted and recovered with the viruses. Each point represents the mean value of three experiments (±SD).

Therefore the occurrence of a short lag phase as observed, for example, for fusion of fibroblasts expressing influenza hemagglutinin or vesicular stomatitis virus with erythrocyte ghosts (Morris et al., 1989; Clague et al., 1990) cannot be ruled out. As a matter of fact, results from flash photolysis experiments (see below) are consistent with a short delay at the onset of fusion.

Influenza Viruses PR8/34 Bind SUVs at 0 °C in a pH-Dependent Manner. It was shown earlier by Stegmann et al. (1987) that at 0 °C influenza viruses X-47 bind liposomes consisting of zwitterionic lipids and not containing a receptor

in a pH-dependent manner. Here, we examined PR8/34 viruses for their ability to bind liposomes at pH 5 and 0 °C. The results (summarized in Figure 3) demonstrate that PR8/34 viruses bind liposomes in a pH-dependent manner similar to that shown for X-47 virions. We have found also that the acid-induced binding could not be reversed by neutralization of the sample. In an attempt to determine the kinetics of binding, we found it to be complete within less than 30 s (pH 5.2; 0 °C).

[^{3}H]PTPC/11: A Photochemical Probe of Membrane Fusion. Physical or chemical probes of membrane fusion (and related vectorial processes) must not undergo spontaneous transfer between membranes. Earlier experiments by Harter et al. (1989) suggested that the spontaneous transfer of [^{3}H]PTPC/11 from liposomes to erythrocyte membranes is slow. Here, additional experiments are described which establish that this lipid does not exhibit any significant exchange between membranes.

LUVs were prepared from different mixtures of phospholipids each containing approximately 10 mol % of [^{3}H]-PTPC/11. To these vesicles were added influenza viruses, and the mixture was incubated first at pH 7.2 (37 °C; 30 min) and then, following addition of acid, at pH 5.2 (37 °C). At various time points, 0.2-mL aliquots of the incubation mixture were subjected to photolysis (10 s) and were then analyzed for ^{3}H radioactivity incorporated into the HA2 polypeptide chain of HA. Figure 4 reports the main results of this experiment. The actual ^{3}H radioactivity associated with the HA2 polypeptide (derived from a total of 30 μg of viral protein) ranged from background levels (30–50 cpm for nonphotolyzed samples) to approximately 40 000 cpm after fusion and is expressed as percentage of the HA2-bound radioactivity to that (total) originally present in the 0.2-mL aliquot (23.5 × 10^6 cpm). Very little labeling of HA2 (150–200 cpm corresponding to 0.0006–0.0009%) was seen after the initial 30-min incubation period (t = 0 min) at pH 7.2, demonstrating that neither spontaneous transfer of [^{3}H]PTPC/11 to the viral membrane nor any significant fusion had occurred during this stage of the experiment. However, upon acidification of the incubation mixture (pH 5.2, 37 °C), there was an immediate and rapid labeling response with a plateau reached after approximately 5–10 min. Both the time course of label incorporation into HA2 and the height of the plateau appeared to be affected by several factors, including temperature and the lipid composition of the target membrane. When the photolysis step was omitted, there was absolutely no labeling of HA2 (30–50 cpm).

It is quite certain that the labeling of HA2 directly reflects fusion of the two membranes and lateral mixing of their constituents. In fact, results of independent fusion measurements by the RET assay revealed a time course for fusion indistinguishable from that found for photolabeling of HA2 (data not shown). Moreover, as predicted, we have found that >90% (data not shown) of the label associated with the HA2 polypeptide chain resides within the C-terminal anchor segment.

Efficient fusion could also be measured with LUVs prepared from POPC/[^{3}H]PTPC/11 (9:1) lacking any acidic phospholipid or ganglioside (G_{D1a}). This is an important result since it was shown previously (Stegmann et al., 1987, 1989b) that the use of liposomes consisting of negatively charged lipids, and of cardiolipin in particular, can result in fusion reactions that do not reflect the biological situation. In agreement with those results, we also found here that the extent of fusion (expressed as percentage of total radioactivity that was covalently incorporated into HA2) was generally higher with vesicles containing negatively charged lipids.

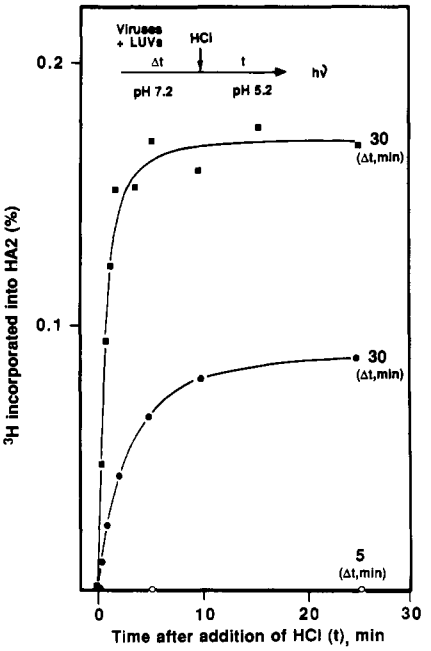

FIGURE 4: Membrane fusion monitored by photolabeling with [^{3}H]PTPC/11. Two-stage experiments were carried out to measure the rate of spontaneous transfer of [^{3}H]PTPC/11 from LUVs into the viral membrane. In stage I of the experiments, LUVs prepared from different lipids containing [^{3}H]PTPC/11 were incubated in fusion buffer with influenza viruses at pH 7.2 and 37 °C for 30 min. The concentration of viruses corresponded to 150 μg of protein/mL and that of the LUVs (phospholipid) to 80 μM. Then (t = 0), the pH of the medium was adjusted to 5.2 by the addition of 1 M HCl (25 μL/mL of mixture), and incubation was continued (stage II). At the time points (t) indicated, 0.2-mL aliquots were photolyzed in Eppendorf tubes for 10 s (control: without photolysis) and then neutralized (5 μL of 1 M NaOH/sample), and the radioactivity was determined, which became covalently associated with the HA2 polypeptide chain of HA. The procedure included precipitation of the protein by adding 3 volumes (0.6 mL) of chloroform/methanol (1:2 v/v), followed by SDS–polyacrylamide gel electrophoresis of the protein under reducing conditions. The Coomassie Blue stained band representing HA2 was excised from the gel and treated with HCS tissue solubilizer prior to scintillation measurements. The ^{3}H radioactivity was normalized to the total radioactivity of [^{3}H]PTPC/11 originally present in each sample, and this ratio (% of total ^{3}H) was plotted as a function of incubation time (t). The LUVs used were prepared from POPC/PE/PS/G_{D1a}/[^{3}H]PTPC/11 (molar ratio 2:1:2:0.25:0.5) (■) and POPC/[^{3}H]PTPC/11 (molar ratio 10:1) (●). Control samples were not subjected to photolysis (○).

The notion that labeling with [^{3}H]PTPC/11 was less efficient (range from 0.1% to 0.2%) than that observed with [^{125}I]TID (0.6%; see above) is not surprising and is likely to reflect the increased lipid/protein ratio in the virus–vesicle fusion system (when compared with viruses alone), and in the case of [^{3}H]PTPC/11 labeling, it could also mean that only a fraction of the available viruses, or of the target vesicles, did actually fuse.

Characterization of the Virus–Liposome Interaction at 0 °C and pH 5. Is the interaction between viruses and liposomes observed at pH 5 and 0 °C (Figure 3) mediated by the (exposed) fusion peptide? We hoped to find an answer to this question by binding viruses to LUVs containing [^{3}H]PTPC/11 and specifically labeling that polypeptide segment or segments penetrating the bilayer of the LUVs. However, as shown in Figure 5, the extent of radioactivity incorporation into the HA2 polypeptide of HA was very low (300–500 cpm corresponding to only approximately 0.001–0.002% of the radioactivity originally present in the target membrane). Essentially the same

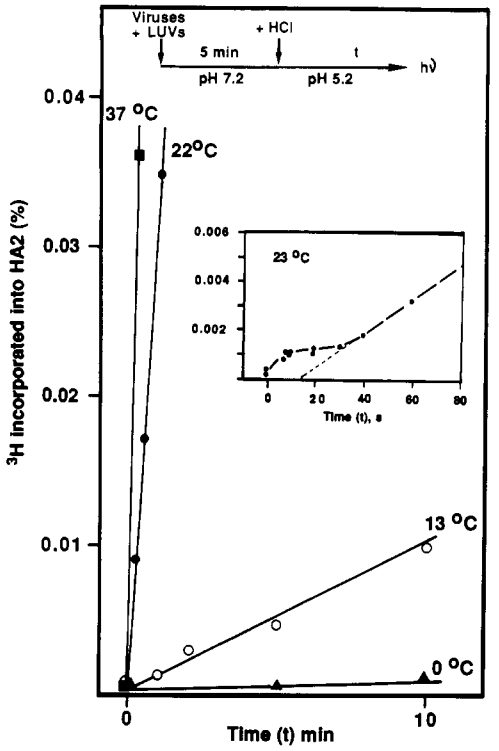

FIGURE 5: Interaction/fusion of influenza viruses with LUVs at different temperatures. Individual incubation mixtures (0.2 mL in fusion buffer, pH 7.2) contained viruses (30 μg of protein) and LUVs prepared from egg PC/[^{3}H]PTPC/11 (molar ratio 10:1) (80 μM). After equilibration at the desired temperature for 5 min, the pH was shifted to 5.2 by the addition of 5 μL of 1 M HCl (t = 0). Then binding/fusion was allowed to occur for up to 10 min. At the time points indicated, individual samples (Eppendorf tubes) were transferred into the thermostated Pyrex glass vessel and were photolyzed for 10 s. The ^{3}H radioactivity covalently bound to the HA2 subunit of HA was determined as described in the legand to Figure 3 and normalized to the original radioactivity in each sample. Inset: Time course of covalent ^{3}H radioactivity incorporation into HA2 during the initial stage of an experiment at 23 °C. In order to achieve increased time resolution, the reagent was activated by means of a flash photolysis device with the following specifications: power supply type PS4302 and xenon air-cooled flash lamp, model 8.150XA, both from Noblelight (Cambridge, England). The lamp was fired at 2.25 kV, which equals 1000 J. The sample (Eppendorf tube) to be activated was placed approximately 4 cm from the flash tube, and a single flash of approximately 0.2-ms duration was used to activate the reagent.

result was obtained when LUVs were used that contained 40% PS and/or virus receptor G_{D1a}. Likewise, the use of small unilamellar vesicles as target membranes did not result in an increased labeling efficiency.

Figure 5 also reports results of experiments performed at 13 and 23 °C (the single data point corresponding to 37 °C was taken from Figure 4). At these temperatures we measured a time-dependent increase in the specific labeling of HA2 that correlates well with the relative (initial) rates of fusion at the corresponding temperatures. In order to analyze the initial phase of the fusion reaction in greater detail, we also performed flash-photolysis experiments at 23 °C (because a single flash was found to activate only about half of the photolabel, the technique was not used routinely). The results (inset, Figure 5) revealed a "biphasic" label incorporation behavior during the early stage of fusion. In fact, they appear to be consistent with the possibility that actual fusion starts only after a short (10–15 s) lag phase. Also during this apparent lag phase we measured a low but nevertheless significant level of labeling (around 200 cpm) corresponding to approximately 0.001% of the original radioactivity.

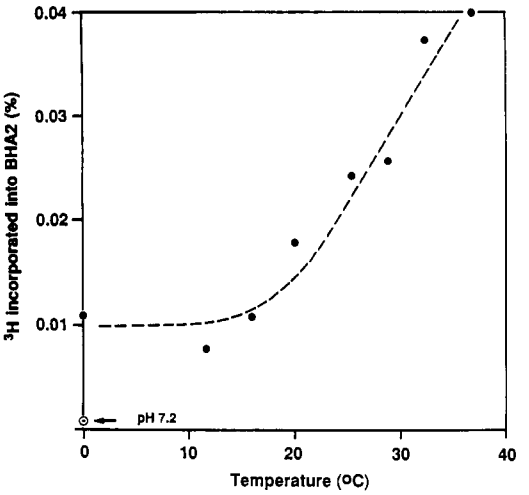

FIGURE 6: Extent of [^{3}H]PTPC/11 labeling of BHA in the presence of liposomes as a function of temperature. LUVs were prepared from a mixture of 0.5 μmol of egg-PC, 50 nmol (1.25 mCi) of [^{3}H]-PTPC/11, and 27.5 nmol (5 mol %) of G_{D1a} in 1 mL of fusion buffer. Individual incubation mixtures (0.2 mL in Eppendorf tubes) contained 90 μL of LUVs (approximately 40 nmol of lipid; 80 × 10^6 cpm of ^{3}H) and BHA (17 μg of protein) in fusion buffer, pH 7.2. Samples were incubated first at pH 7.2 (15 min) at the desired temperature, then the pH was adjusted to pH 5.2 by the addition of 1 M HCl (5 μL), and the incubation was continued for another 15 min. The Eppendorf tubes were then placed into the thermonstated vessel (temperature as before) of the photolysis system and irradiated for 10 s. Following delipidation of the protein by precipitation in chloroform/methanol (1:2), the radioactivity covalently bound to the BHA2 subunit of BHA was determined essentially as described for HA (HA2) (see legand to Figure 3). The arrow denotes a sample that had not been acidified.

Effect of Temperature on the Low pH Conformational Change of Hemagglutinin. It has been shown earlier (Harter et al., 1988) that labeling of BHA at pH 5 and 37 °C is considerably stronger that at pH 5 and 0 °C. Since the interaction of BHA with liposomes may share certain features with that of intact (virus-bound) HA with a target membrane, we have (re)examined, in more detail, the effect of temperature on BHA labeling. The main results are reported in Figure 6. They confirm earlier observations and, interestingly, point to a remarkable correlation between the temperature dependence of label incorporation into BHA2 and the effect of temperature on the fusion and hemolytic activity of intact viruses. Indeed, labeling was found to be strongly increased as the temperature was raised above 15–20 °C, the threshold temperature of fusion and hemolysis. However, while fusion was virtually absent below around 15 °C, some labeling occurred even at 0 °C. Although we have not determined the percentage of BHA trimers (monomers?) that under the conditions of labeling were actually bound to the (excess) liposomes at the various temperatures, the data strongly suggest that the mode of BHA–liposome interaction is not the same at low and at elevated temperatures, and this has implications regarding the molecular mechanism of fusion.

Discussion

The main goal of the present study was to characterize the acid-induced binding between viruses and receptor-free LUVs, which was proposed to involve the exposed hydrophobic fusion peptide (Stegmann et al., 1987). The notion that binding is rapid even at 0 °C where actual fusion is slow, or absent, is also consistent with the view that the extrusion of the fusion peptide is one of the early events in fusion (Yewdell et al., 1983; Bächi et al., 1985; White & Wilson, 1987). By using

the phospholipid-bound carbene-generating probe [^{3}H]-PTPC/11 inserted into the target membrane, we hoped to obtain direct experimental evidence in support of this model.

First, it was necessary to established that [^{3}H]PTPC/11 does not undergo significant spontaneous exchange between membranes. This is a requirement that applies to any reagent designed to probe the vectorial nature of fusion and related processes. Presumably all or most simple hydrophobic photolabeling reagents such as [^{125}I]TID (Brunner & Semenza, 1981) as well as [^{3}H]PTPC, an earlier version of a photoactivatable phospholipid (Brunner et al., 1983), show significant spontaneous transfer between membranes, and therefore their scope for studying fusion is limited. It is quite obvious that a photochemical fusion assay, whose feasibility is demonstrated here, could represent a valuable complement to conventional techniques (based on energy transfer of fluorescent probes or content mixing).

The main question raised by the present study is whether or not the virus–liposome interaction seen at pH 5 and 0 °C involves the fusion peptide. Unfortunately, because of the very weak labeling of the HA2 polypeptide chain, it was not possible to derive a final answer to this question. In addition to the preparation and separation of suitable HA2 fragments, a serious problem is the exclusion of traces of artifactual labeling, or at least a definition of its extent. Moreover, it should be kept in mind that the observed interaction of viruses with LUVs could be a nonspecific effect and perhaps not a related fusion process. As a matter of fact, in an earlier study (Harter et al., 1989) where a similar issue had been addressed, we have obtained labeling evidence that under slightly acidic conditions many, if not most, globular proteins can interact with lipid bilayers. A molten globule-like state of the proteins exhibiting an increased tendency to adhere to membranes may be responsible for this. Therefore, one has to consider also the possibility that the binding may be a result of such general behavior of the ectodomain of HA. In this context it is interesting to note that brief acid treatment (pH 5) of X-47 viruses at 0 °C leads to an irreversible conformational change of HA whereby the *HA1* polypeptide becomes susceptible to proteinase K (Stegmann et al., 1987). It is well conceivable that this pH 5 conformation of the HA1 domain has an increased affinity to membranes.

With the goal of obtaining further insights into the role of the fusion peptide and its interaction with membranes, we have reexamined the effect of temperature on the interaction of isolated BHA with membranes. These experiments provide suggestive evidence that a temperature-induced structural change is required for peptide insertion into membranes and for the expression of fusion activity of intact viruses. In contrast to the view that the acid-triggered exposure of the fusion peptide is rapid even at 0 °C, the present study supports a picture according to which the extrusion of this peptide itself is highly temperature sensitive and does not occur (rapidly) at 0 °C. Such a scheme may have a direct bearing on the virus' behavior and activities.

Junankar and Cherry (1986) have postulated that the temperature dependence of the fusion and hemolytic activity of influenza virus is related to the mobility of hemagglutinin in the membrane, which increases strongly with temperatures above 20 °C. It is known also that when viruses alone are exposed to acid at elevated temperature, they are rapidly inactivated with concomitant loss of mobility of the hemagglutinin. This inactivation has been interpreted to result from lateral aggregation of the hemagglutinin molecules by virtue of their "exposed" hydrophobic fusion peptide (Stegmann et al., 1987). That the virus resists acid inactivation at low temperatures has been explained by the restricted mobility of the glycoprotein in the membrane (Stegmann et al., 1987).

On the basis of our present data, a different scheme may be envisaged in which the functional properties of the virus are correlated to a strongly temperature-dependent structural change within the HA ectodomain. According to this model, treatment of the virus at pH 5 and low temperature is not sufficient to (fully) expose the fusion peptide and, therefore, lateral aggregation will not take place. Thus, inactivation of the virus may be precluded *not* because of the restricted mobility of HA in the viral membrane but rather because of the maintenance of predominantly hydrophilic properties at pH 5 and 0 °C. Such a model would also be consistent with the observations that the spikes of viruses treated at pH 5 and 0 °C have the same, or very similar, morphology as those of untreated (pH 7) viruses. In contrast, viruses exposed to pH 5 at elevated temperature showed a completely distrubed spike layer. The increase in rotational mobility of HA with increasing temperature was interpreted to reflect a temperature-sensitive association–dissociation equilibrium (Junankar & Cherry, 1986). Since it is unlikely that HA trimers dissociate into individual subunits (Doms & Helenius, 1986), that association–dissociation equilibrium may involve solely the ectodomain portion of HA. Our present photolabeling results are consistent with such an interpretation.

The widely accepted view that influenza-induced fusion (and probably fusion of other enveloped animal viruses as well) is triggered by a mechanism involving insertion of the exposed fusion peptide into the target membrane remains to be established. The present data are *not inconsistent* with such mechanism; however, they raise the possibility that these initial steps are slow below 15–20 °C, the threshold temperature for fusion. Although influenza viruses do bind to receptor-free liposomes at pH 5 and 0 °C, the underlying protein–lipid interaction may, therefore, not involve the fusion peptide at all. At least, this apppears to be a reasonable possibility for influenza virus A/PR/8/34.

Since the search for and the characterization of fusion intermediates remain an important task, the present finding may have implications regarding possible future strategies. As pointed out earlier (Harter et al., 1989), one promising approach to obtaining fusion intermediates may be based on the use of mutant hemagglutinins that, as shown by Gething et al. (1986), can associate with a target membrane but do not trigger fusion. An entirely different approach, whose scope we are exploring currently in our laboratory, is based on the use of target membranes that may permit interactions with the virus to occur but will not undergo (full) fusion. The use of membranes consisting of bipolar, membrane-spanning lipids or the use of membranes containing polymerized lipids may offer the possibility of trapping fusion intermediates at elevated temperatures and making them amenable to further characterization.

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