Lipid Interactions of the Hemagglutinin HA2 NH2-terminal Segment during Influenza Virus-induced Membrane Fusion*

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Fusion of influenza viruses with target membranes is induced by acid and involves complex changes in the viral fusion protein hemagglutinin (HA) and in the contact sites between viruses and target membranes (Stegmann, T., White, J. M., and Helenius, A. (1990) EMBO J. 9, 4231-4241). At 0 °C, in a first, kinetically distinct step, target membranes irreversibly adhere to the viruses. Fusion itself starts only after a lag-phase of several minutes (X-31 strain viruses) or after raising the temperature (PR8/34 strain viruses). We now provide evidence that the initial conformational change resulting in virus-target membrane adhesion is restricted to a (minor) subpopulation of the HA molecules. These molecules become susceptible to bromelain digestion, and they could be labeled with the photoactivatable reagent [3H]PTPC/11, a nonexchangeable lipid present in the target lipid bilayer (Harter, C., Bäch, T., Semenza, G., and Brunner, J. (1988) Biochemistry 27, 1856-1864). Only the HA2 subunit was labeled, and analyses of 2-nitro-5-thio-cyanobenzoic acid fragments derived thereof indicate that the HA2 NH2-terminal segment (fusion peptide) inserted into the target membrane bilayer. When the temperature was raised to trigger fusion of PR8/34 viruses, labeling of HA2 increased by a factor of 130. Most (74%) of that label was incorporated into the COOH-terminal membrane anchor region, but there was also a strong increase (about 30-fold) of NH2-terminal fusion peptide labeling. This suggests that fusion is preceded, or accompanied, by further changes in HA which lead to additional extensive lipid insertions of HA2 fusion peptides.

Membrane fusion is an essential step during the infectious entry of enveloped viruses. It is mediated by viral membrane glycoproteins of which hemagglutinin (HA), the fusion protein of influenza virus, is the best characterized (for recent reviews, see Marsh and Helenius, 1989; Stegmann et al., 1989; Wilschut and Hoekstra, 1990; White, 1990). Hemagglutinin is a homotrimeric protein, each subunit consisting of two disulfide-linked polypeptide chains, HA1 and HA2. The protein complex is anchored in the viral membrane through a single membrane-spanning hydrophobic segment located near the COOH terminus of the HA2 polypeptide chain. Treatment of intact viruses with bromelain releases the water-soluble ectodomain of HA (BHA) whose three-dimensional structure has been determined (Wilson et al., 1981).

The fusion activity of HA is triggered by an acid-induced conformational change which leads to the exposure of the so-called fusion peptide, a hydrophobic, 22-residue long NH2-terminal segment of the HA2 subunit of HA. The crucial functional role of this peptide is demonstrated by the fact that site-specific mutations within this region abolish, or severely affect, the fusion activity of HA (Gething et al., 1986). According to a widely accepted view, the HA2 fusion peptide inserts into and somehow destabilizes one or both membrane bilayers to fuse. Evidence in support of such a mechanism was provided by Stegmann et al. (1991) who found that at 0 °C (pH 5.0) viruses rapidly interact with target vesicles but undergo fusion only after a lag-phase of several minutes. Using two photoactivatable lipids incorporated into the target membrane, it could be demonstrated that the HA2 was inserted into the target membrane prior to fusion.

This "insertion model" was recently challenged by Bentz and colleagues (Bentz et al., 1990) who proposed a conceptually different role for the fusion peptide. They speculated that fusion might be promoted by the formation of a protein bridge between adjacent bilayer surfaces with an internal aqueous channel. The role of the fusion peptide could be to dehydrate the internal space of this channel and create a hydrophobic pathway for the lipidic monolayers.

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The abbreviations used are: HA, HA1, and HA2, influenza virus hemagglutinin and the two polypeptides derived therefrom; BHA, bromelain-solubilized ectodomain of HA consisting of HA1 and HA2 polypeptides; [3H]PTPC/11, 1-palmitoyl-2-[11-[[3-(trifluoromethyl) diazirinyl]-phenyl][10-3H]undecanoyl]-sn-glycero-3-phosphocholine; N-Rh-PE, N-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; N-NBD-PE, N-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Tricine, N-tris-(hydroxymethyl)glycine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NTCB, 2-nitro-5-thio-cyanobenzoic acid; NTCB2, and NTCB3, NTCB-bleavage products of HA2 containing the NH2-terminal fusion peptide and COOH-terminal membrane anchor segment, respectively; TUA, 11-[4-[3-(trifluoromethyl)diazirinyl]phenyl]undecanoyl acid; Gnta, disialoganglioside; LUVs, large unilamellar vesicles; MES, 4-morpholinethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PC, phosphatidylcholine.
of the two apposed membranes. This model is attractive because it does not require large alterations to the known neutral pH structure of HA, and could explain how proteins could assemble at the interface of two membranes and promote fusion without actually having to penetrate a lipid bilayer.

Using the same photolabeling approach as Steggman et al. (1991), but working with viruses of a different strain (PR8/34), experiments in our laboratory failed to provide clear-cut evidence for insertion of the HA fusion peptide into the target membrane bilayer (Brunner et al., 1991). This was the more surprising as PR8/34 viruses, like X-31 viruses, were found to bind to receptor-free liposomes in a pH-dependent manner. Thus, our study either suggested that the number of inserted fusion peptides was too small to be readily detected in our experiments, or that, contrary to the model of Steggman et al. (1991), the prefusion interaction did not involve direct membrane insertion at all. Indeed, this second possibility had been considered seriously as virus-target membrane binding could have been the result also of long range attractive forces (Helm et al., 1989). Moreover, as discussed below, demonstration of HA2 labeling alone does not fully prove the insertion model. In view of the relevance of this question, we decided to directly compare the PR8/34 and X-31 strains of viruses and to analyze in more detail the labeling of the HA2 subunit. We now demonstrate that the HA2 NH2-terminal segment (fusion peptide) of PR8/34 and most likely also of X-31 hemagglutinin inserts into the target membrane bilayer prior to fusion. However, our data suggest that additional aspects must be considered in postulating fusion models. Thus, we present evidence that only a minor fraction of the HA molecules actually undergo the rapid acid-induced conformational change and participate in the initial attachment of the viruses to the vesicles. The majority of the viral HA molecules behave in a different manner and expose and insert their fusion peptide more slowly (X-31), or only at elevated temperature (PR8/34). The implications of these findings for the membrane fusion mechanism are discussed.

MATERIALS AND METHODS

Chemicals—[3H]PTPC/11 (13 Ci/rnmol) was synthesized according to the protocol of Harter et al. (1988). The compound was stored as a solution in ethanol-toluene (1:1) at a concentration of <10 mCi/ml. Prior to use, the material was purified by gel column chromatography using the solvent system chloroform/methanol/water (65:25:4, v/v/v). [3H]PTPC was synthesized essentially as described for the nonradioactive TUA (Harter et al., 1989). Moreover, as discussed below, demonstration of HA2 labeling alone does not fully prove the insertion model. In view of the relevance of this question, we decided to directly compare the PR8/34 and X-31 strains of viruses and to analyze in more detail the labeling of the HA2 subunit. We now demonstrate that the HA2 NH2-terminal segment (fusion peptide) of PR8/34 and most likely also of X-31 hemagglutinin inserts into the target membrane bilayer prior to fusion. However, our data suggest that additional aspects must be considered in postulating fusion models. Thus, we present evidence that only a minor fraction of the HA molecules actually undergo the rapid acid-induced conformational change and participate in the initial attachment of the viruses to the vesicles. The majority of the viral HA molecules behave in a different manner and expose and insert their fusion peptide more slowly (X-31), or only at elevated temperature (PR8/34). The implications of these findings for the membrane fusion mechanism are discussed.

Preparation of LU V's—The mixture of phospholipids (<20 μmol) containing freshly purified [3H]PTPC/11 was dried at 10-15 °C for 1 h at room temperature. The dried lipid was then dispersed by vortexing into 1-2 ml of fusion buffer (130 mM NaCl, 15 mM sodium citrate, 10 mM MES, and 5 mM HEPES, pH 7.3). After five freeze-thaw cycles, the liposomes were sized by repeated extrusion through two stacked polycarbonate filters with a pore size of 0.2 μm (Hose et al., 1985; Mayer et al., 1986).

Preparation of Small Unilamellar Vesicles—To solutions of egg-PC or of mixtures of egg-PC and GM1 (5 mol % GM1), 65 mCi/mmol was added as a trace (approximately 0.1 mol % of [3H]PTPC/11 as a nonexchangeable, radioactivity marker. Following evaporation of the solvent, the dried lipid was dispersed in fusion buffer (approximately 1 mM lipid) and pulse-solubilized using a tip sonifier (Branson B30). Residual multilamellar aggregates were sedimented (30 min, 100,000 x g); the supernatant was used for assay.

Liposome Binding to Viruses—Binding assays were performed in the cold room at 3 °C. Incubation mixtures (0.5 ml) containing viruses and small unilamellar vesicles (20 μm phospholipid each) in fusion buffer (pH 7.5) were prepared and diluted with an equal volume of cold fusion buffer (control) or with acidified buffer to give a final pH of 5.0. After appropriate timer incubation for 10, the (acid) filtrates were either first neutralized by the addition of 1 M NaOH or directly subjected to centrifugation (15 min, 14,000 x g) to sediment the viruses and bound liposomes. The extent of virus-liposome binding was calculated from the radioactivity of aliquots of the incubation mixtures (prior to centrifugation) and of the supernatants (after centrifugation). No corrections were made for the residual fraction of viruses in the supernatant.

Fusion Measurements—The resonance energy transfer assay developed by Struck et al. (1981) was used. LUVs contained each 0.6 mol% of N,N'-NBD-PE and N-Rh-PE. Measurements were carried out under continuous stirring, in a thermostatically maintained cell equipped with a solution of fusion buffer adjusted to the desired pH with 1 M HCl. The increase in fluorescence, resulting from dilution of the fluorophores into the viral membrane upon fusion, was recorded continuously at excitation and emission wavelengths of 495 nm (slit 1.5 nm) and 530 nm (slit 4 nm). Accordingly, a decrease in fluorescence (negative fluorescence) was observed for all measurements. For calibration of the fluorescence scale, the initial residual fluorescence of the LUVs was set to zero and the fluorescence intensity was then corrected for the effect of the detergent on the quantum yield of N,NBD-PE (Struck et al., 1981).

Analysis of [3H]PTPC/11-Labeled HA Subpeptides—The photoalyzed samples (see footnotes to Table I) were neutralized by the addition of 1 M NaOH, and the viruses (and bound or fused LUVs) were sedimented in a Sorvall centrifuge (SS 34) at 17,000 rpm (15 min). The sediments (2 ml of fusion buffer) were transferred into polypropylene tubes, and virus protein was precipitated by adding 3 volumes of chloroform/methanol (1:2, v/v). After 1 h at room temperature, the precipitated protein was collected by sedimentation in a Sorvall centrifuge (SS 34, 17,000 rpm (15 min), supernatant was carefully removed and discarded, and the protein dried in vacuo. The protein was dissolved in sample buffer (in boiling water bath for 3 min) and subjected to SDS-polyacrylamide gel electrophoresis (10%) using the Tris/Tricine buffered system according to Schägger and von Jagow (1987). The HA band was visualized directly by a fluorescence quenching technique as described previously (Brunner et al., 1979). HA was electroeluted at 50 mA for 4 h using a homemade apparatus. The elution buffer contained Tris (25 mM), glycine (0.18 M), and 0.1% SDS. The protein solution (1-2 ml) was concentrated by Centricron 30 microconcentrators (Amicon) according to the conditions stated above. HA1 (HA1 and HA2) was stained with Coomassie Brilliant Blue R-250, and the corresponding bands were excised and subjected to scintillation counting. To this end, the polyacrylamide gel slices (approximately 200 mg wet weight) were first treated with 4 ml of 0.1 N NaOH solubilizer for 1 h at room temperature, and then with 4 ml of 0.1 M NaOH, and then washed with distilled water. Then 10 ml of a 2.3-diphenyloxazole-based organic (toluene/Triton X-100) scintillator solution was added, and the samples were subjected to scintillation counting. For quantitation of HA1, HA2, and BHA2 (see below), defined aliquots of reduced and alkylated BHA proteins were subjected to SDS-PAGE and the data calculated on the basis of the molar extinction coefficients of the proteins. The data were corrected for the background. Finally, the data were normalized to the total mass of HA in each sample.
and Skehel (1972) with minor modifications as specified by Harter et al. (1988).

(b) From [3H]PTC/11-labeled virus: [3H]PTC/11-labeled and sedimented viruses were suspended in about 0.7 ml of 100 mM Tris-HCl, 1 mM EDTA, pH 7.2, and subjected to bromelain digestion (3% for 12 h) as described above. After inactivation of bromelain with N-ethylmaleimide (100 mM, 30 min at room temperature), the membranes were solubilized by the addition of detergent C18E9 (final concentration 2 mM). After treatment at 4°C for 1 h, insoluble virus cores were sedimented (100,000 g, 1 h) and the supernatant removed. The sediment was treated with another portion of 2 mM C18E9 and insoluble material sedimented. The combined supernatants (approximately 1.2 ml) were then subjected to Sephacryl S-400 gel filtration (1.5 x 25 cm). As the eluant, fusion buffer containing 2 mM C18E9 was used. Fractions containing BHA were identified by A260 measurements. They were pooled, concentrated (Centricon), and detergent was removed by precipitation of the BHA by the addition of 3 volumes of chloroform/methanol (1:2, v/v). Precipitated BHA was then purified by two-dimensional preparative SDS-PAGE and processed further as described above for HA polypeptides.

**NTCB Cleavage**—Solutions (1-2 ml) of HA2 or BHA2 (1-2 nmol of protein from electrophoresis of Coomassie Blue-stained preparative SDS-polyacrylamide gels) were concentrated by Centricon microconcentrators (Amicon) to approximately 0.2 ml. Protein (plus salt) was precipitated by the addition of 9 volumes of acetone (this step served to remove the Coomassie Blue stain). The dried residues were dissolved in 30 μl of water and the pH adjusted to pH 8.4 by the addition of 1 M Tris-HCl (final concentration 5 mM) and SDS (final concentration 2%) were added, and the solutions were boiled for 3 min. After addition of NTCB (1 mg) and incubation for 1 h at room temperature, the pH was adjusted to 9.5 (addition of 1 M NaOH) and incubation was continued for 5 h at 50-55°C.

**Sequence Analyses**—Sequence analyses were performed on an Applied Biosystems, Inc., model 470 A gas-phase sequenator with an on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems Inc., model 120 A). Polypeptides (HA2 and NTCB fragments derived thereof) separated by SDS-polyacrylamide gel electrophoresis were electroeluted (elution buffer 25 mM Tris, 0.19 M glycerol, 0.05% SDS, pH 8.0). The peptide solutions were concentrated using Centricon microconcentrators (Amicon), and aliquots corresponding to 100-200 pmol of peptide were spotted directly onto polyvinylidenefluoride membranes. After air-drying of the membranes and removal of the buffer salts and excess SDS by washing in 10 mM sodium borate, 25 mM sodium chloride, pH 8.0 (Hirano and Watanabe, 1990), the peptides were subjected to Edman sequence analyses.

**RESULTS**

**Different Fusion Characteristics of PR8/34 and X-31 Influenza Viruses**—PR8/34 viruses were shown previously not to fuse with lipid vesicles at 0°C and pH 5.0 (Brunner et al., 1991). In view of the finding by Stegmann et al. (1990) that fusion of X-31 viruses at 0°C is even more efficient than at 37°C, but starts only after a lag-phase of 4-8 min, we have reexamined the fusion behavior of PR8/34 viruses. In agreement with our data reported earlier, no fusion could be detected even after prolonged incubations of PR8/34 viruses with vesicles prepared from egg-phosphatidylcholine at either pH 5.0 or 4.7 (3°C). Efficient fusion was seen, however, at elevated temperature (Fig. 1). Since fusion is known to be affected by the lipid composition of the target membrane bilayer, we also examined fusion of X-31 and PR8/34 viruses with vesicles containing 40 mol % of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine. X-31 viruses efficiently fused after a lag-phase of several minutes in agreement with the report of Stegmann et al. (1990), but PR8/34 viruses did not undergo detectable fusion within 4 h at pH 5.0, 3°C (Fig. 2).

**Binding of Influenza Viruses to Membranes**—Both PR8/34 and X-31 viruses bind receptor-free liposomes in a pH-dependent manner (Stegmann et al., 1987; Brunner et al., 1991). For X-31 viruses this binding is rapid (within 10-20 s) and irreversible (Stegmann et al., 1987). We now demonstrated that PR8/34 viruses also rapidly bind liposomes. However, under standard assay conditions, PR8/34 viruses exhibited lower liposome binding capacity (15-25%) than measured for X-31 (40-60%).

Additional binding experiments were carried out to study the effect of ganglioside GD1a, in the liposomes. Under neutral conditions, these liposomes were found to bind only weakly (5-10%) to PR8/34 viruses. However, when aliquots of such incubation mixtures were treated briefly with acid (10 s to 5 min at pH 5.0) and then neutralized again, 50-70% of the liposomes now sedimented with the viruses. Thus, the acid-induced (hydrophobic) adhesion of liposomes to viruses is substantially increased by prebinding through this apparently poor virus receptor (GD1a).

Binding measurements were complicated by the fact that viruses (X-31 and PR8/34) exhibit weak interactions with receptor-free liposomes (0-5% binding) even under neutral conditions (pH 7.3). Moreover, these interactions were found to depend on a number of factors including the lipid composition of the liposomes, the incubation time as well as the
concentration of both viruses and liposomes. A similar adsorption phenomenon has been described also for vesicular stomatitis virus (Shaw et al., 1979).

**Acid-induced Conformational Change of HA—Low pH treatment of HA or BHA at 37 °C triggers a conformational change which leads to the exposure of the fusion peptide and to (a partial) dissociation of the globular heads of the trimeric HA structure (Doms et al., 1985; White and Wilson, 1987; Puri et al., 1990). At 0 °C, this transition appears to go only to an intermediate state with the release of the fusion peptide as indicated by protease sensitivity and liposome and antibody binding assays (White and Wilson, 1987; Stegmann et al. 1987). Here, we demonstrate that this rapid transition at 0 °C is restricted to merely a subpopulation of the HA molecules. When X-31 or PR8/34 viruses were treated at pH 5.0, 0 °C, for 2 min and then digested with bromelain, a soluble form of HA was released that was indistinguishable from that obtained by bromelain treatment of native, not acid treated HA; HA1 and BHA2 denote to those of bromelain-solubilized HA polypeptides—Next, we investigated the H+-induced hydrophobic adhesion of viruses to target vesicles by labeling the polypeptide segment(s) penetrating the target bilayer with the photoactivatable lipid [3H]PTPC/11. For both PR8/34 and X-31 viruses, labeling was performed under prefusion conditions (pH 5.0, 0 °C, 2 min) as well as after fusion (pH 5.0, 37 °C, 5 min). With PR8/34 viruses, additional experiments were performed at 23 °C, a temperature at which fusion is preceded by a short lag-phase. For each data point, 2–3 mg of virus (protein) and up to 1 mCi of [3H]PTPC/11 were used. This permitted the very rigorous purification of the HA1 and HA2 polypeptide chains (including SDS-polyacrylamide gel electrophoreses under nonreducing and reducing conditions) and the accurate determination of the specific labeling at a high level of sensitivity.

Regardless of the incubation conditions prior to photoactivation of the reagent, the HA1 polypeptide was not labeled at all (less than 0.05 nCi/nmol). In contrast, the HA2 chain was labeled to extents that exceeded background levels (<0.05 nCi/nmol) by one to four orders of magnitude (Table I). The results can be summarized as follows: first, incubations under neutral conditions (pH 7.3, 0 °C) led to weak, yet not insignificant, labeling of HA2 which was similar for PR8/34 and X-31 viruses (0.2–0.4 and 0.46 nCi/nmol, respectively). Second, acidification (pH 5.0, 0 °C, 2 min) prior to reagent activation resulted in increased labeling. This increase was much higher for X-31 HA2 (about 20-fold) than that measured for PR8/34 HA2 (about 2-fold). Third, for both strains of viruses, a further strong increase in HA2 labeling occurred during fusion, but the final levels were again similar for both viruses (0.90 and 0.26 nCi/nmol, respectively).

**Analysis of Labeled HA2—**It was of interest now to determine the distribution of label between the NH2-terminal fusion peptide and COOH-terminal anchor. Toward this goal, appropriate HA2 fragments had to be obtained. First, we examined bromelain cleavage which, as shown above, releases the HA ectodomain by cleaving off the COOH-terminal anchor at the HA2 subunit. Soluble BHA thus obtained from viruses was then subjected to the same purification procedure as described above for HA and, finally, the specific radioactivities of the HA1 (negative controls) and BHA2 polypeptides were determined. Neither the HA1 nor the BHA2 polypeptide chain contained any detectable radioactivity (<0.05 nCi/nmol).

This result either suggested that labeling of HA2 (Table I) occurred exclusively within the COOH-terminal anchor (this part of HA2 is absent in BHA2), or that labeled HA2 polypeptides were selectively degraded by bromelain. To investigate this point, we studied chemical fragmentation at cysteine residues with NTCB. The positions of the cysteine residues within HA2 polypeptide chain of X-31 and PR834 viral HA are depicted in Fig. 4. Accordingly, NTCB cleavage of both HA2 polypeptides should yield two relatively large fragments one of which (1–136) comprising the fusion peptide and the other (148–210 for PR8/34 and 148–194 for X-31) comprising (most of) the anchor segment. The following part of this

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**Fig. 3.** Comparison of the electrophoretic mobilities in an SDS-polyacrylamide gel of the subunits of BHA generated from native (pH 7) X-31 viruses and from viruses that had been treated with acid (pH 5) at 0 °C for 2 min. Upon digestion of the viruses with bromelain, the virus cores were removed by sedimentation and BHA separated from bromelain by gel filtration. Aliquots of the pooled and concentrated BHA fractions as well as a sample of intact viruses were subjected to SDS-PAGE (reducing conditions), and the proteins were visualized by staining with Coomassie Brilliant Blue. HA1 and HA2 denote to the subunits of intact HA; HA1 and BHA2 denote to those of bromelain-solubilized HA (BHA). M refers to the viral matrix protein.

**Fig. 4.** Positions of the cysteine residues within the HA2 polypeptide chains of HA from PR8/34 and X-31 viruses, respectively. The bold bars refer to the two predominantly hydrophobic segments located at the NH2 terminus (fusion peptide) and near the COOH terminus (anchor peptide), respectively. For sequences see Winter et al. (1981).
TABLE I

Specific radioactivity of the HA2 polypeptide chain upon labeling of PR8/34 and X-31 influenza viruses prior to and after fusion with LUVs containing [H]PTPC/11

<table>
<thead>
<tr>
<th>Incubation*</th>
<th>Temperature</th>
<th>Time</th>
<th>Reagent activation</th>
<th>Specific radioactivity of HA2 polypeptide (nCi/nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR8/34</td>
<td>℃</td>
<td>No. of flashes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
<td>0.3-0.6*</td>
</tr>
<tr>
<td>0</td>
<td>2 min</td>
<td>1</td>
<td></td>
<td>0.4-0.9*</td>
</tr>
<tr>
<td>0</td>
<td>2 min</td>
<td>5</td>
<td></td>
<td>1.35</td>
</tr>
<tr>
<td>0</td>
<td>2 min</td>
<td>5</td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>23</td>
<td>10 s</td>
<td>1</td>
<td></td>
<td>1.3</td>
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<tr>
<td>23</td>
<td>20 s</td>
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<tr>
<td>23</td>
<td>60 s</td>
<td>1</td>
<td></td>
<td>3.6</td>
</tr>
<tr>
<td>37</td>
<td>5 min</td>
<td>5</td>
<td></td>
<td>3.25</td>
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X-31

| 0           | 10 s        | 1    |  | 9     |  |
| 0           | 30 s        | 1    |  | 10    |  |
| 0           | 1 min       | 1    |  | 11    |  |
| 0           | 2 min       | 1    |  | 12    |  |
| 0           | 2 min       | 5    |  | 25    |  |
| 0           | 2 h         | 5    |  | 200   |  |
| 37          | 2 min       | 5    |  | 435   |  |
| 37          | 5 min       | 5    |  | 500   |  |
| 37          | 20 min      | 5    |  | 500   |  |

*In all experiments, mixtures of viruses and LUVs were first equilibrated (2 min) in fusion buffer, pH 7.3 (5 ml) (the virus to vesicle ratio was 1:1 (based on membrane phospholipid target phosphorus) and the concentration of viruses corresponded to 0.6 mg of protein/ml (3 mg of total); the LUVs used consisted of egg-PC/[H]PTPC/[110 ms, pH 5.0]). To trigger binding fusion, the pH was lowered to 5.0 by the addition of an equal volume (5 ml) of acidified fusion buffer to give a final pH of 5.0, and incubation was continued at the temperature and for the time indicated. Samples were subjected to flash photolysis and HA polypeptides were isolated from labeled viruses and analyzed as described under "Materials and Methods."

** For reagent activation, the incubation mixtures (in 17 × 100-mm cylindrical polypropylene tubes (Falcon 2059)) were subjected to flash photolysis using a device with the following specifications: power supply type PS4322 and xenon air-cooled flash lamp, model 8.15XAK, both from Noblelight (Cambridge, United Kingdom). The lamp was fired at 2.25 kV, which equals 1000 J. The polypropylene tube was placed into a double-walled quartz vessel (thermostatted with circulating water) mounted 4 cm from the flash tube, and the reagent was activated by either a single or five consecutive flashes (7 s between individual flashes). Under the present conditions of labeling, one flash (0.2 ms) was determined to photolyze approximately half of the reagent.

Labeling of the HA1 polypeptide chain was consistently within normal background levels corresponding to <0.05 nCi/nmol.

Since incubation mixtures all contained viruses and target lipid vesicles at a defined stoichiometric ratio, the specific radioactivities of labeled HA polypeptides (nCi/nmol) can be used to calculate labeling efficiencies (% of the original (total) radioactivity covalently incorporated into HA polypeptides). Assuming that 30% of the viral protein is HA, a specific radioactivity of 1 nCi/nmol corresponds to a labeling efficiency of 0.001%.

Range of three experiments.

A doublet of well-stainable bands, termed NTCB1,2, as well as a broad, barely visible band, NTCB3, were obtained (Fig. 5). Edman degradation (8 cycles) of each of the two bands of NTCB1,2 gave an amino acid sequence corresponding to that of uncleaved HA2. This demonstrated that both fragments represent amino-terminal peptides of HA2. No amino acid sequence was obtained from a comparable amount of NTCB3 indicating that the NH2 terminus was blocked. Since NTCB cleavage results in a blocked amineterminus (aminothiazolidinyll residue), this latter result was expected. Thus, NTCB3 most likely represents peptides comprising the anchor segment with heterogeneity at both the (blocked) NH2 and COOH terminus. Since it could not be ruled out from this analysis that NH2-blocked COOH-terminal fragments (comprising the anchor segment) coelectrophoresed with NTCB3, a second, independent scheme was devised to identify the NTCB fragments.

This involved (a) differential and selective labeling of the fusion peptide and anchor segment with reagents containing 3H and 14C, respectively; (b) NTCB cleavage of a mixture of the labeled polypeptides; and (c) separation and analysis of the fragments by SDS-PAGE. Selective labeling of the fusion peptide with 3H was accomplished by incubation of BHA with [3H]PTPC/11-containing liposomes at pH 5.0 and subsequent photoactivation of the reagent (Harter et al., 1988). HA2 labeled selectively within the anchor segment with 14C was prepared from viruses following incubation and photolysis of viruses with [14C]TUA, a photactivatable fatty acid. To demonstrate that only the COOH-terminal anchor was labeled with [14C]TUA, a sample of the viruses was digested with bromelain. The BHA generated by this treatment was devoid of any 14C radioactivity (25 cpm/nmol), whereas intact HA2 prepared from the same labeled virus had a specific radioactivity of 1750 cpm/nmol. This demonstrated that >98.5% of the 14C radioactivity was associated with the COOH-terminal portion (anchor plus short cytoplasmic tail) of HA2.
The peptides resulting from NTCB cleavage of the mixture of [3H]PTPC/11-labeled BHA2 and [14C]TUA-labeled HA2 were separated by SDS-PAGE, and the 3H and 14C radioactivity of individual gel slices was determined (Fig. 5). In agreement with the above results (sequence data) (see above), the tritium radioactivity coelectrophoresed with NTCB1,2, whereas the 14C radioactivity was found mainly in NTCB3. Thus, NTCB cleavage in conjunction with SDS-PAGE provides a method to analyze distribution of [3H]PTPC/11 radioactivity in HA2 samples derived from viruses labeled at different stages of fusion.

Fusion Peptide and Anchor Segment Labeling during Fusion—PR8/34 viruses were labeled under prefusion conditions (0 °C, pH 7.4 and 5.0) at the onset of fusion (23 °C, pH 5.0, 10 s) and after fusion (37 °C, pH 5.0, 5 min). Prior to NTCB cleavage, the purified [3H]PTPC/11-labeled HA2 samples were supplemented with a trace of HA2 14C-labeled within the fusion peptide which served as an internal reference.

In addition to the differences in the extents of labeling of HA2 already noted in Table I, the present analysis also revealed clear differences in the distribution of 3H radioactivity between the NTCB fragments (Fig. 6). Most importantly, acidification of the virus-vesicle incubations at 0 °C led to preferential labeling of the NH2-terminal part of HA2 (Fig. 6, A versus B), a result providing further strong evidence that the fusion peptide inserted into the target membrane bilayer and mediates the acid-induced virus-target membrane adhesion. Consistent with the observation that receptor-free liposomes very weakly bind to viruses at even neutral pH, we found very faint labeling of NTCB1,2 indicating that some HA molecules exposed their fusion peptide at pH 7.4 (Fig. 6A). Very brief incubation (10 s) at pH 5.0 and 23 °C resulted in increased anchor segment labeling indicating that at this point some fusion had already occurred (Fig. 6C). Upon extensive fusion, the majority of the label was associated with this segment (Fig. 6D). Following fluorography of a (separate) dried gel (Fig. 7), excision of the labeled NTCB1,2 and NTCB3, rehydration of the gel slices, and scintillation counting, we estimated that 74% of the HA2-associated radioactivity was within the anchor segment and 26% within the fusion peptide. The fluorography (Fig. 7) also confirmed the above finding, namely that fusion versus anchor peptide labeling ratios change during fusion. Thus, at the onset of fusion of PR8/34 viruses (23 °C, pH 5.0, 10 s), the fusion peptide was somewhat more strongly labeled (65% of total radioactivity) than the anchor segment.

Attempts were also made to analyze the label distributions within the HA2 polypeptide derived from X-31 viruses. Unfortunately, however, it has so far not been possible to achieve clean separation of the corresponding NTCB peptides by SDS-PAGE (Fig. 7). Yet, all the available data indicate that the label distribution patterns are similar to those found for PR8/34.

DISCUSSION

Hydrophobic Photolabeling as a Tool to Study Virus-Target Membrane Interactions—A key issue of current discussions

**Fig. 6.** SDS-polyacrylamide gel electrophoretic separation of NTCB fragments derived from [3H]PTPC/11-labeled HA2. PR8/34 influenza viruses and LUVs consisting of egg-PC/[3H]PTPC/11/GD1a (20:2:1) were mixed and incubated as follows: Panel A, 0 °C, pH 7.3 (5 min); panel B, 0 °C, pH 5.0 (5 min); panel C, 23 °C, pH 5.0 (10 s); panel D, 37 °C, pH 5.0 (5 min). After these treatments, [3H]PTPC/11 was flash-phototivated, and the labeled HA2 was isolated and purified by two-dimensional SDS-PAGE (nonreducing and reducing conditions). Individual [3H]PTPC/11-labeled HA2 samples were supplemented with HA2 selectively labeled with [14C]TUA (this served as an internal reference to control subsequent fragmentation and fragment separation) and subjected to NTCB fragmentation, and the resulting labeled peptides were separated by SDS-PAGE. Individual gel tracks were cut into 2-mm slices, and each slice was treated with NCS tissue solubilizer and then subjected to 3H and 14C scintillation counting. The 3H radioactivity profiles mark the positions of residual uncleaved HA2 (gel slice 4) and of NTCB (broad peak centered at slice 11). The 14H radioactivity profiles show peaks corresponding to uncleaved HA2 (slice 4), NTCB1,2 (gel slices 8 and 9), and NTCB3 (gel slices 10–12).

**Fig. 7.** Separation of HA2 NTCB fragments derived from [3H]PTPC/11-labeled influenza virus X-31 and PR8/34. Viruses and LUVs consisting of egg-PC/[3H]PTPC/11/GD1a (20:2:1) were mixed and incubated under the pre- and post-fusion conditions specified. Then, [3H]PTPC/11 was flash-phototivated, and the labeled HA2 was isolated and purified by two-dimensional SDS-PAGE (nonreducing and reducing conditions). Individual [3H]PTPC/11-labeled HA2 samples were then subjected to NTCB cleavage, and the labeled fragments were separated by SDS-PAGE. After brief staining and destaining, the gel was subjected to fluorography using Amplify (Amersham Corp.) and following the procedure recommended by the manufacturer. The gel was exposed to Kodak X-OMAT films for 6 days at −80 °C.
of virus-induced membrane fusion mechanisms concerns the role of the so-called fusion peptide, a conserved, predominantly hydrophobic segment found in most, but not all, viral fusion proteins. The importance of this issue is further emphasized by the recent discovery of a potential fusion peptide in a protein active in sperm-egg fusion (Blobel et al., 1992). Since it is likely that the fusion peptide somehow interacts with one or both membrane bilayers to fuse the characterization of these presumed interactions is a main objective. This study reports a further effort to characterize these interactions by making use of the technique of hydrophobic photolabeling (for a review, see Brunner (1989)).

Since the initial low pH-induced interaction between viruses and target vesicles may be mediated by only a small number of the viral HA molecules the very weak labeling of the HA2 subunit with a reagent confined to the target membrane was not surprising. However, a question remaining was whether this labeling reflected "true" hydrophobic photolabeling, restricted to membrane-embedded portions of HA2, or resulted from artifactual incorporation of radioactivity. To address this question, we now measured and compared the extents of labeling of the HA2 chain with that of the HA1 polypeptide chain, a component lacking any membrane embedded portion. We found that the radioactivity associated with HA1 was consistently within normal background levels, whereas in all cases significant labeling of the HA2 chain was measured. HA2 labeling exceeded background levels by one to four orders of magnitude. From this we can safely conclude that the HA2-associated radioactivity reflects true photolabeling restricted to segments of HA2 directly in contact with the membrane bilayer.

The absence of any detectable radioactivity in the HA1 polypeptide is most remarkable also from a methodological point of view. First, it demonstrates that the procedure used to purify HA1 and HA2 must have been extremely effective in removing noncovalently associated label and other possible radioactive contaminants. Second, it is likely to reflect also particularly favorable physical, chemical, radioclimical, and photochemical properties of the employed photoreagent ['H]PTPC/11. Moreover, it should be kept in mind also that viruses are metabolically inactive and that it is most unlikely therefore that in the course of an experiment any of the ['H]PTPC/11 is degraded and tritium interconverted into amino acids and proteins. In metabolically active systems, such interconversions may occur and contribute to increased "background" labeling.

The 0 °C, pH 5.0, Prefusion Intermediate.—A first main conclusion that can be drawn from this study is that hydrophobic virus-target membrane attachment results from direct insertion of the NH2-terminal portion of the HA2 polypeptide chain into the target lipid bilayer membranes. Indeed, it is this part of the HA2 chain and not the COOH-terminal membrane anchor which upon acid-induced binding became more strongly labeled by the target membrane restricted probe. Although the precise sites of labeling within the NH2-terminal portion have not been determined, it is more than likely that the label resides within the fusion peptide shown previously to be capable of interacting with membranes (Harter et al., 1989). This finding also rules out the possibility that the measured increase in HA2 labeling upon acidification was due to fusion of a tiny fraction of fusion-compotent viruses or resulted from transfer of traces of reagent into the viral membrane. Indeed, both fusion and reagent transfer would have led to increased labeling of the membrane anchor (NTCB,) and not of the NH2-terminal segment.

We also showed that upon brief acid treatment of X-31 and PR8/34 viruses in the presence or absence of a target membrane, the majority of the HA molecules were still in a native conformation or, in other words, that the acid-induced initial adhesion step is mediated by merely a subpopulation of HA molecules which underwent a conformational change and exposed their fusion peptide. We do not know what fraction of the HA became actually involved in binding of the viruses to the target membrane. However, since the yields of BHA obtained from native and acid-treated viruses were similar, we think that only a (small) minority of the HA molecules underwent this change.

That only a subpopulation of the HA molecules are converted into their acid form upon pH 5.0 treatment at 0 °C is not inconsistent with previous data and could explain why viruses treated with acid at low temperature are not inactivated and why the majority of the spikes do not change their structure as viewed by electron microscopy. In contrast, acid treatment at elevated temperature results in the inactivation of the viruses and in the disappearance of the layer of well structured HA spikes (Stegmann et al., 1987).

It is not known why at low temperature only a subpopulation of the HA molecules are converted into the acid form. However, since the initial binding of viruses to the membranes is very rapid (within seconds), it is very unlikely that we are observing a simple kinetic effect. An interesting yet speculative idea is that this rapid change is triggered somehow by prebinding of the HA1 subunit of HA to receptor molecules (G0) on the target membrane. Such a mechanism may have been discovered recently for HIV-1 virions where soluble CD4 (receptor) induces dissociation of gp120, a step that could result in an exposure of the fusion peptide (homologue) of gp41 (Moore et al., 1990). Clearly, alternative mechanisms, e.g. based on a structural heterogeneity in HA, must be considered as well.

Implications for Fusion Mechanism.—A key question arising from the present study is whether those HA molecules involved in initial binding are also responsible for fusion. Arguments in support of this view were provided by Stegmann and colleagues (1991), who interpreted the prefusion lag-phase as reflecting the time needed to rearrange the target membrane bound trimers to assemble into a functional fusion complex. Although such rearrangements may occur and be essential, our results indicate that a possibly important issue may not have been adequately considered so far. Thus, the initial interaction of the viruses with the target membranes involves only a relatively small number of HA2 molecules. However, during further steps in preparation of fusion, or during the process itself, additional changes occur that lead to far more extensive HA2-membrane interactions than observed after initial binding.

In the case of PR8/34 viruses labeling of HA2 during fusion increased about 130-fold (from 2.5 to 325 nCi/nmol (Table 1)). Since 26% of the HA2-associated radioactivity was associated with the NH2-terminal portion (fusion peptide), it follows that this segment was at least 30 times more strongly labeled after fusion than after initial adhesion. We do not think that structural rearrangements of the membrane-embedded fusion peptides alone can account for such an increase in labeling and, therefore, we interpret this to indicate that additional extensive membrane insertion takes place. This is supported also by the observation that after fusion most of the HA molecules were susceptible to bromelain digestion. It is not yet known when this insertion occurs and whether it is directed to the target membrane, the viral membrane, or to the fused membrane. However, it appears, at least, to be a reasonable possibility that the fusion peptides released after
the initial attachment become inserted into the viral rather than into the target membrane. With the present design of the experiments (label within the target bilayer) such interactions would remain invisible until fusion and mixing of the membrane constituents takes place. Insertion of the fusion peptide into the viral membrane has been considered as a possibility also by Ruigrok et al. (1986).

Future Directions—A main task of future work will be the further characterization of the fusion peptide membrane interactions, both in structural and kinetic terms. Initial attempts to incorporate the photoreagent into the viral membrane in order to detect possible (prefusion) interactions of the fusion peptide with this membrane were only partially successful. Although it was possible to incorporate the nonexchangeable reagent [3H]PTPC/11 into the viral membrane by incubation of sonicated [3H]PTPC/11 liposomes and viruses in the presence of phospholipid exchange protein from beef liver, we were not able so far to completely remove the partially [3H]PTPC/11-depleted “donor” liposomes after the exchange procedure, a finding which again illustrates the fact that experiments with model systems cannot accurately reflect the biological situation. Therefore, the search for nonexchangeable photoactivatable reagents of higher specific radioactivity and for techniques to incorporate them into biological membranes remains an important task.

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