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# Interaction of Influenza Virus Hemagglutinin with a Lipid Monolayer. A Comparison of the Surface Activities of Intact Virions, Isolated Hemagglutinins, and a Synthetic Fusion Peptide

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**ABSTRACT:** In the infectious entry pathway of influenza virus, the low pH of the endosomal compartment induces an irreversible conformational change in influenza virus hemagglutinin, leading to fusion of viral and endosomal membranes. In the current report, we characterized the low-pH-induced activation of hemagglutinin of influenza strain X31 by studying its interaction with a lipid monolayer. The surface activities of virions, of isolated hemagglutinins and its proteolytic fragments, and of a synthetic peptide mimicking the amino terminus of subunit 2 of hemagglutinin are compared. The data indicate that the surface activity of both virions and isolated hemagglutinin develop as a result of the low-pH-induced conformational change in hemagglutinin. The surface activity of isolated hemagglutinin is mainly caused by penetration into the lipid monolayer of protein domains other than the amino terminus of subunit 2 of hemagglutinin; domains in subunit 1 may be involved. The surface activity of virions appears to be a secondary effect of the conformational change and is explained by assuming a net transfer of viral lipids to the lipid monolayer.

The fusogenic properties of the fusion protein of influenza virus have been described in the utmost detail (Wiley & Skehel, 1987; Gething et al., 1988). Influenza virus enters and infects a host cell during receptor-mediated endocytosis (Matlin et al., 1981). As a result of the gradual acidification of the endosomal compartment, viral and endosomal membranes fuse, and the viral nucleocapsid is released into the cytoplasm. The influenza hemagglutinin glycoprotein (HA)<sup>1</sup> is solely responsible for the low-pH-activated fusion activity of influenza virus (White et al., 1982a). The molecular structure of the ectodomain of HA (influenza strain X31), released after bromelain treatment of intact virus (BHA), has been determined at neutral pH by X-ray crystallography with 3-Å resolution (Wilson et al., 1981). The HA spike protein is a trimeric complex of identical HA monomers extending approximately 13.6 nm from the viral membrane. The N-terminus of HA<sub>2</sub> (NHA<sub>2</sub>) is thought to be directly involved in the induction of membrane fusion and is located at the trimeric interface approximately 3 nm from the base of the spike protein.

Biochemical, biophysical, immunological, and morphological studies have shown that the HA spike protein undergoes an irreversible conformational change upon exposure to low pH (Daniels et al., 1983; Ruigrok et al., 1986b; Wharton et al., 1988b). Previously hidden parts of HA are exposed; the spike becomes protease-sensitive and acquires amphiphilic properties and the ability to bind detergents and lipid vesicles (Skehel et al., 1982; Doms et al., 1985). Taken together, the data

indicate that at low pH the interactions between the monomers in the trimeric complex are reduced, causing the globular head of the spike to open up; in addition, the N-terminus of HA<sub>2</sub> becomes exposed (Wiley & Skehel, 1987; White & Wilson, 1987). Although the exact fusion mechanism is unknown, several lines of evidence suggest a direct involvement of NHA<sub>2</sub> in the induction of membrane fusion. This 23 amino acid long hydrophobic stretch at the N-terminus of HA<sub>2</sub> has a highly conserved amino acid composition, nonconservative point mutations in NHA<sub>2</sub> strongly affecting fusogenicity (Gething et al., 1986). Synthetic peptides mimicking NHA<sub>2</sub> are capable of inducing lipid mixing of small unilamellar vesicles (Lear & De Grado, 1987; Murata et al., 1987; Wharton et al., 1988a). Recently, experiments using photoaffinity labeling have also shown that NHA<sub>2</sub> mediates the binding of BHA to lipid vesicles at low pH (Harter et al., 1989). On the basis of the aforementioned data, it has been suggested that the induction of membrane fusion by HA involves penetration of NHA<sub>2</sub> into the target membrane.

The results obtained by photoaffinity labeling of vesicle-bound BHA suggested that NHA<sub>2</sub> interacts with the outer lipid monolayer of the target membrane only [Harter et al., 1989; Brunner, 1989; also see Doms et al. (1985)]. In order to gain insight into the mechanism of HA-mediated membrane fusion, we have studied the interaction of HA with a lipid monolayer spread at an air-water interface. Penetration of a protein into a lipid monolayer can be followed by monitoring the resulting change in surface pressure [due to interaction of the protein with the phospholipid headgroups and/or the

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<sup>1</sup> Abbreviations: BHA, ectodomain of HA obtained by bromelain treatment of intact virus; Chol, cholesterol; DOPC, dioleoyl-PC; DOPE, dioleoyl-PE; HA, influenza virus hemagglutinin; HA<sub>1</sub>, subunit 1 of HA; HA<sub>2</sub>, subunit 2 of HA; NHA<sub>2</sub>, amino-terminal "fusion sequence" of HA<sub>2</sub>; PC, phosphatidylcholine; PE, phosphatidylethanolamine;  $\pi$ , surface pressure;  $\pi_e$ , exclusion  $\pi$ ;  $\pi_i$ , initial  $\pi$ ;  $\Delta\pi$ , change in  $\pi$ ; Spm, sphingomyelin; type II, inverted lipid.

glycerol backbone region of the lipid monolayer; see Kimelberg and Papahadjopoulos (1971) and Demel (1982)]. Direct kinetic data on protein insertion into a lipid environment can be obtained, and only small amounts of material (2–10 nmol of lipid, 1–20  $\mu$ g of protein) are required.

Despite the enormous amount of research carried out on virus-membrane fusion, a number of questions concerning the mechanism of HA-induced membrane fusion remain unanswered. One of the most urgent questions concerns the location of NHA<sub>2</sub> after low-pH activation; does it penetrate the target membrane [e.g., see Stegmann et al. (1989, 1990)], the viral membrane [e.g., see Ruigrok et al. (1988)], or no membrane at all (Bentz et al., 1990; White, 1990; Bentz, 1991)? Second, other, as yet unidentified, parts of HA could be involved in the induction of membrane fusion in cooperation with NHA<sub>2</sub> (Gething et al., 1988). Finally, even in its low-pH conformation, HA is still approximately 11 nm long (Ruigrok et al., 1988), and it is not clear how viral and target membranes can come together and fuse without being sterically hindered by HA. In order to address these questions, the surface activity of HA was characterized in some detail using a lipid monolayer spread at an air-water interface for the target. The surface activity of intact virions of influenza X31, of isolated HA spikes, and of proteolytic fragments of HA and the surface activity of a 20 amino acid synthetic peptide mimicking NHA<sub>2</sub> of influenza X31 were compared.

#### MATERIALS AND METHODS

**Materials.** Bovine brain sphingomyelin (Spm) was obtained from Sigma Chemical Co. (St. Louis, MO). Cholesterol (Chol) was from Baker Chemical Co. (Phillipsburg, NJ). Dioleoylphosphatidylcholine (DOPC) was synthesized from egg yolk phosphatidylcholine as described (Van Deenen & De Haas, 1964). Phospholipids were pure as judged by high-performance thin-layer chromatography. All other reagents and chemicals were of analytical grade.

**Viruses.** Wild-type influenza A, strain X31 (H<sub>3</sub>N<sub>2</sub>), and a fusion mutant designated 1a (Daniels et al., 1985) were used in this study. Viruses were grown in the allantoic cavity of embryonated eggs and purified by isopycnic centrifugation using sucrose (Skehel & Schild, 1971) and/or potassium tartrate density gradients (Burger et al., 1988). Viruses (10 mg of protein/mL) were stored at 4 °C in PBS (10 mM phosphate/150 mM NaCl, pH 7.4) containing 0.01% NaN<sub>3</sub>. The protein concentration was determined using the method of Lowry (Lowry et al., 1951).

**HA-Rosettes, BHA, Proteolytic Fragments, and Low-pH Preincubation.** Rosettes of complete hemagglutinin (HA<sub>1</sub> 1–328 + HA<sub>2</sub> 1–221; MW of monomer 71K) were obtained from influenza X31 virus as described before (Wharton et al., 1986). Briefly, virus was solubilized in 0.5% (w/v) Brij36T. After centrifugation, the supernatant was passed down an affinity chromatography column to remove the viral neuraminidase. Brij36T was exchanged for octyl glucoside on a sucrose gradient, and the latter was removed by dialysis in the presence of Amberlite XAD-2 beads to adsorb the detergent. During removal of the detergent, the HA molecules are aggregated into rosettes via interaction of the HA<sub>2</sub> C-terminal membrane-spanning regions (Ruigrok et al., 1986b). Not all detergent can be removed by dialysis, but the amount of residual detergent has been shown to be less than five molecules of octyl glucoside per HA monomer (Wharton et al., unpublished results).

The ectodomain of HA (BHA) was isolated from X31 and mutant 1a virus by bromelain treatment and purified by sucrose gradient centrifugation, following standard procedures

(Skehel et al., 1982; Brand & Skehel, 1972). BHA (HA<sub>1</sub> 1–328 + HA<sub>2</sub> 1–175; MW of monomer 66K) is a water-soluble fragment that lacks the 46 C-terminal amino acid residues of HA<sub>2</sub> which normally link HA to the viral membrane. Upon lowering the pH, HA becomes protease-sensitive, and after protease treatment, several proteolytic fragments of HA can be isolated. The experimental procedures designed to obtain these fragments have been extensively described elsewhere (Skehel et al., 1982; Wharton et al., 1988b). First of all, digestion of low-pH-treated virus (X31) with trypsin at pH 7.4 was used to isolate the upper parts, or tops (HA<sub>1</sub> 28–328; MW 39K), of the HA spike protein. The trypsin digestion was stopped by adding an equal weight of soybean trypsin inhibitor. After removal of the virus by centrifugation, the tops were recovered in the supernatant. At low pH, BHA aggregates into rosettes (Skehel et al., 1982; Ruigrok et al., 1986b), and trypsin digestion was used to remove the tops and obtain rosettes of predominantly BHA<sub>2</sub> ("bottoms", HA<sub>1</sub> 1–27 + HA<sub>2</sub> 1–175; MW of monomer 27K). Bottoms were separated from the tops by sucrose gradient centrifugation. In each case, the samples were concentrated and the sucrose removed by dialysis against PBS. HA-rosettes, BHA, and proteolytic fragments were stored in PBS containing 0.01% NaN<sub>3</sub> at a protein concentration of 0.2–2 mg/mL. The protein concentration was calculated from the absorbance at 280 nm by considering the number of Trp and Tyr residues present (Ruigrok et al., 1986a).

In some experiments, virus or isolated HA was preincubated at low pH for 10–30 min at 37 °C. Acidification and re-neutralization were carried out by adding the appropriate amounts of 100 mM citric acid and 200 mM sodium hydroxide, respectively.

**Synthetic Fusion Peptide.** A 20 amino acid peptide with an amino acid sequence corresponding to the sequence of NHA<sub>2</sub> (X31) was synthesized as described (Wharton et al., 1988a). The sequence was GLFGAIAGFIENGWEGMIDG (MW 1767). Amino acid analysis was performed according to standard procedures and gave the following result (expected number of amino acids is given in parentheses): D 2.09 (2), E 2.11 (2), G 5.80 (6), A 2.17 (2), M 0.95 (1), I 2.75 (3), L 1.04 (1), F 1.97 (2), W not determined (1). The peptide was dissolved in DMSO at a concentration of approximately 1 mg/mL. The protein concentration was determined by performing a (quantitative) amino acid analysis in the presence of a known quantity of norleucine. The peptide may be considered to be >90% pure. By way of a functional assay, the fusogenicity of the peptide toward sonicated lipid vesicles was checked, and confirmed, using the experimental setup described earlier (Wharton et al., 1988a; result not shown).

**Monolayer Measurements.** The surface pressure of the monolayer at the air-water interface was measured by the Wilhelmy plate method, using a Cahn 2000 electrobalance [see Demel (1982) for a concise review on methodology and possibilities]. Constant-area experiments were performed with a 5-mL Teflon trough, 5 cm in diameter. For measurements at constant surface pressure, a 5 × 15 cm Teflon trough (65 mL) and a computer-controlled movable Teflon barrier were used. Stock solutions of lipids were prepared in chloroform/methanol (1:1, v/v). The phospholipid phosphorus concentration was determined using the method of Böttcher et al. (1961). The final lipid mixtures were made at a concentration of approximately 1 mg/mL (w/v) in chloroform and contained less than 10% methanol. Lipid mixtures were spread at the air-water interphase using a small glass capillary. Protein (1–50  $\mu$ g) was injected into the subphase using a

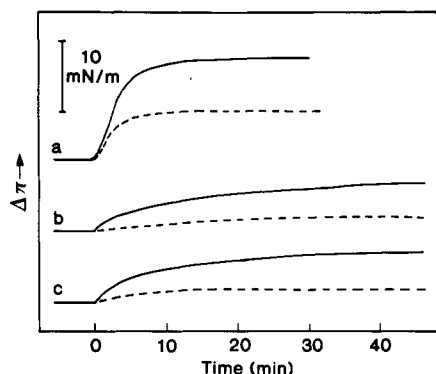


FIGURE 1: Monolayer surface pressure increase induced by intact virions, BHA, and HA-rosettes. Protein was injected ( $t = 0$ ) at pH 5.0 (solid lines) or pH 7.4 (dashed lines). (a) Intact virus (150  $\mu\text{g}$  of protein), (b) BHA (10  $\mu\text{g}$  of protein), and (c) HA-rosettes (10  $\mu\text{g}$  of protein) from influenza X31. Standard composition of the monolayer (DOPC/Spm/Chol, 1:1:2 molar ratio), initial surface pressure  $\pi_i$  of 20 mN/m, 37  $^{\circ}\text{C}$ .

Hamilton syringe, and the equilibrium value of surface pressure increase was determined (time after injection: 10–20 min for intact virus, 30–40 min for isolated HA and proteolytic fragments, 5–10 min for the synthetic fusion peptide). PBS was used, adjusted to the desired pH using 100 mM citric acid. All experiments were performed at 37  $^{\circ}\text{C}$ , under constant stirring.

## RESULTS

The lipid composition of the monolayer was chosen so as to approximate the lipid composition of the outer monolayer of a target membrane. A mixture of phosphatidylcholine (PC), sphingomyelin (Spm), and cholesterol (Chol) in a molar ratio of 1:1:2 was used ("standard composition"), which is close to the lipid composition of the outer monolayer of the erythrocyte membrane (Verkleij et al., 1973). Two different HA spike preparations were used, the water-soluble trimeric ectodomain of HA (BHA) and so-called HA-rosettes. HA-rosettes are obtained by detergent extraction (see Materials and Methods) during which 4–12 intact HA spikes aggregate into a rosette via interaction of the membrane-spanning regions (Ruigrok et al., 1986b). Consequently, HA in these HA-rosettes is in its native orientation, with the top of the spike pointing outward.

The results of a typical monolayer experiment are shown in Figure 1. Injection of virions, BHA, or HA-rosettes into the subphase at pH 5.0 resulted in a large increase in surface pressure of the lipid monolayer (solid lines). In contrast, injection at neutral pH was followed by a relatively small increase in surface pressure (dashed lines).

**Low-pH Activation of HA and Development of Surface Activity.** The pH profiles of the surface pressure increase were determined. In addition to preparations of influenza X31, virions and BHA of a mutant of influenza X31 were tested. This mutant, designated mutant 1a, is able to infect cells grown in the presence of the weak base amantadine, which raises the pH of endosomes (Daniels et al., 1985). A single amino acid substitution, D to G at position 112 of HA<sub>2</sub>, destabilizes the trimeric spike complex and causes HA to change its conformation at approximately 0.5 pH unit higher than wild-type HA (Daniels et al., 1985; Weis et al., 1990).

The pH profiles of the surface activity of influenza X31 and mutant 1a virus are shown in Figure 2. Both X31 and mutant 1a virus had significant surface activity at neutral pH (6.7 and 12.7 mN/m, respectively), but to facilitate comparison, only the low-pH-induced surface pressure increase is considered

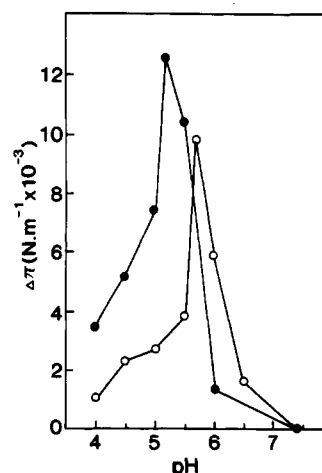


FIGURE 2: pH profiles of surface pressure increase induced by X31 and mutant 1a virus. Values of  $\Delta\pi$  were normalized by subtracting  $\Delta\pi$  measured at pH 7.4 (6.7 mN/m for X31 and 12.7 mN/m for mutant 1a). (●) X31 virus; (○) mutant 1a virus (200  $\mu\text{g}$  of protein). For experimental conditions, see the legend to Figure 1.

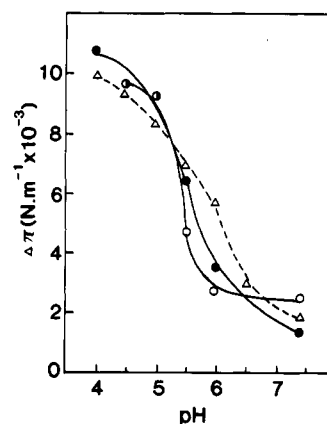


FIGURE 3: pH profiles of surface pressure increase induced by isolated HA of influenza X31 and mutant 1a. (●) HA-rosettes; (○) BHA of X31; (Δ) BHA of mutant 1a (20  $\mu\text{g}$  of protein). Experimental conditions were the same as those described in the legend to Figure 1.

in Figure 2. It should be noted that the fusion activity of influenza virus is absolutely dependent on low pH and therefore only the low-pH-induced surface activity may directly bear relevance to the fusion process. The surface activity of both viruses is characterized by a steep pH dependence and a clear pH optimum. In the case of influenza X31, an optimum is found at approximately pH 5.2. The pH profile of the mutant 1a virus is shifted upward by approximately 0.5 pH unit [(○) in Figure 2]. A steep pH dependence is also observed for the surface activity of BHA and HA-rosettes (Figure 3). The curves are sigmoidal, and closely match the pH profiles of protease sensitivity (for BHA; Doms et al., 1985) and fusion activity (for HA-rosettes; Wharton et al., 1986). The pH profile of BHA obtained from mutant 1a virus is once more shifted upward to higher pH values [(Δ) in Figure 3].

The pH values at which half-maximal fusion activity, half-maximal trypsin sensitivity [see Wharton et al. (1986)], and half-maximal surface activity (this study) are reached are in good agreement (deviations  $\leq 0.2$  pH unit); in the case of mutant 1a, these pH values are shifted upward approximately 0.5 pH unit. These data indicate that surface activity develops as a result of the low-pH-induced conformational change and activation of HA. It is important to note that a direct comparison of the kinetics with which surface activity develops (Figure 1) and the kinetics with which membrane fusion ac-

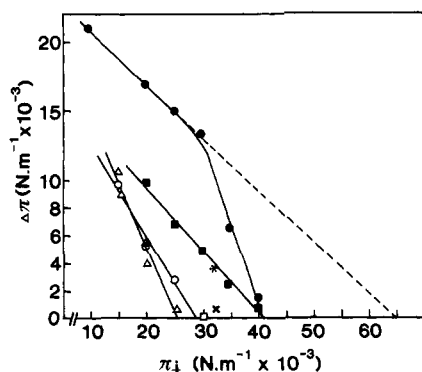


FIGURE 4: Monolayer surface pressure increase as a function of initial surface pressure. A nearly saturating amount of protein was injected at pH 5.0. ( $\Delta$ ) BHA; ( $\circ$ ) HA-rosettes of X31 (20  $\mu$ g of protein); ( $\blacksquare$ ) synthetic fusion peptide (6.4  $\mu$ g of protein); ( $\bullet$ ) X31 virus (150  $\mu$ g of protein). The exclusion pressure of X31 virus is found by extrapolation of the first four points (dashed line). ( $\square$ ) HA<sub>1</sub> 28–328 ("tops", 12  $\mu$ g of protein); ( $\times$ ) large excess of HA-rosettes (200  $\mu$ g of protein), (\*) same molar amount of synthetic fusion peptide (5.3  $\mu$ g of protein). Curves were determined by linear regression analysis. Standard composition of the monolayer, 37 °C.

tivity develops [reaching equilibrium within a few minutes; e.g., see Stegmann et al. (1986)] is not justified. The slower kinetics of surface activity are at least partially due to an unfavorable lipid surface area:buffer volume ratio in the monolayer setup as compared to that in vesicular systems used to monitor virus membrane fusion.

**Dependence of Surface Activity on Initial Surface Pressure: Isolated HA versus Intact Virions and the Fusion Peptide.** The surface activities of HA, intact virions, and the fusion peptide at low pH were determined as a function of the initial surface pressure of the lipid monolayer (Figure 4). A similar dependence on initial surface pressure, of the surface activity of isolated HA, intact virions, and the fusion peptide, would suggest a similar origin of their surface activity.

Influenza X31 virions ( $\bullet$ ) are surface-active, even when high initial surface pressures of the lipid monolayer are used. Extrapolation (dashed line) yields an exclusion pressure ( $\pi_e$ ), the pressure above which virions are no longer capable of inducing a surface pressure increase, of 65 mN/m. This value of  $\pi_e$  is considerably higher than the collapse pressure ( $\pi_c$ ) of the lipid monolayer (about 45 mN/m; not shown), and is therefore only of theoretical importance. The biphasic shape of the curve may be explained by the fact that the sum of  $\pi_i$  and  $\Delta\pi$  will not exceed  $\pi_e$ . Indeed, if much less virus was injected (30  $\mu$ g of protein instead of 150  $\mu$ g), a linear relationship between  $\pi_i$  and  $\Delta\pi$  was found (not shown). In contrast, BHA ( $\Delta$ ) and HA-rosettes ( $\circ$ ) are incapable of inducing a surface pressure increase at initial surface pressures higher than 30 mN/m. Finally, the synthetic fusion peptide mimicking NHA<sub>2</sub> of influenza X31 ( $\blacksquare$ ) is surface-active at high  $\pi_i$ 's, and  $\pi_e$  was about 41 mN/m, a value intermediate between those of isolated HA and intact virions.

These data reveal remarkable differences in surface activity between isolated HA, intact virions, and the fusion peptide and suggest that the origin of their surface activity is in fact different.

**Surface Activity of Isolated HA: Low-pH Preincubation and Proteolytic Fragments.** A number of experiments were performed to reveal the direct cause of the HA-induced increase in surface pressure of the lipid monolayer. In this context, it should be noted that the increase in surface pressure of the monolayer is due to protein-lipid interaction and not simply to the surface activity of HA itself. This follows from the fact that in the absence of a lipid monolayer, the maximally

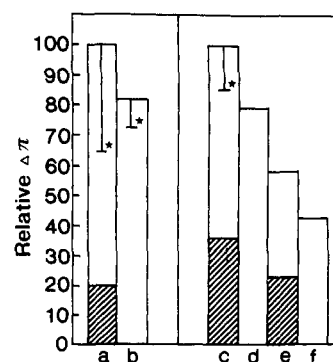


FIGURE 5: Relative surface activity of proteolytic fragments of BHA and effect of low-pH preincubation. Protein (0.3 nmol of monomer) was injected at pH 7.4 (striped bars) or pH 5.0 (open bars).  $\Delta\pi$  is expressed relative to  $\Delta\pi$  measured at pH 5.0 for HA-rosettes (in a and b) or BHA (in c–f). (a) HA-rosettes of X31; (b) HA-rosettes of X31 preincubated for 10 min at pH 5.0 (37 °C) and reneutralized; (c) BHA of X31; (d) BHA of X31 preincubated and reneutralized [as in (b)]; (e) tops (HA<sub>1</sub> 28–328) of influenza X31; (f) bottoms (HA<sub>2</sub> 1–175 + HA<sub>1</sub> 1–27) of influenza X31. (★) SEM as indicated (4–5 determinations); in all other cases, the SEM is estimated to be  $\pm 15\%$ . The large spread in (a) is due to batch differences. For experimental conditions, see the legend to Figure 1. It should be noted that the surface activity of HA-rosettes at pH 5.0 is slightly higher than that of BHA (on the average by approximately 10%).

attainable surface pressure was found to be only 22 mN/m (BHA at pH 5.0; results not shown), whereas much higher final surface pressures were reached in the presence of a lipid monolayer (e.g., Figure 3).

First, the effects of low-pH preincubation on the surface activity of BHA and HA-rosettes were examined. Upon low-pH preincubation in the absence of a target membrane (e.g., 10 min, pH 5.0, 37 °C), BHA aggregates via NHA<sub>2</sub> into rosettes containing 6–12 BHA spikes (Ruigrok et al., 1986b, 1988). Similarly, low-pH preincubation of HA-rosettes leads to movement of NHA<sub>2</sub> to the center of the HA-rosettes (Ruigrok et al., 1986b). Assuming a spherical shape of BHA and HA-rosettes, the presence of NHA<sub>2</sub> near the center of these rosettes after low-pH preincubation should result in a strongly reduced possibility for NHA<sub>2</sub> to interact with the lipid monolayer. However, only a slight reduction by about 20% in the surface activity of HA-rosettes (cf. Figure 5a,b) and BHA (cf. Figure 5c,d) is found. We conclude that the surface activity of BHA and HA-rosettes is (almost) completely due to insertion of parts of HA other than NHA<sub>2</sub> into the lipid monolayer. This conclusion is corroborated by the results mentioned earlier (see Figure 4); if the surface activity of isolated HA would arise from penetration of NHA<sub>2</sub> into the lipid monolayer, surface activity should not have been confined to low initial surface pressures of the monolayer (recall that the fusion peptide is surface-active up to 41 mN/m).

In order to determine which part(s) of HA is (are) responsible for its low-pH-induced surface activity, proteolytic fragments of BHA were isolated, and their surface activity was determined (Figure 5). The data clearly show that not only the BHA<sub>2</sub>-containing fragment (f) but also the HA<sub>1</sub>-containing fragment (e) is quite surface-active. Interestingly, the surface activity of the HA<sub>1</sub>-containing fragment ("tops") is strongly pH-dependent (e). Consequently, the surface activity of isolated HA at low pH appears to be at least partially due to protein domains in HA<sub>1</sub>. The latter domains (and possibly also parts of HA<sub>2</sub> other than NHA<sub>2</sub>) could play an important role in HA-mediated membrane fusion, in addition to the role of NHA<sub>2</sub>.

On the basis of the results of photoaffinity labeling experiments (Harter et al., 1989), one would expect a penetration

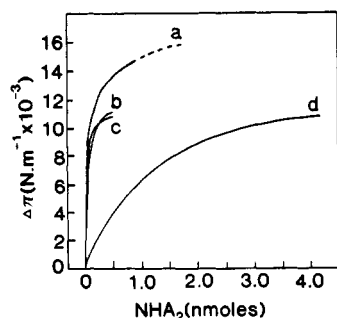


FIGURE 6: Comparison of the surface activities of virus, isolated HA, and the (synthetic) fusion peptide of X31. Protein was titrated in at pH 5.0. The surface pressure increase is shown as a function of the amount of  $\text{NHA}_2$  present. The HA content of intact virus was taken to be 15% of the total protein. (a) X31 virus; (b) HA-rosettes, (c) BHA of X31; (d) synthetic fusion peptide. Experimental conditions as described before (see Figure 1).

of  $\text{NHA}_2$  into the lipid monolayer at low pH (see the introduction). The surface activity of  $\text{NHA}_2$  is not simply obscured by a much larger surface activity of other parts of HA, e.g., in  $\text{HA}_1$ . A large excess of HA-rosettes added at high  $\pi_i$  (32 mN/m;  $\text{HA}_1$  is not surface-active at this pressure, see Figure 4) did not result in a significant increase in surface pressure, whereas the same molar amount of synthetic fusion peptide did induce a clear surface pressure increase [cf. (X) and (\*) in Figure 4]. This result suggests that  $\text{NHA}_2$  of HA-rosettes does not penetrate into the lipid monolayer (also see Discussion).

**Surface Activity of Intact Virions: Comparison with Isolated HA and the Synthetic Fusion Peptide.** The characteristics of surface activity observed for intact virions greatly differ from those observed for isolated HA or for the synthetic fusion peptide. As mentioned before, differences are found in the dependence on the initial surface pressure of the lipid monolayer. Second, if the surface pressure increase is plotted against the amount of  $\text{NHA}_2$  (or fusion peptide) present (Figure 6), virions appear to be more surface-active than isolated HA (compare curve a with curves b and c), and much more surface-active than the synthetic fusion peptide (d). Third, low-pH preincubation only results in a small decrease in the surface activity of isolated HA (Figure 5), whereas the surface activity of intact virions is greatly reduced (i.e., reduced to a value close to the surface activity at neutral pH; see Figure 7). These data suggest that in analogy to isolated HA, the surface activity of intact virions is not a direct effect of the penetration of  $\text{NHA}_2$  into the lipid monolayer (if it occurs). Moreover, it does not seem to be directly caused by the interaction of activated HA with the lipid monolayer at all.

In the intact virion, low pH will lead to the characteristic conformational change in HA, but in addition virions will become fusion-active. We propose that the surface activity of intact virions results from a net transfer of constituents of the viral membrane to the lipid monolayer. In order to get some idea of the amount of viral protein (HA) and/or viral lipids that might insert into the lipid monolayer, an experiment was performed in which the surface pressure of the monolayer (32 mN/m) was kept constant throughout the experiment. The increase in surface area of the lipid monolayer necessary to maintain the surface pressure was monitored after injection of influenza X31 virions into the subphase at low pH (Figure 8). Considering the HA content of intact virus, 10–15% of total protein, the increase in surface area is 42–62  $\text{\AA}^2$  per HA monomer. If one assumes that the monolayer at its final surface area is completely saturated with virions, 110 nm in diameter, and having 400 HA spikes per virion (Ruigrok et

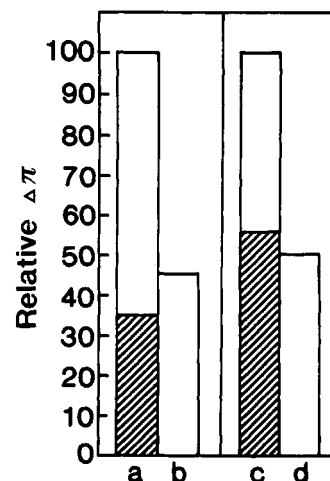


FIGURE 7: Low-pH preincubation of influenza X31 and mutant 1a virus. Virus (150  $\mu\text{g}$  of protein) was injected at pH 7.4 (striped bars) or low pH [open bars; pH 5.2 in (a) and (b); pH 5.7 in (c) and (d)].  $\Delta\pi$  is expressed relative to  $\Delta\pi$  measured at low pH for X31 (a and b) or mutant 1a (c and d). (a) X31 virus; (b) X31 virus preincubated at pH 5.0, 10 min, 37  $^\circ\text{C}$ ; (c) mutant 1a virus; (d) mutant 1a virus preincubated at low pH [as in (b)]. The accuracy of the data is estimated to be  $\pm 10\%$  (SEM). Experimental conditions as described before (see Figure 1).

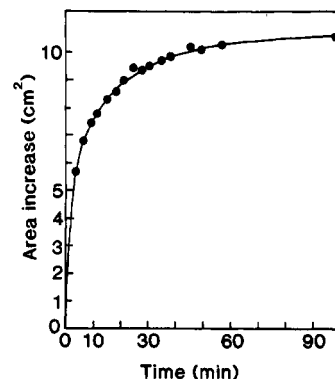


FIGURE 8: Monolayer surface area increase induced by X31 virus. Virus (2 mg of protein) was injected ( $t = 0$ ) at pH 5.0. The surface pressure (32 mN/m) was kept constant, and the surface area increase of the monolayer was followed in time ( $\bullet$ ). The initial surface area of the lipid monolayer was 31.3  $\text{cm}^2$ . Standard composition of the monolayer, 37  $^\circ\text{C}$ .

al., 1984), it can be calculated that 20–30% of the added virions are bound to the monolayer. These spherical virions interact with a planar monolayer, and no more than 10% of their HA spikes should be involved in this interaction. With these assumptions, the area increase of the monolayer amounts to 1400–3100  $\text{\AA}^2$  per interacting HA monomer. This simple estimation once more does not favor the penetration of  $\text{NHA}_2$  into the lipid monolayer as the direct cause of surface pressure increase; penetration of the 23 amino acid  $\text{NHA}_2$  would not be expected to induce an increase in surface area of this magnitude. The latter is supported by the results of experiments in which pure peptide monolayers were compressed, and the limiting molecular area of the synthetic fusion peptide was found to be 290  $\text{\AA}^2$  (at pH 5.0; Burger et al., 1991). Instead, the large increase in surface area is likely due to activation of the fusogenic properties of HA followed by a net transfer of viral lipids to the lipid monolayer. Assuming a mean molecular area of 45  $\text{\AA}^2$  for a lipid molecule (phospholipid/Chol mixture in a 1:1 molar ratio; Demel et al., 1972), a surface area increase of 1400–3100  $\text{\AA}^2$  per HA monomer could then be the result of a net transfer of 30–70 lipid molecules per interacting HA monomer.

## DISCUSSION

**Influenza HA: Surface Activity and Biological Activity Are Closely Related.** The aim of the current study was to gain insight into the initial interaction of influenza HA with its target membrane. This interaction appears to be confined to the outer leaflet of the membrane bilayer (Doms et al., 1985; Brunner, 1989), and therefore a lipid monolayer spread at the air–water interface is an attractive model system to use. The data thus obtained on HA–lipid interaction are only valuable if they can be correlated with the well-known properties of HA, i.e., the low-pH-induced conformational change and resulting fusion activity. This appears to be the case; the pH profiles of surface activity, protease sensitivity, and fusion activity closely match, indicating that the development of surface activity, the conformational change, and the fusion activity of HA are interrelated phenomena. An important clue concerning the origin of surface activity is found upon studying the dependence of surface activity on the initial surface pressure of the lipid monolayer. Marked differences are observed between isolated HA, intact virions, and a 20 amino acid synthetic peptide mimicking NHA<sub>2</sub>. In particular, isolated HA is not surface-active if the initial surface pressure of the lipid monolayer exceeds 30 mN/m. This result strongly suggests that the direct cause of surface activity is different for isolated HA as compared to intact virions.

It should be noted that, on the basis of indirect evidence, the surface pressure that exists in a leaflet of a biomembrane is assumed to be about 32 mN/m [Demel et al., 1975; also see Blume (1979) and Seelig (1987)]. The fact that isolated HA is not surface-active at this pressure of the lipid monolayer thus suggests that the surface activity measured at lower surface pressures may be of little relevance to HA–membrane interaction. However, direct data on the surface pressure in a biomembrane are not available, and since the exclusion pressures measured for isolated HA are close to 32 mN/m, we conclude that the surface activity of (isolated) HA may very well turn out to be important in HA-mediated membrane fusion.

**Surface Activity of Isolated HA: Role for Parts of HA Other than the N-Terminus of HA<sub>2</sub>.** Low-pH preincubation and proteolytic digestion of isolated HA have been used to determine which part of HA is responsible for its surface activity. Low-pH preincubation in the absence of a lipid target results in aggregation of BHA via NHA<sub>2</sub> (Ruigrok et al., 1988) and in the movement of NHA<sub>2</sub> toward the center of an HA-rosette (Ruigrok et al., 1986b); in both cases, the possible interaction of NHA<sub>2</sub> with the lipid monolayer should be greatly hindered. The fact that the surface activity of BHA and HA-rosettes is hardly affected by a low-pH preincubation therefore strongly discounts the involvement of NHA<sub>2</sub>. The latter is corroborated by a remarkable difference in the dependency on the initial surface pressure between isolated HA and the synthetic fusion peptide (see Results). The relatively high surface activity of an HA<sub>1</sub>-containing proteolytic fragment of BHA instead suggests that domains in HA<sub>1</sub> become surface-active at low pH. It is worthwhile mentioning that circular dichroism and infrared spectroscopy have shown that the secondary structure of the proteolytic fragments used in this study is almost identical to their secondary structure in intact BHA (Wharton et al., 1988b).

The possible presence of surface-active sites in HA<sub>1</sub> is also suggested in experiments using a hydrophobic, but not lipid-linked, photoaffinity label to study BHA–vesicle interaction (Boulay et al., 1987). At low pH, BHA<sub>2</sub> is labeled, and sometimes labeling of HA<sub>1</sub> is observed [Boulay et al., 1987;

Wharton et al., unpublished results; cf. Harter et al. (1989)]. If lipid-linked probes are used, only (N)HA<sub>2</sub> is labeled (Harter et al., 1989). These results suggest that low pH exposes hydrophobic sites in HA<sub>1</sub> but that these sites do not deeply penetrate the vesicle bilayer. These sites could play an important role in HA-mediated membrane fusion, e.g., by cross-linking viral and target membrane (see later).

Lowering the pH would be expected to result in penetration of NHA<sub>2</sub> into the lipid monolayer at the air–water interface. It is important to note that the monolayer data do not exclude such a penetration but merely indicate that if NHA<sub>2</sub> penetrates it does not contribute much to the observed increase in surface pressure. In fact, quantitation suggests that the number of N-termini of HA<sub>2</sub> expected to penetrate the lipid monolayer may be too low to allow detection. The maximal binding of BHA to the lipid monolayer can be estimated using the experimental value for maximal binding of BHA to lipid vesicles at low pH; a BHA trimer-to-phospholipid ratio of 1:700 was found with respect to total phospholipid (Doms et al., 1985), giving a ratio of 1:350 with respect to the phospholipids present in the outer lipid monolayer. Using this ratio and a mean area for a lipid molecule of 45 Å<sup>2</sup> (phospholipid/Chol mixture in a 1:1 molar ratio; Demel et al., 1972), only 0.01 nmol of BHA trimers would be expected to bind to the lipid monolayer, and a maximum of 0.03 nmol of NHA<sub>2</sub> might then penetrate the lipid monolayer. Judging from the results obtained with the synthetic fusion peptide, the penetration of 0.03 nmol of NHA<sub>2</sub> would not result in a significant increase in surface pressure (see Figure 6).

**Surface Activity of Intact Influenza Virions: Possible Lipid Transfer Activity.** The pH profile of surface activity (Figure 2) clearly indicates that the surface activity of intact virus is due to activation of HA by low pH. However, the remarkable differences that are observed in the characteristics of surface activity between isolated HA and intact virus (see Results) suggest that the surface activity of intact virions is not due to the initial interaction of activated HA with and partial penetration into the lipid monolayer. Instead, it is likely to be a secondary effect of this interaction and penetration. This suggestion is corroborated by the similarities between the characteristics of surface activity and fusion activity of influenza virus. The pH profiles of surface activity (Figure 2) and fusion activity toward large unilamellar zwitterionic vesicles [e.g., see Stegmann et al. (1986)] are almost identical; in both cases, a sharp pH optimum is found and a decrease in activity at pH values below the pH optimum. Furthermore, a low-pH preincubation in the absence of a lipid target strongly reduces low-pH-induced surface activity (Figure 7) and fusion activity (White et al., 1982b; Sato et al., 1983; Stegmann et al., 1987). Membrane fusion activity (Stegmann et al., 1986) and surface activity can be stopped and reinitiated by neutralization and reacidification (not shown). Finally, fusion activity toward pure lipid vesicles (White et al., 1982b; Van Meer et al., 1985) and surface activity are greatly stimulated by the incorporation of phosphatidylethanolamine (PE) in the lipid target; going from PC/Spm/Chol 1:1:2 to PC/Spm/Chol/PE 1:1:4:2 molar ratio, an up to 2-fold increase in surface activity was observed (results not shown; it should be noted that the pressure–area curves of monolayers of these lipid mixtures were found to be almost identical). If the surface activity of intact virions is a secondary effect of the activation of HA at low pH, what then is the direct cause of the observed surface pressure increase? The possibility of fusion of the viral membrane bilayer and the lipid monolayer at the air–water interface is hard to envisage following current theories of the



mechanism of membrane fusion, and can be virtually excluded. However, two possibilities remain. The surface activity of intact virus could result from semifusion, during which the outer lipid monolayer of the viral membrane would become continuous with the lipid monolayer, or alternatively, it could result from lipid transfer activity. In both cases, a net transfer of viral material to the lipid monolayer could explain the observed increase in surface pressure. The transfer of membrane constituents to a lipid monolayer at the air-water interface has probably been observed before in a study of the interaction of proteoliposomes with a lipid monolayer [final surface pressures of 39–43 mN/m were reached; see Davies and Jones (1986)]. The driving force behind such a net transfer of membrane constituents could be a difference in surface pressure. Interestingly, a difference in membrane surface pressure was recently proposed as an explanation for the net membrane transfer that occurs during reversible fusion of secretory granules with the plasma membrane in secreting mast cells (Monck et al., 1990).

The hypothesis explaining surface activity by assuming a net transfer of constituents of the viral membrane to the lipid monolayer at the air-water interface is based on circumstantial evidence and should, as yet, be regarded as being speculative. However, it would explain the differences found in the characteristics of surface activity between isolated HA and intact virions; only in the latter case could activation of HA result in a net transfer of membrane constituents to the lipid monolayer. It would also explain the magnitude of surface activity observed for intact virions (Figure 8; see Results). Furthermore, preliminary experiments using octadecylrhodamine B chloride (R18) labeled virus and video-enhanced fluorescence microscopy in a monolayer setup showed a steep increase in surface fluorescence upon lowering of the pH in the presence of virus. At neutral pH, the fluorescence was negligible, even in the presence of a virus receptor in the lipid monolayer (the disialoganglioside  $G_{D1a}$ ; Burger et al., unpublished results). These results could be due to low-pH-induced transfer of viral lipids (and R18) to the lipid monolayer.

**Mechanism of HA-Mediated Membrane Fusion.** How do the monolayer data presented here and the proposed lipid transfer activity of influenza HA fit in with current models of influenza HA-induced membrane fusion? The central enigma is the movement of  $NHA_2$  after the conformational change and the mechanism by which viral and target membranes finally join (see the introduction). Unfortunately, the monolayer experiments failed to reveal the penetration of  $NHA_2$  into the lipid monolayer, and the direction of movement of  $NHA_2$  after low-pH activation could not be studied. Since hard evidence on the movement of  $NHA_2$  is as yet lacking, one can only consider indirect data favoring or not favoring a particular model for HA-induced membrane fusion. In particular, the location of  $NHA_2$  at neutral pH should be considered: 3 nm away from the viral membrane but more than 10 nm away from the target membrane. In doing so, a model in which  $NHA_2$  penetrates neither viral nor target membrane seems very attractive (Bentz et al., 1990; Bentz, 1991; Ellens et al., 1990; White, 1990). In their model, a number of activated HA spikes associate; their N-termini are extruded sideward and provide a hydrophobic surface along HA, allowing a flow of lipids between the viral and the target membrane. At first, only the outer lipid monolayers of viral and target membranes join, and at this intermediate stage an inverted lipid micelle [a type II lipid structure; see Verkleij (1984) and Siegel (1986)] is sandwiched between the two interacting membranes at the center of the HA spike complex.

Breakthrough of this intermediate results in the formation of a small fusion pore [see Bentz et al. (1990)].

The results obtained in monolayer experiments using isolated HA and intact virions (this study) fit in well with this model. First of all, the monolayer experiments using isolated HA suggest that parts of HA other than  $NHA_2$  may be involved in the interaction of HA with the lipid target at low pH; in particular,  $HA_1$  was implicated. If cross-linking of viral and target membranes at low pH is not due to the penetration of  $NHA_2$  into the target membrane, as proposed in the last model, other parts of HA must be involved. These parts could be responsible for the surface activity of isolated HA as measured in a monolayer setup. The presence of additional surface-active sites in HA, besides  $NHA_2$ , could also play a supporting role in establishing the "lipid bridge" between viral and target membranes. In this context, it is important to note that HA-mediated membrane fusion can occur at 0 °C and that under this condition fusion probably occurs without major dissociation of the tops of HA [Stegmann et al., 1990; see also Bentz et al. (1990)]. The concept of lipid flow between viral and target membranes proposed in this model is directly in line with the hypothesis we put forward to explain the surface activity of intact virions toward a lipid monolayer. Application of the model proposed for HA-mediated membrane fusion to the interaction of a virion with a lipid monolayer predicts lipid flow between the viral membrane and the lipid monolayer at the air-water interface. Finally, the presence of an inverted lipid micelle would explain why both virus-membrane fusion and the surface activity of virus are stimulated by incorporating a type II lipid, like phosphatidylethanolamine, into the lipid target.

Although the model proposed by Bentz and co-workers is supported by the results of the monolayer experiments described in this study, it does not exclude other possibilities. The monolayer data do not, for example, rule out the possibility of penetration of  $NHA_2$  into the lipid target. Both the fusion model and the putative involvement of parts of HA other than  $NHA_2$  should be tested in future experiments. The current report clearly illustrates the great potential of using lipid monolayers in studies of pH-dependent protein-lipid interactions, and emphasizes the molecular complexities of "simple" biological fusion processes.

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