The EMBO Journal, Vol. 1 No. 2, pp.217–222, 1982

Membrane fusion activity of influenza virus

Judy White[1]*, Jürgen Kartenbeck[2], and Ari Helenius[1]

[1]European Molecular Biology Laboratory, Postfach 10.2209, 6900 Heidelberg, FRG, and Yale University School of Medicine, Section of Cell Biology, P.O. Box 3333, 333 Cedar Street, New Haven, CT 06510, USA; and [2]Institute of Cell and Tumor Biology, German Cancer Research Center, 6900 Heidelberg, FRG

Communicated by K. Simons
Received on 26 January 1982

A simple assay is described to monitor fusion between fowl plague virus (FPV, an avian influenza A virus) and liposomes which allows the simultaneous quantitation of both lytic and non-lytic fusion events. As in fusion between viruses and the plasma membrane and in FPV-induced cell-cell fusion, the reaction only occurs at pH 5.5 or below, and it is fast, highly efficient, and essentially non-lytic when fresh virus and liposomes are used. The fusion occurs over a broad temperature range, and has no requirement for divalent cations. The fusion factor of influenza virus is the hemagglutinin (HA) spike which protrudes from the virus membrane and which is also responsible for virus binding to the host cell. The finding that fusion occurs as efficiently with liposomes containing or lacking virus receptor structures, further emphasizes the remarkable division of labor in the HA molecule: the receptor-binding sites are located in the globular HA_1 domains and the fusion activation peptide is found at the N-terminal of HA_2 in the stem region of the protein. The mechanism of fusion is discussed in terms of the three-dimensional structure of the HA and the conformational change which the protein undergoes at the fusion pH optimum.

Key words: membrane fusion/fowl plague virus/liposomes/low pH-dependent fusion/virus entry

Introduction

Low pH-dependent membrane fusion activity has now been documented for members of three major families of enveloped animal viruses – togaviruses (Helenius *et al.*, 1980a; White and Helenius, 1980; Väänänen and Kääriäinen, 1980), orthomyxoviruses (White *et al.*, 1981; Matlin *et al.*, 1981; Huang *et al.*, 1981, Maeda *et al.*, 1981), and rhabdoviruses (White *et al.*, 1981; Matlin *et al.*, 1982). Fusion has been demonstrated using the plasma membrane of tissue culture cells (White *et al.*, 1980; Matlin *et al.*, 1981; Matlin *et al.*, 1982), red blood cells (Väänänen and Kääriäinen, 1979; Yamamoto *et al.*, 1981; Maeda and Ohnishi, 1980; Lenard and Miller, 1981), and liposomes (White and Helenius, 1980; Maeda *et al.*, 1981) as target membranes. Several lines of evidence indicate that the fusion activity is of central importance in the pathway of productive viral infection. After endocytosis, the virus particles are routed into the lysosomal compartment where the low pH triggers fusion between the viral and lysosomal membranes, thereby releasing the viral genome into the cytoplasm for replication (Marsh and Helenius, 1980; Marsh *et al.*, 1982; Helenius *et al.*, 1980a,b; Matlin *et al.*, 1981; Helenius *et al.*, 1982; Matlin *et al.*, 1982). The viral spike glycoproteins mediate the fusion and, in the case of influenza virus, the hemagglutinin (HA) has been identified as the fusion factor (White *et al.*, 1981; Huang *et al.*, 1981; Maeda *et al.*, 1981). The HA is synthesized as a precursor which is proteolytically cleaved to the active (fusogenic and infective) form consisting of two disulfide-bonded glycopolypeptide chains, HA_1 and HA_2 (mol. wts. 58 000 and 26 000, respectively, Lazarowitz and Choppin, 1975; Klenk *et al.*, 1975; Skehel and Waterfield, 1975).

We have characterized the low pH-dependent fusion between fowl plague virus (FPV, an avian influenza A virus) and liposomes. The ability of influenza A virus to fuse with liposomes has recently been demonstrated by Maeda *et al.* (1981) using electron spin resonance spectroscopy. We report here a simple quantitative assay to study the fusion of FPV with liposomes. The assay, which allows the quantitation of both membrane fusion and membrane lysis, was used to characterize the properties and requirements of the low pH-induced fusion reaction.

Results

The fusion assay

In initial experiments, mixtures of trypsin-containing liposomes [phosphatidylcholine (PtdCho)/phosphatidylethanolamine (PtdEtn)/sphingomyelin (Sph)/cholesterol; 1:1:1:1.5] and [^{35}S]methionine-labeled FPV were briefly acidified (pH 5.2, 5 min, 37°C), to allow fusion between the virus membranes and the liposomes to occur, and then neutralized. After a further 15 min, the amount of acid-precipitable radioactivity was determined. The maximum possible level of trypsin degradation (about half of the total radioactivity) was established in parallel samples treated with excess detergent (Triton X-100) and trypsin. It was reproducibly found that 60–70% of the maximum level of degradation occurred in the experimental samples subjected to low pH, whereas no (<1%) degradation was observed in controls kept at neutral pH. This result indicated that access of the trapped trypsin to the trypsin-sensitive virus proteins was pH-dependent. Although this observation was consistent with fusion of the viruses with the liposomes, it could also be explained by lysis of liposomes and viruses.

To differentiate between lysis and fusion, a membrane-impermeable inhibitor, soybean trypsin inhibitor (STI), was added to the bulk solution of the assay mixtures to inhibit any trypsin that became accessible to the bulk solution through lytic reactions. Figure 1 shows that the STI clearly decreased the amount of low pH-induced protein degradation in the assay with a stable level obtained at 5 mg STI/ml. The STI-resistant component was defined as "tight" fusion and the STI-sensitive fraction as "leaky" (or lytic) fusion. Although the total amount of protein degradation measured in the absence of inhibitor remained essentially constant, the ratio of "tight" and "leaky" fusion varied widely depending on the history of the virus and liposomes (Figure 1). When freshly prepared liposomes were used with fresh virus (which had not been harshly pelleted during isolation), leakiness was

*To whom reprint requests should be sent at the Yale University address.

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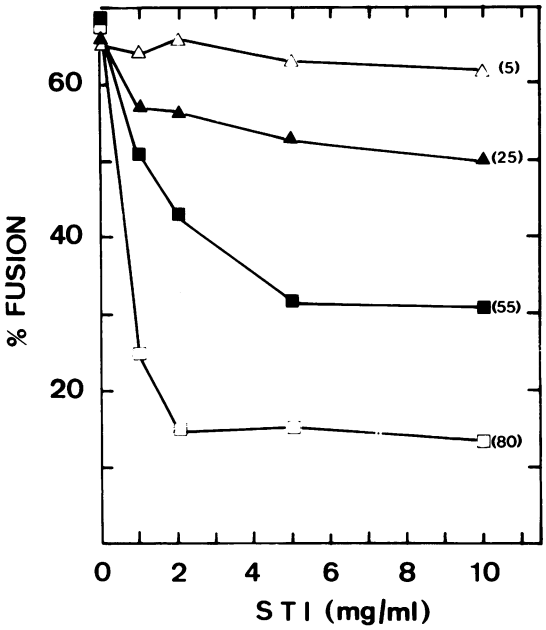

Fig. 1. Leaky and tight fusion of FPV with liposomes. [^{35}S]Methionine-labeled FPV and trypsin-containing liposomes were mixed at pH 7.0 with the indicated amount of STI in the bulk solution and the pH lowered to 5.2 for 5 min at 37°C. After neutralization and a further 15 min incubation, the trypsin was inactivated, the samples solubilized with SDS, and the percentage of total fusion determined as described in Materials and methods. Percentage leaky fusion is the component inhibited by STI (numbers in parentheses), whereas percentage tight fusion is the component which is unaffected by high concentrations of inhibitor (see Materials and methods for quantitative definitions). (△), freshly prepared virus and liposomes; (▲) aged virus and fresh liposomes; (■) freeze-thawed virus and fresh liposomes; (□) fresh virus and liposomes stored overnight.

minimal (<5%). When viruses stored for more than two months at −70°C, "aged virus", harshly pelleted viruses, liposomes stored overnight, or freeze-thawed viruses were used, the leaky component could be as large as 80% (Figure 1). For all subsequent experiments, fresh virus and liposomes were employed.

To determine which virus proteins were degraded, the virus preparations and the fusion mixtures were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A fluorogram of a heavily loaded gel displays the viral proteins (Figure 2, lane a). Densitometer scanning of a less heavily loaded sample showed that ~75% of the label was present in two of the internal protein components – the nucleocapsid (NC) protein (37%) and the matrix (M) protein (37%). HA_2 contained 22% of the label, but HA_1, the RNA polymerases ($P_{1,2,3}$) and the neuraminidase (NA) protein were hardly detectable (Figure 2). When [^{35}S]methionine-labeled FPV was solubilized with Triton X-100 in the presence of trypsin (either at neutral or acid pH), the M protein was totally degraded whereas the NC and, apparently, the HA_2 proteins were partially degraded (Figure 2, lane e). The proteins of FPV thus displayed individual differences in trypsin sensitivity. When a low pH-treated mixture was analyzed, the pattern of degradation of the internal virus proteins was virtually identical to the detergent-treated digest with one exception – a large (~52 000 dalton) trypsin-resistant fragment of the NC protein was observed between the NC and HA_2 bands (Figure 2, lane d). It is quite significant, however, that both in the fused samples and in the Triton X-100 control, the most sensitive internal protein component, the M protein, was fully degraded. This indicates that the fusion was practically 100% efficient. The fact that the total amount of radioactivity rendered acid soluble in the fused samples is only 65% of that in the Triton X-100 control (see Figure 1) can therefore be attributed to the observation that the NC and HA_2 proteins are further digested in the presence of Triton X-100 than in its absence (Figure 2, lanes d and e). Our subsequent measurements of the percentage fusion are thus low estimates.

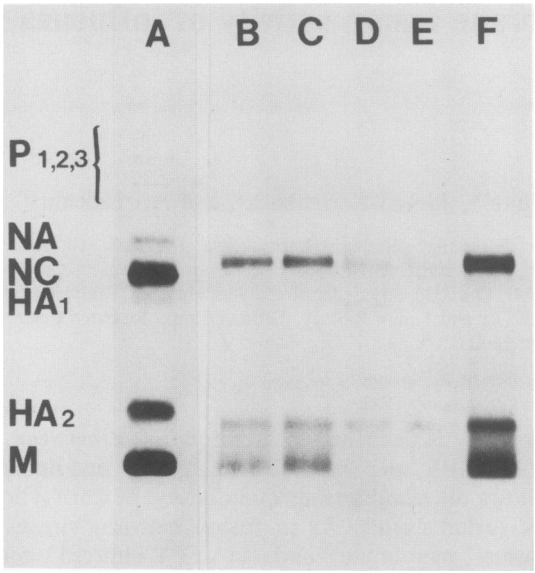

Fig. 2. Fluorographic identification of the virus proteins degraded during the fusion assay. Lane **A**, [^{35}S]FPV. Lanes **B**–**F**, mixtures of [^{35}S]FPV and trypsin-containing liposomes incubated as follows: lane **B**, 4°C, pH 7.0; lane **C**, 37°C, pH 7.0; lane **D**, 37°C, pH 5.2; lane **E**, 37°C in the presence of 1% Triton X-100 and 10 mg/ml added trypsin; lane **F**, start virus. The numbers of c.p.m. loaded per lane were: **(A)** 60 000; **(B–E)** 8000; **(F)** 20 000. Autoradiography was for: lane **A**, 24 h; lanes **B–F**, 1 week.

Properties of the fusion reaction

The assay was next employed to determine the pH-, lipid-, divalent cation-, time-, and temperature-dependence of the fusion reaction. Fresh FPV and fresh liposomes were used, and most of the assays were performed both with and without 5 mg/ml STI in the medium so that the "leaky" and "tight" components of the fusion could be assessed.

Figure 3 shows the pH-dependence of fusion. Fusion starts at pH 5.8, with 50% activity at pH 5.5 and full activity at 5.2. We have observed an identical pH-dependence in FPV fusion with the plasma membrane of intact tissue culture cells (Matlin *et al.*, 1981) and in FPV-induced cell-cell fusion (White *et al.*, 1981).

The kinetic studies shown in Figure 4 indicate that fusion reaches completion 2–3 min after acidification. At lower temperatures the fusion is slower, requiring >2 min (but <5 min) for maximum activity (Figure 5). The proportion of "leaky" fusion is also significantly greater at lower temperature (88% at 0°C *vs* 7% at 37°C, Figure 5).

Prior treatment of the virus with pH 5.2 buffer for 1 min at 37°C did not greatly affect its subsequent fusion activity; a 13% inactivation was observed (Table I). However, if the time of acid pretreatment was increased to 5 min, the inactivation was 85%, indicating irreversible changes in the virus particles which decreased their fusion activity.

The fusion efficiency observed with our standard

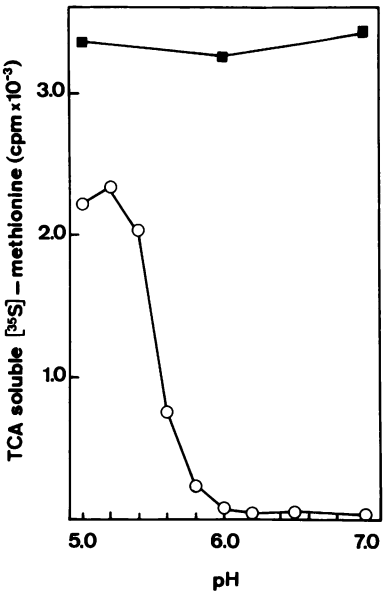

Fig. 3. pH dependence of fusion. Samples of [^{35}S]methionine FPV and trypsin-containing liposomes were mixed in the absence of STI and incubated at 37°C for 5 min at the indicated pH. After neutralization and a further 15 min incubation, the acid-soluble radioactivity was determined as described in the legend to Figure 1. In this experiment the total added radioactivity per sample was 7 x 10^3 c.p.m. (○), fusion mixtures; (■) TX controls.

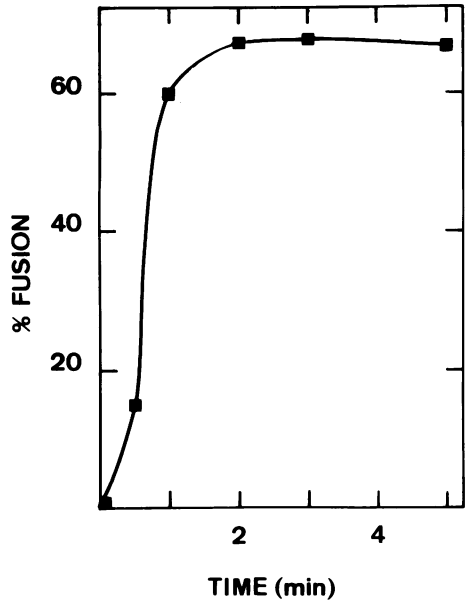

Fig. 4. Time dependence of fusion. Fusion reactions were initiated by adjusting the mixtures to pH 5.2. After incubation for the indicated time, the samples were neutralized and then further incubated at 37°C for a total of 20 min. At this time, the samples were processed and the percentage total fusion determined as described in the legend to Figure 1.

liposomes composed of PtdCho, PtdEtn, Sph, and cholesterol (1:1:1:1.5), was 64 ± 10% (which, as discussed above, is an underestimate). Omission of PtdCho, Sph, or cholesterol, or addition of gangliosides (18 mol %) had little effect on the overall fusion activity, although in some cases a slight elevation of the lytic component could be recorded (Table II). A 50% reduction in the fusion activity was observed when PtdEtn was omitted (Table II). Tests with different concentrations of PtdEtn indicated that the fusion acivity increased linearly with the PtdEtn concentration until the maximum level was reached at 22 mol %. Qualitatively the lipid dependence of FPV fusion is similar to that reported by Maeda *et al.* (1981) for a human influenza virus strain A_0PR8.

Table I. Virus inactivation by low pH in the absence of liposomes

Pretreatment	TCA-soluble radioactivity % TX control	
	pH 7.0	pH 5.2
None	0.7	58.9
1 min, pH 7.0	1.4	56.8
1 min, pH 5.2	1.3	43.6
5 min, pH 7.0	0.9	56.7
5 min, pH 5.2	2.9	8.7

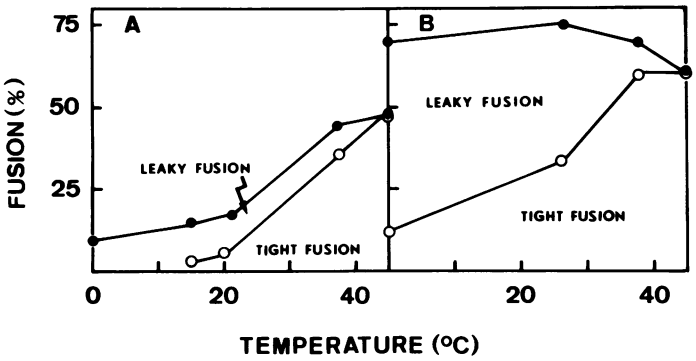

Fig. 5. Temperature dependence of fusion. Fusion mixtures were set up and incubated at pH 5.2 for either 2 min **(A)** or 5 min **(B)** at the indicated temperature. Following neutralization, the samples were further incubated at 37°C for a total of 20 min. At this time, the percentage fusion was determined as described in the legend to Figure 1. The leaky and tight components of the total fusion are indicated. Percent fusion measured in the absence (●) or presence (○) of 5 mg/ml STI.

To study the divalent cation requirement, the lipids (standard mixture) were passed through a Chelex column prior to the preparation of liposomes. In addition, chelators were added to some of the final fusion mixtures. As shown in Table III, none of these treatments significantly affected the fusion activity or the leakiness. Divalent cations are, therefore, not essential in the FPV fusion reaction.

Negative-staining electron microscopy

Electron microscopy after negative staining with phosphotungstic acid confirmed the fusion between liposomes (in this case trypsin-free) and FPV. In control samples which had been kept at pH 7.0 throughout (Figure 6c), the viruses could be seen as ~100 nm diameter particles with the familiar 13 nm spikes on their surface. Weakly-staining liposomes were seen in the background. There was no indication of interaction between the two particle populations. In a second control (Figure 6d), the viruses had been treated at low pH in the absence of liposomes. Again no evidence for fusion was seen, but the particles appeared slightly damaged, and the spikes somewhat disorganized. In the actual samples, where the mixture of FPV and liposomes had been briefly acidified (Figures 6a and b), two types of particles were seen: liposomes devoid of detectable protein, and large vesicles studded with spike glycoproteins. No unfused viruses remained. The spiked surface of the fused vesicles frequently displayed an undulating surface (see arrows) not seen on the unfused vesicles. The spike glycoproteins were best detected along the edges of the vesicles, but in rare instances (see arrowheads) they were seen

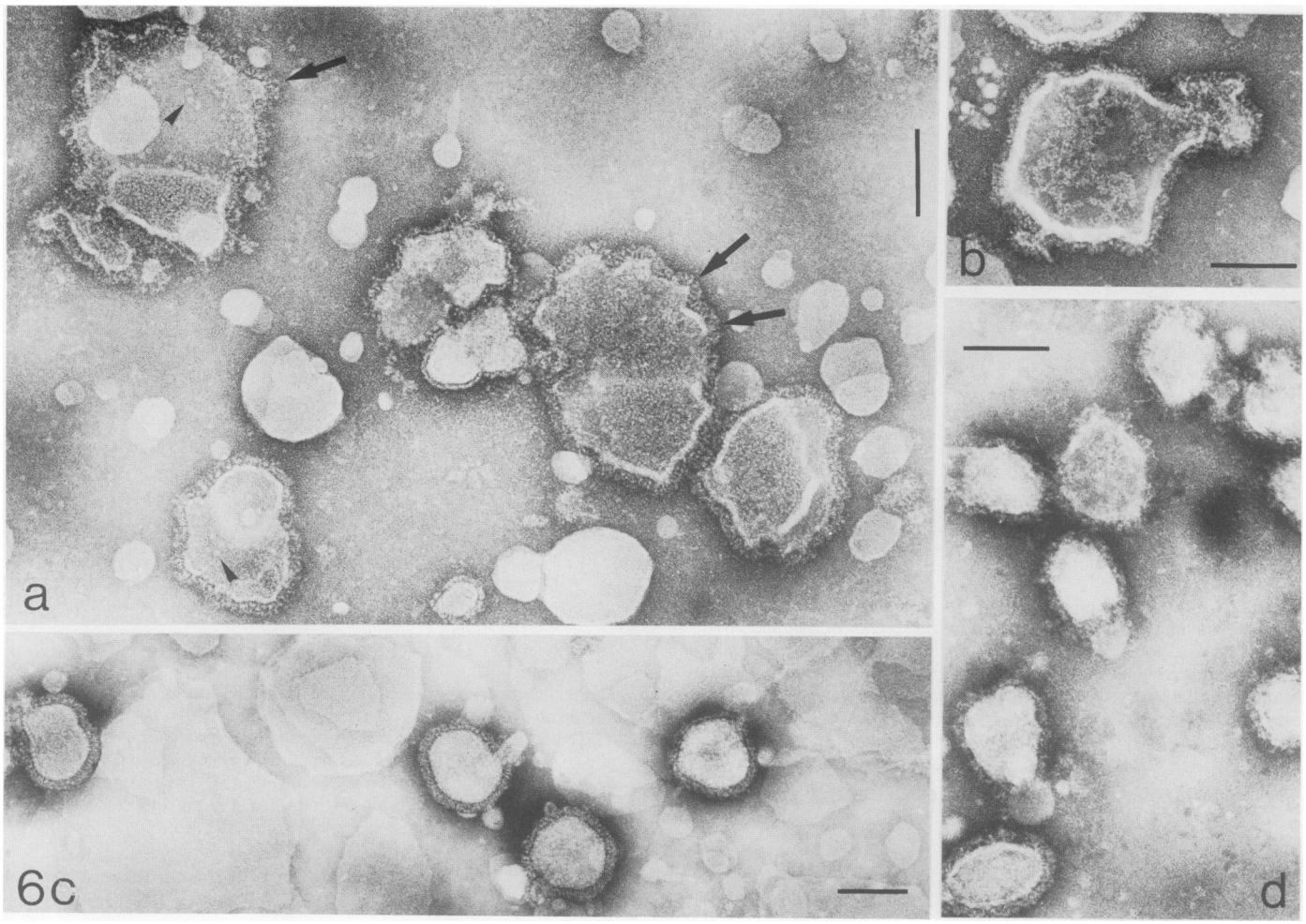

Fig. 6. Electron microscopy of the interaction of FPV with liposomes at pH 5.2 (**a** and **b**) or pH 7.0 (**c**). Samples containing 0.2 mg/ml egg-grown FPV and 0.5 mM lipid were incubated for 5 min at 37°C, neutralized, and then stained with 2% phosphotungstic acid. The image in **d** is from a sample treated as in **a** and **b** but lacking liposomes. Arrows, Arrowheads, see text. Bars: 0.1 μm.

Table II. Lipid requirement

Lipid composition	% Total fusion average ± s.d.	% Leaky fusion average ± s.d.
PtdEtn/PtdCho/Sph/cholesterol (1:1:1:1.5)	64 ± 10 (9)[a]	5 ± 7 (6)
PtdEtn/Sph/cholesterol (1:1:1)	69 ± 3 (2)	17 ± 18 (2)
PtdEtn/PtdCho/cholesterol (1:1:1)	79 ± 19 (2)	20 ± 9 (2)
PtdEtn/PtdCho/Sph (1:1:1)	78 ± 19 (2)	37 ± 2 (2)
PtdCho/Sph/cholesterol (1:1:1)	37 ± 6 (3)	3 ± 1 (2)
PtdEtn/PtdCho/Sph/cholesterol/ganglioside (1:1:1:1.5:1)[b]	66 ± 3 (2)	7 (1)

[a]Number of determinations.
[b]2.5 mg ganglioside/5.5 μmol lipid.

Table III. The fusion does not require divalent cations

Treatment of lipids	Chelator	% Total fusion	% Leaky fusion
None	None	65.0	4.0
None	1 mM EDTA	70.0	9.4
None	10 mM EGTA	69.6	12.1
Chelex[a]	None	64.1	N.D.[b]
Chelex	1 mM EDTA	66.6	N.D.
Chelex	10 mM EGTA	71.6	N.D.

[a]Lipids in chloroform/MeOH containing a tracer amount of [^{32}P]BHK lipids were passed over a 2.5 ml cation exchange column (Chelex-100) to remove divalent cations (Carter and Weber, 1966).
[b]Not determined.

in top view. The overall appearance of the spikes on the vesicle surface was less orderly than on control viruses; this could reflect the irreversible conformational change caused by acid treatment (Skehel *et al.*, 1982) or it may simply be a result of the looser packing of the spikes in the fused vesicles. In some cases (see Figure 6b), where stain penetrated into the vesicles, the strand-like ribonucleoproteins could be visualized.

Discussion

The HA spike is a trimer composed of three molecules each of HA_1 and HA_2. The structure of the external polar moiety of the protein, isolated after bromelain cleavage, has recently been described at 3 Å resolution by Wilson *et al.* (1981). It extends 135 Å from the viral membrane with a long highly α-helical stem region composed mainly of HA_2 supporting a large globular domain composed of HA_1. Both of the viral functions required for entry – attachment to the cell surface and low pH-dependent membrane fusion – reside in the

polar moiety. The sialic acid-binding receptor sites are located in the globular domain, whereas the region involved in fusion, the N-terminal region of HA_2 (Huang *et al.*, 1981; Maeda *et al.*, 1981; White *et al.*, 1981; Skehel *et al.*, 1982; Richardson *et al.*, 1980), is part of the stem (Wilson *et al.*, 1981). In the three-dimensional structure (presently available only for the neutral pH form), the predominantly apolar N-terminal segment of HA_2 wraps around the stem of the molecule ~35 Å from the viral membrane. The second and third residues are buried in the interface of the HA trimer, inaccessible from the surface of the molecule; the next 14 residues wrap around the stem.

Recent studies by Skehel *et al.* (1982) with the water-soluble bromelain fragment have shown that, at pH values needed to trigger fusion, a conformational change occurs exposing the HA_2 N-terminal segment and conferring amphiphilic properties to the polar molecule. The evidence is as follows: (1) the near ultraviolet c.d. spectrum changes; (2) two trypsin cleavage sites in HA_1 become accessible in the previously trypsin-resistant molecule; (3) the protein acquires the ability to bind to liposomes and to micelles of nonionic detergents; (4) in the absence of lipids and detergents, it aggregates to form rosettes; and (5) the aggregated material can be dissociated by thermolysin which removes the N-terminal 23 residues of HA_2 (J. Skehel, personal communication). The present evidence thus implies that fusion requires the exposure of a hydrophobic domain on the external surface of the spike. In most strains of influenza virus, the highly conserved apolar N-terminal HA_2 sequence contains acidic residues at positions 11, 15, and 19 (for example, see Min-Jou *et al.*, 1980; Fang *et al.*, 1981; Gething *et al.*, 1980). Protonation of these residues may be required to trigger the conformational change or to render the exposed segment hydrophobic enough to insert into a bilayer during fusion (Maeda and Ohnishi, 1980). The generation of the conformational change may also involve protonation of other groups in the molecule.

It is still not obvious how fusion of the viral and target membranes occurs following the low-pH induced conformational change in the HA. The finding that influenza virus can fuse equally well with artificial liposomes devoid of sialic acid as with sialic acid-containing liposomes or cellular membranes demonstrates that the receptor-binding function of HA_1 is not required for fusion. The virus membrane and the target bilayer must therefore be brought into close contact by other means. This most likely occurs by insertion of the HA_2 N-terminal segment into the target membrane. The HA_2 may, at this stage of fusion, actually serve as an integral component of both membranes with its C-terminal segment anchored in the virus membrane and its N-terminal region in the target membrane. The role of the spike glycoproteins in virus fusion may simply be to bring the bilayers close enough to perturb their hydration shells, thereby permitting contact between the lipids in the two membranes (Rand, 1981). How the HA_2 N-terminal segment, which at neutral pH is located 100 Å from the tip of the spike, gains access to the target membrane remains a puzzle. It is possible, but as yet unsubstantiated, that the HA_1 subunits move away from the central axis of the molecule to facilitate the HA_2-target membrane interaction.

Several other conclusions regarding the mechanism of viral-membrane fusion can be drawn. The fusion reaction of FPV with liposomes is essentially unleaky provided that the virus and liposomes are carefully prepared. Factors which influence the degree of leakiness include the age and physical state of the virus, the age of the liposomes, the temperature at which the reaction is performed, and, to some extent, the lipid composition of the target liposomes. The results presented here (and additional unpublished data) indicate that lytic fusion can result even if the virus membrane and the target membrane do not contain pre-existing holes. Hemolysis, which has been used as a convenient semiquantitative assay for virus fusion (Huang *et al.*, 1981; Lenard and Miller, 1981; Maeda and Ohnishi, 1980; Väänänen and Kääriäinen, 1979, 1980), monitors only leaky fusion events and is therefore of limited use in measuring the total fusion activity. During the course of a normal infection, the cellular conditions; e.g., temperature, age of the virus, composition of the lysosomal membrane (Bode *et al.*, 1975) etc., would favor a non-lytic fusion process.

For optimal fusion, we found that the target liposomes had to be present at the time of the pH shift. The results suggest that even though the conformational change in HA is irreversible (Skehel *et al.*, 1982), low pH treatment does not lock the virus into a fusogenic form that can later be triggered by adding membranes. It seems more likely that, in the absence of membranes, the virus is inactivated at low pH. Whether the inactivation occurs by further conformational changes in the HA, or by a general change in the virus membrane, such as HA aggregation, remains to be determined.

Another result which bears on the mechanism of viral fusion is that, with both FPV and Semliki Forest virus (SFV), fusion with liposomes does not depend on either divalent cations or on charged lipids. This suggests that electrostatic interactions are not important in the initial bridging reaction. In this respect, viral membrane fusion differs from the Ca^{2+}-induced fusion of artificial lipid bilayers (for review, see Papahadjapoulos, 1978). In most respects, the fusion activity of SFV, which has been studied in great detail (Väänänen and Kääriäinen, 1979, 1980; White and Helenius, 1980; White *et al.*, 1980, 1981), is similar to that of influenza virus. The main differences are: (a) SFV fusion has an absolute cholesterol dependence; (b) it occurs at somewhat higher pH; and (c) the virus does not appear to be inactivated by pre-incubation at low pH (A. Helenius, unpublished results). Further investigations using these model fusion systems should help clarify the molecular events which lead to infection and to membrane fusion. Few membrane fusion reactions are presently as amenable to biochemical and biophysical analysis.

Materials and methods

Materials

[^{35}S]Methionine was from Amersham. PtdCho from egg yolk (type V-E), PtdEtn from soybean (Type IV), Sph from bovine brain (Type I), gangliosides (Type II) from bovine brain, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma. Cholesterol from Merck was recrystallized four times from ether. Trypsin was obtained from Worthington, STI from Boehringer Mannheim, EN^{3}HANCE from New England Nuclear, and Chelex 100 from Biorad.

Virus growth and labeling

Unlabeled FPV was grown in embryonated chicken eggs as described (White *et al.*, 1981) and [^{35}S]methionine-labeled FPV in Madin-Darby canine kidney cells as described (Matlin *et al.*, 1981).

Preparation of liposomes

Liposomes containing PtdCho, PtdEtn, Sph, and cholesterol in the molar ratio 1:1:1:1.5 (unless otherwise stated) were freshly prepared essentially as described (White and Helenius, 1980), with the following modifications. The buffer used throughout was 20 mM 2-(morpholine)ethanesulfonic acid (Mes)/0.13 M NaCl (Mes buffer). For all quantitative experiments, trypsin-containing liposomes were prepared as follows: the lipid films were resuspended in 0.5 ml of a solution of 10 mg/ml trypsin in Mes buffer (pH 7.0). After

removal of the largest vesicles by centrifugation at 10 000 *g* in a Wifug (Stockholm, Sweden) table-top centrifuge for 60 s, the supernatant was placed in an Eppendorf tube containing 5 mg of STI to inactivate the untrapped trypsin. This mixture was then passed over a column of Sephadex G-150 (0.7 x 20 cm) to remove the external trypsin-STI complexes. Fractions (0.3 ml) were collected and the three most opalescent fractions pooled. A tracer amount of [^{32}P]-labeled lipids from BHK-21 cells (Helenius *et al.*, 1978) was added to the lipid films when quantitation of lipid concentration was necessary.

Fusion assay

For a typical fusion assay, virus and trypsin-containing liposomes were mixed together on ice in a total volume of 650 μl. Each mixture contained 0.5–1.0 mM lipid, 2.25 μg of unlabeled FPV, and ~4–5 x 10^5 c.p.m. [^{35}S]FPV. When the lytic component of the fusion reaction was being determined, a parallel mixture was also set up containing 5 mg/ml STI. To initiate fusion, these mixtures were split into 6 x 200 μl aliquots and added to duplicate tubes containing either Mes buffer (pH 7.0 control), Triton X-100 and trypsin, such that the final concentrations were 1% and 10 mg/ml, respectively (TX control), or dilute acetic acid to adjust the mixtures to the indicated pH value (5.2 unless otherwise stated). The samples were placed at 37°C for 5 min at which time the pH-adjusted samples were neutralized by addition of dilute NaOH and the incubation continued for a further 15 min at 37°C. The samples were then placed on ice and quenched with 100 μl of a solution containing 3% bovine serum albumin and 1 mM PMSF in Mes buffer, pH 7.0. Next they were treated at 95°C for 5 min with 1% SDS, chilled on ice, and mixed with an equal volume (225 μl) of ice-cold 20% trichloroacetic acid (TCA). After 30 min on ice, the samples were spun for 10 min in an Eppendorf centrifuge at 4°C, and 230 μl of the supernatant was counted with 10 ml of Triton X-100/Toluol based scintillation fluid. The percentage "total" fusion is defined as (a/b) x 100, where a = the ^{35}S c.p.m. rendered TCA soluble at pH 5.2 and b = the ^{35}S c.p.m. rendered TCA soluble in the TX control. The TCA-soluble c.p.m. in the pH 7.0 control were always $<1\%$ and were therefore neglected. The percentage "tight" fusion as defined as $[(a/b)(100)]$ where a is the percentage fusion measured in the presence of 5 mg/ml STI and b is the percentage fusion measured in its absence. The percentage "leaky" fusion is 100 – percentage "tight" fusion.

Electron microscopy

Fusion mixtures were set up on ice containing 0.5 mM lipid and 0.2 mg/ml unlabeled FPV at pH 7.0. The mixtures were either maintained at pH 7.0 or adjusted to pH 5.2 and then incubated for 5 min at 37°C. The low pH samples were then neutralized and negatively stained with 2% phosphotungstic acid as described (Helenius *et al.*, 1978). Control samples without liposomes were similarly treated. The grids were visualized with a Siemans electron microscope.

Other methods

SDS-PAGE was conducted with a modified Laemmli system (Blobel and Dobberstein, 1975) and for fluorography the gels were fixed with 10% TCA, treated with EN^{3}HANCE and then dried. Autoradiography was carried out with Fuji film and for the scanning of gels a Joyce-Loeble densitometer was used. Phospholipid phosphorous was quantitated by the method of Ames (1966) and protein concentrations by the method of Lowry *et al.* (1951).

Acknowledgements

We thank Karl Matlin and Kai Simons for many helpful discussions, Margaret Kielian and Mark Marsh for critically reading the manuscript, and Linda Herzman for typing. J. White was supported by an NIH postdoctoral fellowship. The work was, in part, supported by the National Institute of Health (AI 18599).

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