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Fusion of Influenza Hemagglutinin-Expressing Fibroblasts with Glycophorin-Bearing Liposomes: Role of Hemagglutinin Surface Density†

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ABSTRACT: Influenza virus gains access to the cytoplasm of its host cell by means of a fusion event between viral and host cell membrane. Fusion is mediated by the envelope glycoprotein hemagglutinin (HA) and is triggered by low pH. To learn how many hemagglutinin trimers are necessary to cause membrane fusion, we have used two NIH 3T3 fibroblast cell lines that express HA protein at different surface densities. On the basis of quantitations of the number of HA trimers per cell and the relative surface areas of the two cell lines, the HAb-2 cells have a 1.9-fold higher plasma membrane surface density than the GP4F cells. The membrane lateral diffusion coefficient and the mobile fraction for HA is the same for both cell lines. A Scatchard analysis of the binding of glycophorin-bearing liposomes to the cells showed 1700 binding sites for the GP4F cells and 3750 binding sites for the HAb-2 cells, with effectively the same liposome–cell binding constant, about 7×10^{10} M^{-1}. Binding was specific for glycophorin on the liposomes and HA expressed on the cells. A competition experiment employing toxin-containing and empty liposomes allowed us to quantitate the number of liposomes that fused per cell, which was a small constant fraction of the number of bound liposomes. For the HAb-2 cells, about 1 in every 70 bound liposomes fused and for the GP4F cells about 1 in every 300 bound liposomes fused. Hence, the HAb-2 cells showed 4.4 times more fusion per bound liposome, even though the surface density of HA was only 1.9 times greater. We conclude the following: (i) One HA trimer is not sufficient to induce fusion. (ii) The HA bound to glycophorin is not the HA that induces fusion. That is, even though each HA has a binding and a fusion function, those functions are not performed by the same HA trimer.

Hemagglutinin, the major envelope glycoprotein of influenza virus, is responsible for binding the virus to sialic acid containing receptors on the host cell surface (Hirst, 1941) and for initiating the fusion event between viral and cellular membranes [e.g., Maeda and Ohnishi (1980), Matlin et al. (1981), and White et al. (1982)]. The ectodomain of HA[1] has been crystallized (Wilson et al., 1981) and shown to undergo a conformational change at the pH associated with viral fusion (Skehel et al., 1982; Doms et al., 1985; Ruigrok et al.,

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[1] Abbreviations: HA, hemagglutinin; HAO, the fusion-incompetent hemagglutinin precursor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PBS CMF, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PMSF, phenylmethanesulfonyl fluoride; RBC, red blood cell; RIA, radioimmunoassay; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

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1988). The conformational change results in exposure of the highly conserved apolar N-terminal segment of the HA2 subunit, the "fusion peptide" (White & Wilson, 1987). Interaction of the newly exposed fusion peptide with the target bilayer is thought to trigger the fusion event (Gething et al., 1986; Lear & DeGrado, 1987; White & Wilson, 1987; Harter et al., 1989; Brunner, 1989). For recent reviews on the role of HA in viral fusion, see Stegmann et al. (1989b), Bentz (1990), and White (1990).

While the behavior of the isolated HA trimer is rather well understood, little is known about the mechanism by which the newly exposed apolar sequence actually promotes fusion. A very important question is how many HA trimers are required at the fusion site. With this knowledge we can begin to elucidate the architecture of the fusion site, which is required to understand the mechanism of fusion at the level now available for fusion of phospholipid vesicles (Bentz & Ellens, 1988; Ellens et al., 1989a; Siegel et al., 1989a,b).

Following several lines of indirect evidence, it has been speculated that fusion may require several HA spikes (Doms & Helenius, 1988; Morris et al., 1989), although radiation inactivation experiments have suggested that a single HA may be sufficient (Gibson et al., 1986; Bundo-Morita et al., 1987). Determining the number of HA trimers needed to promote fusion requires measuring the fusion efficiency as a function of HA surface density (Bentz, 1990).

As a first step toward this goal, we have used two NIH 3T3 fibroblast cell lines (denoted GP4F and HAb-2) that express the HA protein at different surface densities. One reason for this choice of system was that our theoretical analysis implied that the minimal number of HA trimers in the fusion site cannot be determined at high HA surface densities, such as may be found on the intact viral envelopes (Bentz, 1990). To measure the HA fusion efficiency we used glycophorin-bearing liposomes, in which glycophorin functions as a specific receptor for HA. We counted the number of liposomes fusing per cell by doing a competition experiment between "empty" liposomes and liposomes containing the toxin gelonin. Our data show that individual HA trimers cannot induce fusion and that an aggregate of two or more HA trimers is required at the fusion site. We argue that the upper bound for the number of HA trimers at the fusion site is about five.

Materials and Methods

Materials. Egg phosphatidylcholine (PC) and plant phosphatidylethanolamine (PE) (from soy bean) were purchased from Avanti Polar Lipids, Inc. (Pelham, AL). Cholesterol, glycophorin (from human blood, type B), gelonin, trypsin (TPCK treated), soybean trypsin inhibitor, and neuraminidase (from Clostridium perfringens) were obtained from Sigma Chemical Co. (St. Louis, MO). The nonexchangeable lipid marker cholesteryl[1-^{14}C]oleate (57 mCi/mmol) and mixed ^{14}C-labeled amino acids were from NEN Research Products (Boston, MA). Cell culture media and other tissue culture reagents were purchased from GIBCO Life Technologies, Inc. (Grand Island, NY). Fetal calf serum was from Hazelton Research Products, Inc. (Lenexa, KS).

Liposomes. The liposomes were prepared essentially according to the procedure of MacDonald and MacDonald (1975). Briefly, 1.6 μmol of PC, 1.6 μmol of PE, 1.6 μmol of cholesterol, and 0.033 μmol of ^{14}C-labeled cholesteryl oleate were dissolved in 3 mL of chloroform/methanol (2:1). To this was added 86 μL of an aqueous solution of glycophorin (5 mg/mL). The solvent was evaporated on a rotary evaporator and residual solvent was removed under high vacuum for 2 h. The lipid/glycophorin film was hydrated overnight with 700 μL of the solution to be encapsulated, either Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS CMF) for "empty" liposomes or a solution of gelonin in PBS CMF for gelonin-containing liposomes. After hydration of the lipid/glycophorin film, the mixture was vortexed vigorously. The resulting vesicle suspension, a mixture of glycophorin liposomes, liposomes devoid of glycophorin, and glycophorin rosettes, was layered on 5% sucrose in PBS CMF and centrifuged for 3 h at 100000*g* in an AH627 rotor in a Sorvall ultracentrifuge. Under these conditions, only the glycophorin liposomes pelleted, while liposomes devoid of glycophorin collected at the 5% sucrose interface and glycophorin rosettes remained distributed throughout the sucrose solution. The absence of glycophorin rosettes in the glycophorin liposome pellet was demonstrated by chromatography of ^{125}I-labeled glycophorin liposomes over a Sepharose CL-2B column [results not shown; see also MacDonald and MacDonald (1975)]. The glycophorin liposomes were washed twice with PBS CMF by centrifugation in a TLA 100.3 rotor in a Beckman tabletop ultracentrifuge for 35 min at 75000*g*. Under these conditions about 10–40% of the lipid is recovered in the final pellet and the lipid to glycophorin molar ratio in the pellet is about twice the starting ratio, i.e., about 450:1, which approximates the glycophorin surface density on the erythrocyte. The *Z*-average diameter of these liposomes, determined by dynamic light scattering, is about 550 nm. We calculate 2.6×10^8 unilamellar liposomes per nanomole of phospholipid, assuming a surface area of 20 Å^2 for cholesterol and 70 Å^2 for PE and PC. The liposomes were used within 2–5 days of their preparation.

Neuraminidase Treatment of the Glycophorin Liposomes. The glycophorin liposomes were incubated for 1 h at 37 °C in citrate/phosphate buffer (0.1 M citrate/phosphate pH 6.0 and 48 mM NaCl to adjust the osmolarity to 300 mosm) at a concentration of 220 nmol of phospholipid/mL with 0.1 unit of neuraminidase (from Clostridium perfringens)/mL, which amounts to an estimated 4 units of neuraminidase/mg of glycophorin. This is more than sufficient to remove all of the sialic acid residues from glycophorin (de Kroon et al., 1985). After the incubation the liposomes were washed twice and the final pellet was resuspended in PBS CMF.

Hemagglutinin-Expressing Cells. The HA-expressing murine 3T3 fibroblast cell line (BV1-MTHA/NIH) was originally established by Sambrook et al. (1985). This cell line was generated by cotransfecting cells with a bovine papilloma virus vector containing the HA gene from the Japan strain of influenza virus (A/Japan/305/57) and a plasmid containing the gene for neomycin resistance. Cells expressing high levels of HA were isolated from this line by using a fluorescence-activated cell sorter on the basis of their ability to bind fluoresceinated red blood cells (RBCs) [HA-b, Doxsey et al. (1985); GP4, J. Sambrook and M.-J. Gething, personal communication]. Single cell clones of the HAb and GP4 lines were then selected on the basis of their ability to remain adherent throughout the trypsin treatment needed to activate the HA from its precursor form (HAO) and their ability to bind a maximum number of RBCs. Briefly, to clone the GP4F cells, cultures of GP4 cells were treated with 10 μg/mL of TPCK-treated trypsin in PBS CMF for 15 min at room temperature. They were then washed twice with PBS CMF and further incubated in PBS CMF for 15 min at room temperature. After several more washes with PBS CMF, the remaining cells were removed from the dish with 0.05% trypsin/0.02% EDTA and plated at single-cell density (50 cells/100 mm dish). Random clones were selected, expanded, and then compared

for their ability to bind RBCs. The clone GP4F was selected for further use because it bound RBCs over its entire surface (20–25 RBCs per cell). The HAb-2 cell line was isolated similarly (S. Doxsey and A. Helenius, unpublished results; Ellens et al., 1989b). By indirect immunofluorescence, these cells express uniformly high levels of HA (not shown). These two cell lines were used in the experiments described in this paper.

NIH3T3, GP4F, and HAb-2 cells were grown in Dulbecco's modified Eagles' minimal essential medium (DMEM H21) supplemented with 10% fetal calf serum, 10 mM HEPES, 100 units/mL penicillin, and 100 μg/mL streptomycin sulfate. The cells were grown on plastic dishes at 37 °C in a 5% CO_2 atmosphere.

Determination of HA Surface Density and Mobility. The amount of HA per microgram of cell protein was quantitated essentially as described previously (Gething & Sambrook, 1981). Briefly, dilutions of lysates of the GP4F and HAb-2 cells containing up to 10μg of cell protein were analyzed for their HA content by radioimmunoassay (RIA) using a polyclonal antibody specific for HA from the Japan strain of influenza virus (gift from M.-J. Gething). The slopes of the linear portions of the RIA curves were determined by a least-squares fit to the data ($R > 0.97$ for each data set). Three different cell lysates were used. The ratio of the slopes indicated that the HAb-2 cells contain (1.9 ± 0.2)-fold more HA per microgram of cell protein than the GP4F cells. The proportion of HA expressed at the surface of each cell line was determined, following steady-state labeling with [^{35}S]-methionine (Amersham, Arlington Heights, IL), as described previously (Matlin & Simons, 1983). For both cell lines, 85% of the HA was present at the cell surface.

We use the ratio of HA per microgram of cell protein for the two cell lines to estimate their ratio of HA surface densities, i.e., 1.9 ± 0.2. To fortify this claim, we measured the amounts of HA per cell and the relative cell surface areas by using whole cell capacitance measurements. There was about 10 ng of HA/μg of HAb-2 cell protein and 5 ng of HA/μg of GP4F cell protein. The amount of protein per cell was determined to be 226 ± 10 μg/10^6 GP4F cells and 283 ± 42 μg/10^6 HAb-2 cells. Whole cell capacitance measurements yield 18 ± 6 pF ($n = 15$) for the GP4F cells and 25 ± 10 pF ($n = 15$) for the HAb-2 cells (Al Franco and Judith White, unpublished data). The standard deviations for the capacitance measurements within a population (i.e., GP4F or HAb-2) reflect cell size differences, most likely due to the cells being at differents stages of the cell cycle. With a molecular weight of 2×10^5/HA trimer, 85% of the HA on the surface, and assuming 100 μm^2/pF of capacitance, we obtain (2.9 ± 1.9) × 10^3 HA/μm^2 of HAb-2 cell membrane and (1.6 ± 0.8) × 10^3 HA/μ^2 of GP4F cell membrane. The ratio is 1.8, which agrees well with the value of 1.9 determined simply by the ratio of HA per microgram of cell protein. The relatively large standared deviations are due to cell size differences, not HA surface density inhomogeneities between the cells. Thus, while these measurements are more direct, we believe that the standard deviation for the ratio of HA per microgram of cell protein is a better estimator for the standard deviation of the ratio of HA cell surface densities. For these data, the absolute amount of HA per cell was calculated by reference to a standard curve by using the bromelain-released fragment of the HA (BHA) from the Japan strain of influenza virus purified as described previously (Doms et al., 1985). The amount of BHA in the standard was determined by quantitative amino acid analysis.

In order to demonstrate that the HA expressed on these two cell lines differs only with respect to surface density, we used fluorescence recovery after photobleaching, FRAP, to measure the lateral mobility of the HA in the cell membranes and the mobile fraction of HA (Scullion et al., 1987). In these experiments, rhodamine-labeled anti-HA Fab fragments were used. For the GP4F cells the lateral diffusion coefficient was (10.3 ± 2.5) × 10^{-10} cm^2/s ($n = 15$) and the mobile fraction of HA was 68 ± 18%. For the HAb-2 cells, the coefficient was (9.7 ± 2.5) × 10^{-10} cm^2/s ($n = 19$) and the mobile fraction of HA was 75 ± 13% (F. Zhang and K. Jacobson, unpublished data). Therefore, within the precision of the measurement, both the lateral diffusion coefficients and the mobile fraction of HA were the same on both cell lines. The fact that the lateral diffusion coefficients are at the upper end of the range found for integral membrane proteins is convincing evidence that the majority of the HA is freely diffusing in both cellular plasma membranes. Our fusion experiments were done at pH 4.8, which is low enough to activate all known strains of influenza HA.

Liposome Binding and Fusion. For the liposome binding and fusion experiments the cells were grown in 12-well cluster dishes (NUNC). The cells were seeded at 34000 cells/mL per well and were used 3 days later. At this time, they were approximately 70–80% confluent.

The hemagglutinin on the HAb-2 and GP4F cells was expressed as the HA0 precursor. Before liposome binding and fusion, the HA on the cells was cleaved into HA1 and HA2 by treating the cells for 4 min at room temperature with 10 μg/mL trypsin in serum-free DMEM H21 supplemented with 10 mM HEPES. Neuraminidase (1 mg/mL) was included in this treatment as it was found to promote red blood cell binding to HA-expressing fibroblasts. The cells were then washed twice with serum-containing growth medium supplemented with 20 μg/mL soybean trypsin inhibitor. The cells were allowed to recover from this treatment for 1.5–2 h in a 37 °C CO_2 incubator. During this time there was minimal endocytosis of HA (Lazarovits & Roth, 1988).

For liposome binding, the liposomes were suspended in bicarbonate-free RPMI 1640 medium, supplemented with 10 mM HEPES, 0.2% bovine serum albumin, and 35 mM NaCl (pH 7.4), and 0.5 mL of the liposome suspension was added per well. The liposomes were allowed to bind to the cells for 30 min. at room temperature. During this incubation period the dishes were spun twice for 3 min at 500g, with a 180° rotation between spins. The centrifugation step enhances the binding of liposomes about 3-fold (results not shown). For liposome binding experiments, the cells were washed four times with PBS CMF to remove unbound liposomes and dissolved in 0.5% Triton X100 in PBS CMF and the cell-associated radioactivity was measured. For liposome fusion experiments, the RPMI pH 7.4 binding medium was removed and fusion between bound liposomes and cells was induced by a 90-s incubation of the cells with fusion medium (RPMI 1640, supplemented with 10 mM succinate, 0.2% bovine serum albumin, and 35 mM NaCl, pH 4.8) in a 37 °C water bath. The fusion medium was then replaced with regular growth medium and the cells were returned to the 37 °C CO_2 incubator. Protein synthesis activity was measured 4 h after the fusion step. Following a 2-h incubation with ^{14}C-labeled mixed amino acids in growth medium (2 μCi/mL) the cells were washed several times with PBS CMF and dissolved in lysis buffer (0.5% Triton X100 in PBS CMF, with 10 μg/mL soybean trypsin inhibitor, 20 μg/mL aprotinin, and 180 μg of PMSF/mL). The nuclei were removed by centrifugation. The

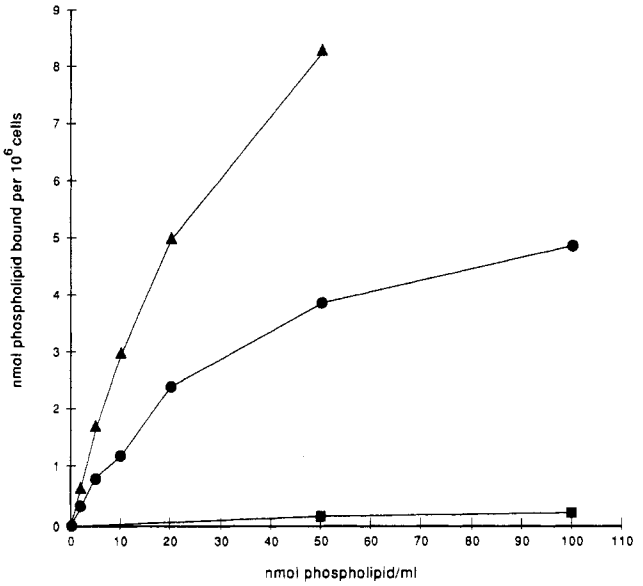

FIGURE 1: Binding of ^{14}C-labeled cholesteryl oleate glycophorin liposomes to NIH 3T3, GP4F, and HAb-2 cells. The cells were treated with trypsin and neuraminidase as described in Materials and Methods. After the recovery period the cells were incubated with various concentrations of glycophorin liposomes for 30 min at room temperature as described in Materials and Methods. The values shown are the means of three measurements: (■) NIH 3T3; (●) GP4F; (▲)HAb-2. These same symbols are used in all the figures.

lysates were precipitated overnight at 4 °C with a final concentration of 10% trichloroacetic acid. The precipitates were pelleted in an Eppendorf centrifuge and the pellets were washed twice with cold acetone. The final pellets were dissolved in 125 mM TRIS/4% SDS, pH 8–9, and the TCA-precipitable radioactivity was measured.

RESULTS

Binding of Glycophorin Liposomes to Hemagglutinin-Expressing Cells. The HA-expressing GP4F and HAb-2 cells and untransfected NIH 3T3 cells were prepared for liposome binding as described in Materials and Methods. The glycophorin liposomes used in this binding experiment were labeled with 1 mol % ^{14}C-labeled cholesteryl oleate. The cells were incubated with liposomes at concentrations varying between 2 and 100 nmol of phospholipid/mL, as described in Materials and Methods. Figure 1 shows that there was very little binding of glycophorin liposomes to the untransfected NIH 3T3 cells. At a concentration of 50 nmol/mL, there was 0.15 nmol of phospholipid bound/10^6 cells. The binding to the hemagglutinin-expressing GP4F and HAb-2 cells was 20–50-fold greater. At 50 nmol/mL, the GP4F and the HAb-2 cells bind 3.9 and 8.3 nmol of phospholipid/10^6 cells, respectively. When neuraminidase-treated glycophorin liposomes were incubated with GP4F and HAb-2 cells the binding was reduced by more than 90% (results not shown).

These experiments show that the glycophorin liposomes bind specifically to the hemagglutinin expressed on the surface of these cells and that they do not recognize any other receptor on these cells. The binding data for the GP4F and HAb-2 cells was graphed as a Scatchard plot (Figure 2), i.e., according to the equation

$$r/[V] = -Kr + nK \quad (1)$$

where r = number of liposomes bound per cell, [V] = concentration of free or unbound liposomes, K is the liposome–cell binding constant, and n is the number of binding sites available per cell (Heath et al., 1984).[2]

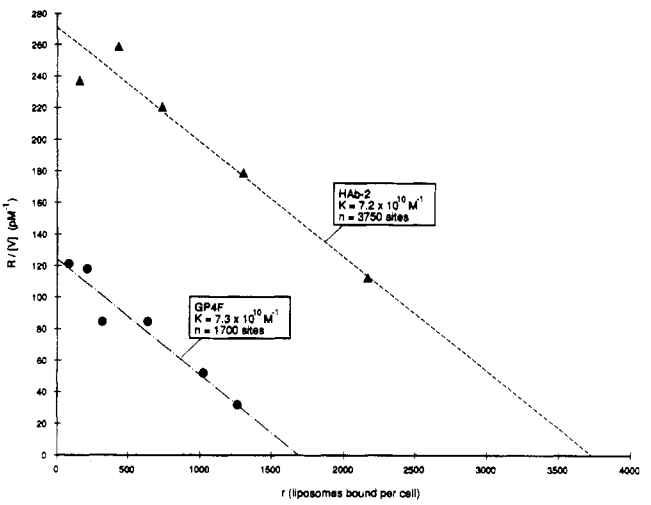

FIGURE 2: Scatchard plots of the liposome binding data from Figure 1. The straight lines are linear least squares fits from eq 1 to the data, yielding 1700 sites per cell for the GP4F cell line and 3750 sites per cell for the HAb-2 cell line. For both cell lines, the liposome–cell binding constant was nearly the same: 7.3×10^{10} M^{-1} for the GP4F cells and 7.2×10^{10} M^{-1} for the HAb-2 cells.

For both cell lines, the data fit a linear plot, with the same binding constant $K = 7 \times 10^{10}$ M^{-1}. Given that the binding constant was the same for both cell lines and that the binding was specific to the HA on the cell membrane, it must be that the number and the affinity of the HA–glycophorin interactions for each liposome were the same for both cell lines. Cell counting yielded 5.7×10^5 GP4F cells per well and 3.1×10^5 HAb-2 cells per well, on average. Using these numbers, we calculated 1700 binding sites per GP4F cell and 3750 binding sites per HAb-2 cell.

Fusion of Glycophorin Liposomes with Hemagglutinin-Expressing Cells Requires Mature HA. In order to demonstrate HA-mediated fusion between glycophorin liposomes and the plasma membrane of HA-expressing cells, liposomes were loaded with the toxin gelonin (Stirpe et al., 1980). Gelonin is a single-chain molecule with a molecular weight of about 30 000. It catalytically inactivates the 60-S ribosomal subunit, very much like the A chains of abrin, ricin, and modeccin. It has been estimated that in a cell-free system from rabbit reticulocytes, one molecule of gelonin inactivates about 200 ribosomes per minute. Gelonin is not toxic to intact HeLa cells, presumably because there are no receptors for gelonin on the surface of these cells (Stirpe et al., 1980).

We determined that gelonin is also nontoxic to the HA-expressing 3T3 fibroblasts. When these cells were incubated with free gelonin at a calculated 30-fold higher solution concentration (10 μg/mL) than was encapsulated in the added liposomes (under otherwise identical conditions), there was no effect on the protein synthesis activity. Therefore, the only way in which liposome-encapsulated gelonin can inhibit protein synthesis activity in intact fibroblasts is following a fusion event

[2] A rigorous application of the Scatchard analysis would require that binding be checked at a later time point to assure that equilibrium binding conditions are satisfied. However, due to the linearity of the graph in Figure 2, we believe that the binding reaction was sufficiently close to equilibrium to apply this analysis (Bentz & Nir, 1981a,b). Even if the binding is nonequilibrium, our conclusions are the same. In this case, we would speak of attachment rate constants, rather than binding constants, and the data would show that both cell lines have the same rate constants for liposome attachment. Since the attachment depends solely upon the HA trimer–glycophorin interactions, it must be that the number of HA trimer–glycophorin interactions per liposome are the same for both cell lines.

Table I: Low-pH-Induced Delivery of Liposome-Encapsulated Gelonin Requires Mature HA[a]

hemagglutinin	% inhibitn of protein synth act.[b] pH 7.4	pH 4.8
HAO	0	0
mature HA	0	89

[a] HAb-2 cells were treated with neuraminidase (1 mg/mL) or with a mixture of trypsin (10 μg/mL) and neuraminidase (1 mg/mL) for 4 min at room temperature, resulting in expression at the cell surface of HAO or mature HA, respectively. After the recovery period the cells were incubated for 30 min at room temperature with glycophorin liposomes at 50 nmol of phospholipid/mL. Fusion was induced by a 90-s incubation of the cells with the bound liposomes at pH 4.8 and at 37 °C. Control cultures were incubated for 90 s in pH 7.4 binding medium at 37 °C. The cells were then incubated in growth medium for an additional 4 h at the end of which the protein synthesis activity was measured. The level of protein synthesis activity at pH 7.4 was defined as the 0% inhibition value. [b] The values shown are the means of three measurements with standard deviations for protein synthesis activity of about 12%.

between the liposomal membrane and the membrane of the cells.

Table I shows that the mature form of HA is required for the low-pH-induced fusion between liposomes and cells. The incorporation of labeled amino acids at pH 7.4 was taken as 100% activity, i.e., 0% inhibition. Following the low-pH treatment, protein synthesis activity was inhibited by 89% in HAb-2 cells expressing the mature HA, demonstrating that essentially all the cells in the population received gelonin. The protein synthesis activity in cells expressing the precursor form of HA (HAO) was unaffected. Similar results were obtained with the GP4F cells (not shown).

When the effect of gelonin-containing liposomes was compared with that of empty liposomes on cells maintained at pH 7.4, we found that at high concentrations the gelonin liposomes caused some inhibition even without a low-pH treatment. This inhibition was presumably due to a small amount of endocytic uptake of the liposomes and fusion at the level of the endosomal membrane. This affects our measurements only slightly. In a repeat of the experiment shown in Table I (with cells expressing the mature HA), the inhibition following the pH 4.8 treatment was 82% relative to the pH 7.4 control treated with gelonin liposomes and 87% relative to the pH 7.4 control treated with empty liposomes. Unless mentioned otherwise, in the following experiments the low-pH-induced inhibition was measured relative to a control incubation with empty liposomes, although protein synthesis activity was the same with empty liposomes as it was without any liposomes.

Only a Fraction of the Liposomes Bound to HA Are Induced to Fuse at Low pH. Figure 3 shows the inhibition of protein synthesis activity as a function of liposome concentration in both the GP4F and HAb-2 cell lines. Note the different scales on the *x*-axes. At high liposome concentrations, i.e., 50 nmol/mL for the GP4F cells and 10 nmol/mL for the HAb-2 cells, the inhibition is essentially complete. Half-maximal inhibition occurs at 5 nmol/mL for the GP4F cells and at 0.5 nmol/mL for the HAb-2 cells, i.e., a 10-fold difference. This is consistent with the fact that delivery of mRNA encoding chloramphenicol acyltransferase by glycophorin-bearing liposomes was more efficient with the HAb-2 cells than with the GP4F cells (Ellens et al., 1989b; Glenn et al., 1990).

The gelonin was encapsulated in the liposomes at a high concentration (0.35 mg/mL). It can be calculated that a liposome with a diameter of 550 nm contains approximately 600 gelonin molecules. In cell-free systems, gelonin is as active as the A-chains of abrin and ricin (Benson et al., 1975; Stirpe et al., 1980). For abrin and ricin there is evidence that one molecule can completely inhibit protein synthesis activity in a given cell (Eiklid et al., 1980). Therefore, it is expected that the gelonin contained in one liposome is enough to completely inhibit protein synthesis activity in a given cell. That this is indeed the case will be shown below.

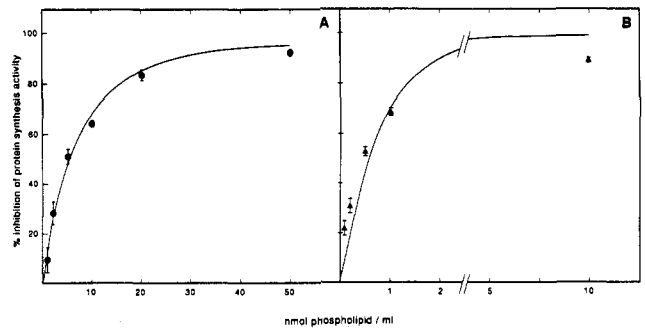

FIGURE 3: Liposome concentration dependence of protein synthesis inhibition in GP4F and HAb-2 cells. GP4F cells (panel A) and HAb-2 cells (panel B) were incubated with various concentrations of gelonin-containing liposomes. Maximal protein synthesis activity (i.e., 0% inhibition) was measured in cultures that were not incubated with liposomes but were otherwise treated identically. The values shown are the means of three measurements. The solid lines are calculated from eq A.5 in the Appendix, which assumes that 0.0033 of the liposomes bound to the GP4F cells fuse and 0.0145 of the liposomes bound to the HAb-2 cells fuse. The values for bound liposomes are calculated from the parameters obtained in the Scatchard plot, Figure 2.

Our binding data (Figure 1) show that at 5 nmol/mL there are about 200 liposomes bound per GP4F cell and at 0.5 nmol/mL there are about 35 liposomes bound per HAb-2 cell. Hence, the fact that we see half-maximal inhibition of protein synthesis activity under these conditions (Figure 3) indicates that only a small fraction of the bound liposomes are able to fuse, even though all the liposomes are bound specifically to HA. The solid lines in Figure 3 show theoretically calculated values of protein synthesis inhibition from eq A.5 in the Appendix. In essence, the closeness of the fit of the data points to the theoretical curves implies that any bound liposome can fuse but that only a small fraction actually do fuse, for reasons we will discuss below.

Quantitation of the Number of Liposomes Fusing per Cell. In order to quantitate the number of liposomes fusing per cell, we performed a competition experiment between gelonin-containing and empty liposomes. The HA-expressing cells were incubated with varying ratios of gelonin-containing and empty liposomes at a constant total liposome concentration. When all the liposomes contain gelonin, the inhibition will be the maximal attainable, at that particular liposome concentration. Likewise, when all the liposomes are empty, there will be no inhibition of protein synthesis activity. At intermediate ratios of gelonin-containing and empty liposomes there will be a partial inhibition of protein synthesis activity, since only a fraction of the fusing liposomes contain gelonin.

A simple probabilistic analysis of the dependence of the measured protein synthesis inhibition on the ratio of gelonin-containing and empty liposomes enabled us to calculate the number of liposomes that fused per cell. For example, if three liposomes fuse per cell and half of the liposomes contain gelonin (with the other half being empty), then only 12.5% of the cells will show protein synthesis activity, as that is the probability that all three fusing liposomes are empty. The calculation of the number of liposomes that fuse per cell is derived in the Appendix.

Quantitation of the number of liposomes fusing per cell based on this analysis assumes that one liposome contains

Table II: One Liposome Contains Enough Gelonin to Completely Inhibit Protein Synthesis Activity in a Given Cell within 4 h[a]

% gelonin-containing liposomes	gelonin concn (mg/mL)	% inhibitn of protein synth act.[b]	no. of liposomes fusing/ cell[c]
100	0.35	100	
20		72	5.7
10		46	5.8
100	1.75	100	
20		73	5.9
10		55	7.6
100	3.50	100	
20		67	5.0
10		34	4.0

[a] GP4F cells were incubated with various ratios of empty liposomes and liposomes containing three different concentrations of gelonin as indicated in the table. The total lipid concentration in each case was 100 nmol of phospholipid/mL. [b] The values shown are the means of three measurements with standard deviations for protein synthesis activity of about 15%. The maximal inhibition of protein synthesis, i.e., the inhibition of 100% gelonin-containing liposomes relative to the inhibition of 100% empty liposomes, is 90 ± 1%. [c] The number of liposomes fusing per cell is calculated from the protein synthesis inhibition data as described in the Appendix and Figure 4.

enough gelonin to give maximal inhibition of protein synthesis activity in a given cell. To prove that this is indeed the case, we prepared liposomes containing different concentrations of gelonin (0.35, 1.75, and 3.5 mg/mL) and used each of these three preparations in a competition experiment with empty liposomes. If, for example, the liposomes prepared with the lowest concentration of gelonin (0.35 mg/mL) do not contain enough of the toxin per liposome to give maximal inhibition of protein synthesis activity in a given cell, then, in the competition experiment, cells treated with liposomes containing higher concentrations of gelonin will indicate a larger number of liposomes fusing per cell.

The result of this experiment is shown in Table II. GP4F cells were incubated with various ratios of gelonin-containing and empty liposomes at a total lipid concentration of 100 nmol/mL. The maximal inhibition in the absence of empty liposomes (100% gelonin-containing liposomes) was 89%, 91%, and 88%, respectively, for the liposomes prepared with 0.35, 1.75, and 3.5 mg of gelonin/mL. For the purpose of the quantitation, these levels were defined as 100% inhibition in the table. The percent inhibition of protein synthesis activity with 20% and 10% gelonin-containing liposomes was calculated relative to this 100% level. As seen in Table II, for a given ratio of gelonin-containing to empty liposomes, the extent of inhibition of protein synthesis activity was the same within experimental error. The number of liposomes fusing per cell was calculated using eq A.4 in the Appendix. It can be seen that the three preparations of gelonin-containing liposomes give essentially the same number of liposomes fusing per cell. Therefore, in all three preparations one liposome contained enough gelonin to completely inhibit protein synthesis activity in a given cell.

Figure 4 shows the result of a competition experiment like the one described in Table II. GP4F cells were incubated with seven different ratios of gelonin-containing and empty liposomes at a total lipid concentration of 100 nmol/mL. The percent inhibition of protein synthesis activity at each ratio was measured and used to calculate the number of liposomes fusing per cell. This analysis gave an average number of 4 (± 1) liposomes fusing per cell. For comparison, we show theoretically calculated curves for 2, 4, and 8 liposomes fusing per cell. The data fit the theoretical inhibition curve for 4

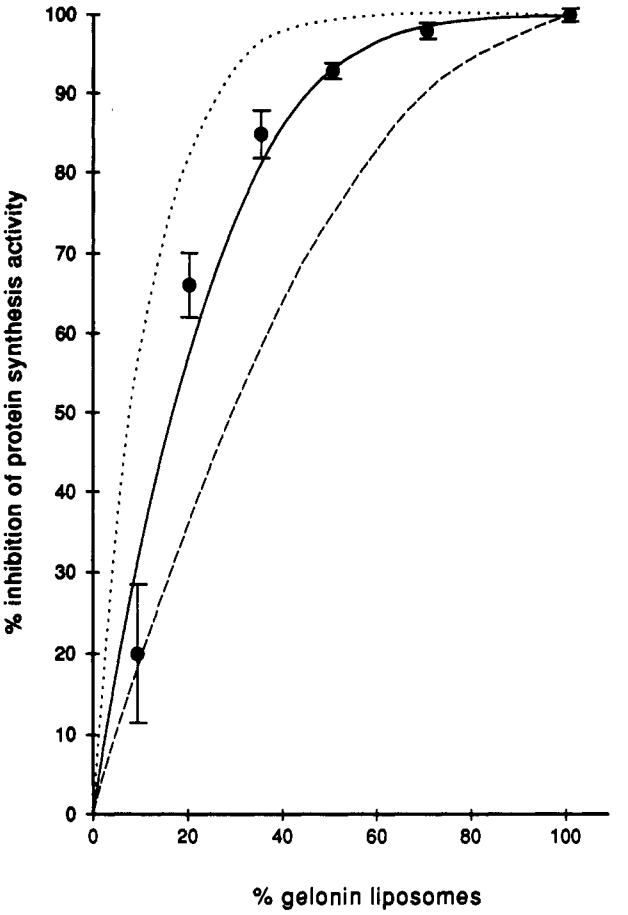

FIGURE 4: Determination of the number of liposomes fusing per cell upon incubation of GP4F cells with glycophorin liposomes at 100 nmol of phospholipid/mL. The cells were incubated with various ratios of gelonin-containing and empty liposomes at a total concentration of 100 nmol of phospholipid/mL. The closed circles are the mean values of three measurements of inhibition of protein synthesis activity. The data can be best fit from eq A.3, in the Appendix, by assuming that 4 liposomes fuse per cell. The solid line shows the theoretical inhibition curve for the fusion of 4 liposomes per cell. For illustrative purposes, we also show the theoretical inhibition curves for the fusion of 2 liposomes per cell (- - -) and 8 liposomes per cell (···).

liposomes fusing per cell over the complete range of ratios of gelonin-containing and empty liposomes.

A 1.9-Fold Increase in HA Density Leads to a 4.4-Fold Increase in Fusion Efficiency. We subsequently determined the number of liposomes fusing per cell at different liposome concentrations for both the GP4F cells and the HAb-2 cells. The results are shown in Table III. At 5 nmol of phospholipid/mL there are on average 0.6 liposomes fusing per GP4F cell and 6 liposomes per HAb-2 cell. For the GP4F cells this means that 60% of the cells show fusion with 1 liposome and 40% of the cells show no fusion at all. At 10 nmol of phospholipid/mL, the GP4F and HAb-2 cells show, on average, 1 and 12 liposomes fusing per cell, respectively. In general, we find that the HAb-2 cells have about 10 times more liposomes fusing per cell and about 2 times more liposomes bound per cell than do the GP4F cells (Figure 2); i.e., about 5 times more of the bound liposomes fuse to the HAb-2 cells than to the GP4F cells.

Surprisingly, we found for both cell lines that the number of liposomes that fused was a small constant fraction of the total number of bound liposomes, regardless of the lipid concentration. On average the GP4F cells showed a ratio of fused to bound liposomes of 0.0033 ± 0.0007 (n = 5) and the HAb-2 cells showed a ratio of 0.0145 ± 0.0009 (n = 4) (some cases

Table III: The HAb-2 Cells Fuse with 4.4 Times More Bound Liposomes Than the GP4F Cells

liposome concn (nmol of phospholipid/mL)	GP4F			HAb-2		
	liposomes bound/cell[a]	liposomes fused/cell[b]	ratio	liposomes bound/cell[a]	liposomes fused/cell[b]	ratio
5	206	0.6	0.0029	439	6	0.0137
10	310	1	0.0032	774	12	0.0156
100	1264	5	0.0040	nd[d]	nc[c]	nd[d]

[a] Determined from the data in Figure 1. [b] Determined from the data as in Figure 4 and Table II. [c] nc: This number was not calculated, since at this concentration the liposomes cause formation of extensive syncytia of the HAb-2 cells, so that now one liposome can deliver its contents into more than one cell and consequently inhibit protein synthesis activity of more than one cell. This becomes especially noticeable at the small percentages of gelonin-containing liposomes. [d] nd: not determined.

shown in Table III). In other words, for the GP4F cells, roughly 1 out of every 300 bound liposomes fused, and, for the HAb-2 cells, 1 out of every 70 bound liposomes fused.

We can use these data to show that each bound liposome has the same probability of fusing to a given cell. The solid lines in Figure 3 are theoretical values for protein synthesis inhibition, assuming that the fusion of any bound liposome is random, with a probability of 0.0033 for the GP4F cells and of 0.0145 for the HAb-2 cells. The total number of bound liposomes at any liposome concentration was calculated from the parameters of the Scatchard plot given in Figure 2. The fit of the curves in Figure 3 to the experimental data implies that any bound liposome can fuse and that the probability of that fusion depends solely on the HA surface density; i.e., the fusion is a simple random event.

At 100 nmol of phospholipid/mL the GP4F cells show 5 liposomes fusing per cell. At this lipid concentration the HAb-2 cells showed extensive syncytia formation (results not shown). The formation of syncytia occurred only after incubation of the HAb-2 cells with high concentrations of liposomes and then only after low-pH incubation. Therefore syncytia formation is promoted by liposome fusion, presumably by one or more liposomes fusing with 2 neighboring cells. The extensive syncytia formation at this lipid concentration prohibited us from calculating the number of liposomes fusing per cell, since one fused liposome could deliver its contents into more than one cell and therefore inhibit protein synthesis activity in two or more cells. It has been shown previously that HA-expressing cells can show cell–cell fusion when the cells express high levels of HA (Gething et al., 1986, 1988). The HAb-2 cells do not express enough HA to show cell–cell fusion in the absence of liposomes, but do so at high liposome concentrations.

HA Expressed on 3T3 Fibroblasts Is Not Inactivated by a Low-pH Preincubation. Incubation of influenza virus at low pH (for 5 min at 37 °C) in the absence of a target membrane leads to inactivation of viral fusion and/or hemolytic activity (White et al., 1982; Sato et al., 1983; Junankar & Cherry, 1986; Stegmann et al., 1986, 1987, 1989a,b). Under these conditions the X31 and X47 strains of influenza virus and fowl plague virus (an avian influenza virus) are more than 85% inactivated. Interestingly, the Japan strain HA expressed on the GP4F and HAb-2 cells did not show any low-pH-induced inactivation in terms of the number of liposomes fusing per cell. Table IV shows the result of an experiment in which the cells were preincubated (in the absence of liposomes) for 5 min at pH 4.8 in a 37 °C water bath. The cells were then allowed to recover from the low-pH treatment for 15 min in regular tissue culture medium. After this recovery period the cells were incubated with various concentrations of liposomes and treated at low pH to induce fusion. It was clear from the results of this experiment that there was no low-pH-induced inactivation occurring in a 5-min preincubation at pH 4.8 and 37 °C. Recently, it has been reported that intact virions of the Japan strain of influenza virus (A/305/57) also appear to be resistant to inactivation by acid pretreatment (Puri et al., 1990).

Table IV: The HA-Expressing Cells Do Not Exhibit Low-pH Inactivation of Hemagglutinin

cell line	liposome concn (nmol of phospholipid/mL)	% inhibitn of protein synth act.[a]	
		control cultures	low-pH pretreated cultures[b]
HAb-2	10	90	92
	1	69	67
	0.1	22	17
GP4F	50	93	93
	5	40	46
	2	15	21

[a] The values shown are the means of three measurements with standard deviations for protein synthesis activity of about 7%. [b] The cultures were pretreated with the pH 4.8 fusion medium for 5 min in a 37 °C water bath. After 15 min at neutral pH the liposomes were added and the fusion protocol was executed.

Discussion

Although the behavior of the influenza HA trimer in response to low pH is fairly well understood, it is not known how the low-pH form of the HA with its exposed fusion peptides actually promotes fusion of the apposed membranes. The central issue addressed in this paper is whether more than one HA trimer are required to form a fusion site. To do this, we compared the binding and fusion of glycophorin-bearing liposomes to two fibroblast cell lines, which express HA at different surface densities.

Liposome Binding. The first step was to determine the liposome–cell binding constants and the number of liposome binding sites. For both cell lines the linearity of the fits to the Scatchard equation was very good. The GP4F cell line shows 1700 liposome binding sites per cell, while the HAb-2 cell line shows 3750 sites per cell, or just over 2.2 times as many sites. These values imply that essentially all of the exposed cell surface area must be available for binding. The liposome Z-average diameter from dynamic light scattering is 0.55 μm. Thus, 3750 liposomes would cover at least 1000 μm^2 of cell surface area (Bentz et al., 1988), which is 80% of the top surface of the HAb-2 cells, as estimated by capacitance (Materials and Methods).

The fact that the liposome–cell binding constants were the same for both cell lines was unexpected. There are about 3000 glycophorin molecules in the outer monolayer of the liposome and several hundred HAs underneath each bound liposome, assuming a liposomal cross-sectional area of 0.24 μm^2. The opportunity for multivalent binding, i.e., several HA–glycophorin bonds per liposome, should be possible. Multivalent liposome–cell binding has been found between smaller liposomes (ca. 0.2-μm diameter) with antifluorescein IgG conju-

gated to their surface and erythrocytes with fluorescein conjugated to their membranes (Heath et al., 1984).

Since the liposome–cell binding constant is independent of HA surface density, it must be that the HA–glycophorin binding is saturated with respect to the HA surface density for both cell lines. It is interesting that the binding constant for sialic acid to HA is ca. 10^3 M^{-1} (Pritchett et al., 1987; Sauter et al., 1989), so that the simultaneous binding of three to four sialic acids residues (possibly from different glycophorin molecules) to the sialic acid binding sites (possibly on different HAs) would have a maximal binding constant of about 10^9–10^{12} M^{-1} (Heath et al. 1984), which is comparable to the measured value of 7×10^{10} M^{-1}. It is also possible that many more, much weaker binding attachments form.

Quantitation of Liposome Fusion. In order to count the number of liposomes fusing per cell, we needed an assay sensitive enough to measure small amounts of fusion. Furthermore, we wanted a contents mixing assay, which measured the delivery of the liposome's encapsulated contents to the cytoplasm of the cell. This is the only reliable means of verifying that fusion actually has occurred (Bentz & Ellens, 1988).

Previous studies have used liposome-encapsulated diptheria toxin A chain or ricin A chain to demonstrate cytoplasmic delivery of liposome contents (Gitman et al., 1985; Collins & Huang, 1987). The sensitivity of these fusion assays stems from the fact that one toxin molecule can completely inhibit protein synthesis in a given cell. Whether this is true also for gelonin is not rigorously known. However, Table II shows that one liposome does contain enough gelonin to completely inhibit protein synthesis activity in a given cell in 4 h.

In this study, the fusion efficiency of the HA-containing membrane has been calibrated by the extent of fusion between the cell and prebound liposomes. We have stressed that fusion mechanisms are best studied by using initial rates and time courses of fusion, rather than final extents (Bentz & Ellens, 1988; Bentz et al., 1988). With gelonin as the marker for fusion, it is only possible to measure the total number of fused liposomes. Given the small fraction of fused to bound liposomes, it was not feasible to use fluorimetric fusion assays. However, for the system used in this study, we expect no discrepancy between monitoring initial rates or final extents. Because fusion is defined by the contents mixing of a large soluble protein, every liposome that starts to fuse, which would be monitored by an initial rate, completes the process, which would be monitored by the final extent. Our measurements could differ from initial rate data if during the 90-s low-pH incubation some of the liposomes dissociate from the membrane and then reattach to fuse at a later time. It is likely that the number of bound liposomes remains constant during the 90-s low-pH treatment, and thereafter the pH is raised to neutral where no additional fusion will be initiated (Stegmann et al., 1985; Morris et al., 1989). Thus the only other concern with using final extents to count fusion sites or efficiencies is that the fused liposomes will alter the target membrane and thereby affect the fusion of subsequent liposomes (Bentz et al., 1988). This is unlikely to be a problem in this system, given the small number of liposomes that fuse per cell.

Different HAs Mediate Binding and Fusion. Analysis of variance shows that the fractions of fused to bound liposomes for both cell lines are significantly different, 0.0145 ± 0.0009 and 0.0033 ± 0.0007 for HAb-2 and GP4F cells, respectively. Also, the ratio of these fractions ($4.4 = 0.0145/0.0033$) is significantly different from the 1.9 ± 0.2 increase in the HA surface density (with $p > 0.98$). These facts have two important implications. Firstly, the HA bound to glycophorin is not involved in fusion. Secondly, one HA trimer cannot induce the fusion between a liposome and the plasma membrane of the cell.

Since the liposome–cell binding constant is the same for both cell lines, i.e., the binding involves the same number of HA–glycophorin interactions, the HAs involved in binding are not the HAs involved in fusion. If the HAs bound to glycophorin were involved in the fusion process, then the HAb-2 cells should have fused with the same fraction of bound liposomes (0.0033) as the GP4F cells.

The fact that the HAs involved in binding are not the HAs involved in fusion and that only a small fraction of the bound liposomes fuse is consistent with the electron micrograph in Doxsey et al. (1985) of an erythrocyte showing a small area of fusion within a very extensive binding area. Therefore, even though each HA has a binding and a fusion function, those functions are not performed by the same HA.

The HAb-2 cells, with a 1.9 times higher surface density of HA than the GP4F cells, show 4.4 times as much fusion per bound liposome. This shows that one HA trimer (which is not involved in binding) is not able to trigger a fusion event. If one HA trimer were sufficient to induce fusion, then the HAb-2 cells would be expected to show only 0.0063 ($=1.9 \times 0.0033$) of the liposomes fusing per bound liposome, rather than the observed 0.0145. This is a statistically significant difference ($p > 0.98$). Furthermore, since there are on the order of several hundred HA trimers in the membrane area under each bound liposome, if one HA trimer could induce fusion, then we would expect to see much more fusion.

Our experimental methodology was designed to accommodate the situation where some of the liposomes would bind to sites incapable of fusion. As it turned out, the theoretical curves in Figure 3 show that all the binding sites can support fusion in a random fashion. The solid lines are theoretical calculations for percent inhibition assuming that (randomly) 0.0033 of the liposomes bound to the GP4F cells fuse and 0.0145 of the liposomes bound to the HAb-2 cells fuse, where the amounts bound are calculated from the binding constants and the number of sites per cell obtained from the Scatchard plot in Figure 2. Given that these data represent several separate experiments, the concordance is quite good. Recall that in these experiments all of the liposomes contain gelonin, so that the less than complete inhibition of protein synthesis is due simply to the small probability for fusion. We believe that this probability is small because fusion occurs only if an HA aggregate happens to be or to form beneath the liposome during the low-pH treatment.

HA Aggregates and Fusion. The results presented show that fusion involves an aggregate of HA trimers on the cell surface. Since the fusion does not involve HAs bound to glycophorin, these aggregates are not induced by liposome binding but rather are products of mass action interactions on the cell surface. A simple analysis of this hypothesis is presented in Bentz (1990), which illustrates how the minimal fusion unit aggregate size can be determined.

In this study, we have determined that this minimal fusion unit is 2 or larger. We believe that this fusion unit is not very large. Sarkar et al. (1989) have estimated an initial pore size for fusion (measured by contents mixing of NBD–taurine) between erythrocytes and GP4F cells of about 9 nm (or less) in diameter, while Spruce et al. (1989) have estimated an initial pore size for fusion (measured by conductance and capacitance changes) of 4 nm between erythrocytes and the HAb-2 cells. HA on the virus in the neutral pH form occupies

a patch about 6 nm in diameter (Wilson et al., 1981; Doms & Helenius, 1986). Thus, about five HAs would suffice to form the perimeter of a 4-nm fusion site.

There have been a few previous studies aimed at deducing the minimal functional protein unit for fusion of influenza virus. Gibson et al. (1986) and Bundo-Morita et al. (1987, 1988) have used radiation inactivation analysis to estimate the size of the functional unit (in the influenza virus) that is responsible for virus fusion (monitored by lipid mixing) with cardiolipin liposomes and with erythrocytes. Hemolysis and liposomal leakage were also analyzed. They found that a ca. 60–70 kDa target size, consistent with one HA1 + HA2 subunit of one HA trimer, was the minimal inactivation unit for inhibition of lipid mixing. In other words, inactivation of each and every HA1 + HA2 subunit on the virus was required to eliminate its lipid mixing capacity. However, liposome leakage or hemolysis could be inhibited after inactivation of a target 3–8 times larger.

Radiation inactivation is an interesting technique for probing minimal activity units. However, the results are difficult to interpret in a system as complex as virus–liposome fusion. For example, Bundo-Morita et al. (1987) found that the virus undergoing the radiation protocol, but without irradiation, was 25–100% more active than the native virus, with respect to lipid mixing. Evidently the experimental protocol is producing changes in the viral envelope proteins that also "activate" their fusion capacity. More importantly, it is possible that the data fitted could correspond to the regime of high HA surface density, where the size of the minimal fusion unit is underestimated. If this were the case, it would be necessary to probe the system at higher radiation dosage levels, where the surface density of fusogenic HA would be reduced (Bentz, 1990).

These reservations notwithstanding, the results could imply that lipid mixing proceeds from a smaller functional unit than does leakage. This might mean that leakage happens after membrane mixing and involves some postfusion complex or collapse of the fusion product. An alternative is that the initial lipid mixing represents destabilization of only the outer monolayer of the liposome or erythrocyte, i.e., hemifusion with the outer monolayer of the viral envelope, which is known to occur in a few liposome systems (Duzgunes & Bentz, 1988). Leakage, on the other hand, certainly requires destabilization of the entire membrane. Although influenza virus–liposome fusion involves contents mixing of large molecular weight markers (White et al., 1982; Stegmann et al., 1985), there has never been a kinetic analysis of virus–liposome fusion comparing contents mixing to lipid mixing, in order to determine the squence of events (Bentz et al., 1988).

Morris et al. (1989) have studied the fusion of erythrocyte ghosts with GP4F cells, using a lipid mixing assay. The erythrocyte ghosts were bound to the GP4F cells and then the cells (with bound erythrocyte ghosts) were removed from the culture dish by trypsin treatment, so that the fusion experiments were done with suspension cells. The initial kinetics of lipid mixing were measured as a function of pH and it was suggested that nine protonations may be involved at the fusion site. While the basic idea of deducing minimal fusion units by altering pH in order to vary the surface density of "active" fusion proteins is valid (Blumenthal, 1988), supplementary data (such as the intrinsic pK_a of the protonated site) are required before reliable estimates can be obtained (Bentz, 1990). It is of interest that Henis et al. (1989) suggest that the fusion of Sendai virus involves more than one of the envelope proteins.

The absolute fusion capacity of the cell membrane decreases rapidly as the surface density of HA decreases. Nevertheless, the value of the minimal fusion unit can be fitted rigorously by using cell lines with lower HA surface densities than that found on the GP4F cells (Bentz, 1990). It is worthwhile to note here how much fusion can be expected. If a cell line has half the HA surface density and the same number of liposome binding sites (1700) as the GP4F cell line, then, with 1700 bound liposomes, we would expect a maximum of three liposomes fusing per cell if the minimal fusion unit is 2. If the minimal fusion unit is 10, then only 0.9 liposomes would fuse per cell; i.e., fusion activity would become quite small at lower bound liposome levels. In this sense, it would be difficult to prove that the minimal fusion unit is 10 or greater.

Mechanism of Membrane Destabilization. Our finding that more than one HA is required at the fusion site has led us to reconsider the molecular mechanism by which HA destabilizes membranes. Knoll et al. (1988) have speculated that HA-induced liposome–cell fusion occurs via lipidic particle intermediates. This is based upon electron micrographs showing the presence of ca. 30-nm diameter fusion-correlated particles on the freeze–fracture faces. For pure lipid systems, lipidic particles are observed that are also ca. 30 nm in diameter and that are known to arise from fusion intermediates that are much smaller (Ellens et al., 1989a, and references therein). Thus, the actual fusion site could be smaller than 30 nm, as has been proposed by Sarkar et al. (1989) and Spruce et al. (1989).

The liposome–liposome fusion mechanism associated with the appearance of lipidic particles applies only to lipids capable of subsequently transforming to inverted cubic phases (Ellens et al., 1986, 1989a). The fusion intermediate of this mechanism, a so-called interlamellar attachment (ILA; Siegel, 1986), has been imaged recently by using time-resolved cryo-transmission electron microscopy and found to be the same object as the lipidic particle imaged via freeze-fracture (Frederik et al., 1989; Siegel et al., 1989b). The involvement of an ILA-like structure in HA-mediated fusion, while speculative, can explain why two or more HAs are required for fusion. The lipids in the glycophorin-containing liposomes, as well as those in the membrane of the cells, are not capable of forming nonlamellar phases when they are fully hydrated. However, the close approach of apolar peptide groups of the HAs would dehydrate the intermembrane space between them. It is known that reducing the water content of lipids will facilitate the formation of nonlamellar phases (Gruner et al., 1988). We have shown that the initial contact of two liposomes can respond to this inverted phase mechanism even when only the outer monolayer of *one* of the liposomes is composed of inverted phase forming lipids (Bentz et al., 1987). Thus the fact that the inner monolayers of the cell and the liposome are not tempted toward inverted phases does not rule out this proposed mechanism. Further discussion on the architecture of the HA fusion site can be found in Bentz (1990).

Appendix: Calculation of the Number of Liposomes Fusing per Cell

Let J denote the average number of liposomes that fuse per cell. Clearly J will depend upon the number of bound liposomes. In order to fit the value of J for a given total lipid concentration, we employ a competition experiment by varying the fraction of liposomes that contain gelonin, denoted p. Thus $1 - p$ is the fraction of liposomes that are empty. For example, a fusion experiment with 50 nmol/mL total phospholipid and $p = 0.2$ implies 10 nmol/mL gelonin-containing liposomes and 40 nmol/mL empty liposomes.

We have shown that the fusion of one gelonin-containing liposome is sufficient to produce complete protein synthesis inhibition, Table II. Thus after the fusion of J liposomes, the only cells capable of normal protein synthesis are those for which none of the fused liposomes contained gelonin. If (assumption I), the gelonin-containing and empty liposome bind and fuse equally well, then

$$\% \text{ cells not containing gelonin} = 100(1-p)^J \quad \text{(A.1)}$$

Note that $(1-p)^J$ is just the probability that of J randomly chosen liposomes, none contain gelonin.

After fusion, the cells are incubated for 4 h. This is sufficient time for the injected gelonin to completely inhibit protein synthesis, as shown by the insensitivity of protein synthesis inhibition to the concentration of encapsulated gelonin. Thus the incorporation of labeled amino acids 4 h after the low-pH treatment will occur only in those cells that did not receive gelonin. Let i_1 denote the amino acid incorporation level of the cells when $p = 1$; i.e., all liposomes contained gelonin and protein synthesis is inhibited maximally. Let i_0 denote the level when $p = 0$; i.e., all liposomes are empty. Finally, let i_p denote the level when a fraction p of the liposomes contain gelonin. Then

$$\% \text{ inhibition} = \% \text{ protein synthesis inhibition} = 100\frac{i_p - i_1}{i_0 - i_1} \quad \text{(A.2)}$$

Now, if (assumption II) all cells without gelonin grow equally well, then

$$\% \text{ inhibition} = 100 - \% \text{ cells not containing gelonin initially} = 100[1-(1-p)^J] \quad \text{(A.3)}$$

As long as assumption II is valid, it is not necessary to consider whether cell division occurred. Figure 4 shows the graph of eq A.3 for several values of J. Equation A.3 can be solved for J as

$$J = \frac{\log(1 - \% \text{ inhibition}/100)}{\log(1-p)} \quad \text{(A.4)}$$

and J can be fitted as the numerical average of the data.

The two assumptions made thus far cannot be rigorously proven. We do know that the overall binding of gelonin-containing and empty liposomes is the same; data not shown. However, the binding at the fusion site involves only a few percent of the total binding. With respect to their fusion, it would be instructive to quantify the fusion kinetics of the gelonin-containing and the empty liposomes to intact virus.

These equations hold when some of the binding sites will not sustain fusion. However, if a constant fraction, ϕ, of all the binding sites are fusogenic and there are r gelonin-containing liposomes bound per cell, then

$$\% \text{ inhibition} = 100[1-(1-\phi)^r] \quad \text{(A.5)}$$

r is calculated from the Scatchard equation, eq 1, using the parameters fitted in Figure 2. We have found that $\phi = 0.0033$ for the GP4F cells and $\phi = 0.0145$ for the HAb-2 cells, Table III. The solid lines in Figure 3 are from this equation. The fact that the curves fit the data as well as they do implies that any binding site can be a fusion site provided there is an HA aggregate formed beneath the liposome during the 90-s low-pH treatment. Note that eqs A.5 and A3, while similar in form, represent completely different experiments.

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