Articles

Fusion of Influenza Virus with Sialic Acid-Bearing Target Membranes†

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Received July 13, 1993; Revised Manuscript Received November 8, 1993®

ABSTRACT: We have monitored the fusion of intact A/PR/8/34 influenza virus with glycophorin-bearing liposomes and with ganglioside- (GD1a-) containing liposomes. The lipid bilayers of the glycophorin-bearing liposomes had several compositions, including pure dioleoylphosphatidylethanolamine (DOPE), pure egg phosphatidylethanolamine (EPE), and pure dioleoylphosphatidylcholine (DOPC). Examination of the temperature dependence of fusion for these and other compositions showed that even if the lipids are competent to form inverted hexagonal phases (H_{II}), there is no enhancement of the fusion rate constant at the L_α–H_{II} phase transition temperature of the lipids, T_H. Thus, the H_{II} phase transition is not involved in the HA-mediated fusion mechanism. However, this mechanism is sensitive to lipid composition, in that PC bilayers fused more slowly than PE-containing bilayers above 20 °C. These results show that the HA-mediated fusion mechanism depends primarily upon specific lipid–protein interactions, although the fundamental parameters of lipid phase stability (interstice stabilization and monolayer spontaneous radius of curvature) may also be important. The fact that H_{II} phase-competent lipid bilayers in the glycophorin liposomes do not enhance the HA-mediated fusion rate strongly suggests that substantial bilayer–bilayer contact is not involved in HA-mediated fusion. Previously, we have shown that glycoprotein-bearing liposomes bind to HA-expressing cells specifically through HA-glycophorin interactions and that fusion is mediated by HAs not bound to glycophorin. Thus, with respect to the target membrane, the fusion site involves just the lipid bilayer. Our results with GD1a-containing liposomes strongly suggest that HAs bound to this sialic acid-bearing molecule are likewise incapable of participating in the fusion site. This could be due to a diminished lateral mobility of the HAs simultaneously bound to both closely apposed membranes. Finally, we find that the low-pH-induced viral inactivation is inhibited by binding to either glycophorin- or GD1a-containing target membranes.

Hemagglutinin (HA)[1] is the major envelope glycoprotein of influenza virus. It 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 & Ohnishi, 1980; Matlin et al., 1981; White et al., 1982). The ectodomain of HA has been crystallized (Wilson et al., 1981) and shown to undergo conformational changes at the pH associated with viral fusion, including the exposure of the highly conserved apolar N-terminal segment of the HA2 subunit (Skehel et al., 1982; White & Wilson, 1987; Ruigrok et al., 1988).

Generally held beliefs about HA-mediated fusion include the following: only sialic acid (on either a protein or a ganglioside) is required for physiological binding to the target membrane (Pritchett et al., 1987); fusion is initiated at low pH after exposure of the N-terminus of HA2 (Skehel et al., 1982; Gething et al., 1986; White & Wilson, 1987; Ruigrok et al., 1988; Stegmann et al., 1990; Godley et al., 1992); there is more than one HA trimer at the fusion site (Ellens et al., 1990); and the trimer remains largely intact during fusion (Doms & Helenius, 1986; Puri et al., 1990; Stegmann et al., 1990). While the mechanism by which various conformational changes actually promote membrane fusion is not known, recent work has elucidated several possibilities (Bentz et al., 1990, 1993; Stegmann et al., 1990, 1991; Spruce et al., 1991; Guy et al., 1992; White, 1992; Tsurudome et al., 1992; Brunner & Tsurudome, 1993; Clague et al., 1993; Carr & Kim, 1993; Siegel, 1993a,b; Stegmann & Helenius, 1993; Wilschut & Bron, 1993).

In Ellens et al. (1990), we used HA-expressing fibroblasts and glycophorin-bearing liposomes to show that two or more influenza hemagglutinin trimers were required at the fusion site. The glycophorin-bearing liposomes bound to the HA-expressing cells specifically through HA-glycophorin interactions, and the data strongly suggested that the fusion was mediated by HAs *not* bound to glycophorin. While each HA has both a binding a fusion function, being bound to glycophorin appears to preclude the fusion function for that HA. Thus, the fusion site involves just the lipid bilayer of the glycophorin-bearing target membrane.

† Supported in part by National Institutes of Health Research Grant GM31506 (J.B.).

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® Abstract published in *Advance ACS Abstracts,* February 1, 1994.

[1] HA, hemagglutinin; HA0, the fusion-incompetent hemagglutinin precursor; DOG, dioleoylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; EPE, phosphatidylethanolamine derived from eggs; TES, 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid, CHES, (cyclohexylamino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; $C_{12}E_8$, octaethylene glycol monododecyl ether; GD1a, disialoganglioside; PR8, influenza virus A/PR/8/34; Luv, large unilamellar vesicle(s); ODR, octyldecylrhodamine B; DMPC, dimyristoylphosphatidylcholine; ESR, electron spin resonance; MDCK, Madin–Darby canine kidney; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl-PE; Rh, rhodamine-PE.

0006-2960/94/0433-1977$04.50/0

While intact influenza virus can fuse with virtually any target bilayer, including liposomes without sialic acid moieties (Stegmann et al., 1985; Stegmann, 1993), we show that careful analysis of the fusion kinetics as a function of the target bilayer composition is fruitful. In this study, we have examined the temperature dependence of fusion of influenza virus with two different sialic acid-bearing membranes: glycophorin-bearing liposomes and ganglioside- (GD1a-) containing liposomes. Fusion was monitored by the dequenching of a fluorescent probe and care was taken to find preincubation conditions where the dequenching rate was due solely to fusion of preaggregated membranes.

It is known that influenza virus will fuse with membranes composed of lipids which, in isolation, are not susceptible to the formation of nonlamellar phases [for a review of this literature, see Stegmann (1993)]. However, none of these studies resolved whether the fusion being measured was rate-limited by virus–target membrane aggregation or by fusion, per se, which makes it impossible to reach significant conclusions about fusion mechanisms and the inverted phase behavior of the membrane lipids. At best, these data support the suggestion that this susceptibility is not necessary for fusion. In fact, since the effect of HA on the "local" lipid phase behavior (at the fusion site) is unknown, it is possible to argue that even this suggestion is too strong. Our approach proves that increased susceptibility of the lipids to form nonlamellar phases is not even sufficient to affect the fusion rate, which resolves this question for this influenza virus. Whether this is the case for other viruses [e.g., Sendai virus: see Yeagle (1993)] remains to be seen.

The lipid mixing rate constant was lower with phosphatidylcholine (DOPC) bilayers than with phosphatidylethanolamine-containing (PE) bilayers, below 20 °C. Clearly, the lipid headgroups are important to the fusion intermediates. However, these observations cannot be explained simply by the effects of either monolayer spontaneous radius of curvature or void volume stabilization. These findings support the idea that fusion is not simply the consequence of the spontaneous merging of closely apposed bilayers; rather, the architecture of the proteins at the fusion site creates the surface which the lipids must follow (Bentz et al., 1990).

We also found that the lipid mixing rates for 10 and 15 mol % GD1a-containing DOPC liposomes were much smaller than the rates found for 5 mol % or smaller GD1a surface densities. We believe that these data show that while the virions bind more strongly to the liposomes with higher GD1a surface densities, presumably through more HA-sialic acid contacts per virion, part of this reduction in lipid mixing rate is due to fewer nonbound HAs being available to participate at the fusion site. As with glycophorin, HAs bound to GD1a appear to be precluded from simultaneously executing their fusion function. This can explain the observations made here and elsewhere (Nir et al., 1990, 1993; Ramalho-Santos et al., 1993) that virions inactivate more slowly in the presence of target membranes. Preliminary data on this work was presented previously in Alford et al. (1991).

MATERIALS AND METHODS

Influenza A/PR/8/34 (H1N1) (catalog no. VR-95) inoculum was purchased from American Type Culture Collection (ATCC) (Rockville, MD). Virus was grown in the allantoic cavity of 9–11-day-old fertilized chicken eggs. Eggs were inoculated with 0.1 mL of a 1:40 000 dilution of the original ATCC stock in sterile 10 mM TES buffer and incubated for 48 h at 37 °C. After 12 h at 4 °C the allantoic fluid was harvested and centrifuged for 30 min at 1000*g* to remove debris. The virus was pelleted through a 20% sucrose step at 125000*g* for 90 min. This pellet was resuspended overnight, homogenized, and placed atop a 0/20/60% sucrose density step gradient. The purified virus was collected from the 20/60% sucrose interface. The virus was assayed for protein, quick-frozen, and stored at −80 °C.

Dioleoylphosphatidylcholine (DOPC), egg phosphatidylethanolamine (EPE), dioleoylphosphatidylethanolamine (DOPE), monomethyl dioleoylphosphatidylethanolamine (DOPE-Me), and dioleoylglycerol (DOG) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Glycophorin A (human blood type B negative) was obtained from Sigma Chemical Co. (St. Louis, MO), and the ganglioside GD1a was purchased from Calbiochem (La Jolla, CA). Octyldecylrhodamine B, chloride salt (ODR), was purchased from Molecular Probes (Eugene, OR) and dissolved in 100% ethanol. Ultrol grade TES (2-{[tris(hydroxymethyl)methyl]-amino}ethanesulfonic acid), and CHES [(cyclohexylamino)-ethanesulfonic acid] were from Calbiochem.

Buffers consisted of (a) 150 mM NaCl, 10 mM TES, and 0.1 mM EDTA (pH 7.5), and (b) 150 mM NaCl, 10 mM CHES, and 0.1 mM EDTA, (pH 9.6). Both were adjusted to an osmolarity of 290 mmol/kg. Sucrose step gradients were prepared by diluting a 60% (w/w) sucrose/10 mM TES with buffers at the appropriate pH.

Glycophorin Liposomes. Glycophorin A is a 29 000 molecular weight membrane glycoprotein found on red blood cells. It is heavily glycosylated, has multiple sialic acid residues, and has a single transmembrane domain. The sialic acid residues serve as a binding receptor for the HA of influenza virus (Pritchett et al., 1987).

Glycophorin vesicles with different lipid compositions were prepared by modification of the procedure of MacDonald and MacDonald (1975). Briefly, 3 μmol of lyophilized lipid was dissolved in 3.75 mL of MeOH (HPLC grade), followed by addition of 80 μL of a 5 mg/mL glycophorin A stock in a suitable buffer. The glycophorin A stock buffer was pH 7.5 (TES) for PC liposomes or pH 9.6 (CHES) for PE-containing preparations. The MeOH was removed via rotary evaporation. To this almost-dry film, 300 μL of additional buffer was added, and the sample was rotated slowly overnight at 4 °C. The sample was then vortexed vigorously and centrifuged on a 20/4/0% sucrose density step gradient at 127000*g* for 1.5 h at 4 °C. The glycophorin-containing liposomes were collected from atop the 20% sucrose step and assayed for protein (Bradford, 1976) and phospholipid (Bartlett, 1959). Ratios of lipid to protein were within the range of 115–150 (mol/mol) and these liposomes were generally 300–700 nm in diameter. Following the gradient, PE-containing preparations were stable at pH 7.5. The stability of glycophorin-bearing PE liposomes has been documented by Taraschi et al. (1982a,b) and Pinnaduwage and Huang (1989).

Ganglioside Liposomes. DOPC liposomes with varying amounts of GD1a were prepared by the reverse-phase evaporation procedure (Szoka & Papahadjopoulos, 1980). The vesicles were prepared in pH 7.5 buffer and extruded through 0.1-μm polycarbonate membranes (Poretics Corp., Livermore CA). The phosphate content was determined according to Bartlett (1959). For each composition, the lipid content was approximated by correcting for GD1a, e.g., 10 nmol of 10 mol % GD1a liposomes was the product of mixing 9 nmol of DOPC with 1 nmol of GD1a, as determined by weight.

Virus Labeling. Aliquots of frozen virus were quick-thawed and labeled with ODR (in EtOH) at 5% of the total virus phospholipid. No more than 1.5% EtOH was added to the sample volume. Following addition of the label, the virus was allowed to equilibrate at 20 °C for 30 min, and then the sample was layered onto a 20/40/60% sucrose density step gradient and centrifuged at 127000*g* for 90 min at 4 °C. The virus was recovered at the 40/60% interface and assayed for protein. The ratio of 175 nmol of phospholipid/mg of viral protein was used to determine the approximate lipid concentration (Stegmann et al., 1985).

Fluorescence Measurements. Fusion of virus to target membranes was measured as the dequenching of ODR. Measurements were made on a PTI Alphascan fluorometer (South Brunswick, NJ) in a thermostated, stirred cuvette. The excitation and emission wavelengths were 565 and 595 nm, respectively. Fluorescence emission was further refined with an OG 590 filter (Schott Glass Technologies, Inc., PA). In most instances, concentrated stocks of virus and liposomes (20–25×) were preincubated at 4 °C for at least 30 min prior to dilution to the preequilibrated cuvette, which was adequate since longer incubations did not affect the lipid mixing kinetics. This fluorescence level was set as 0% lipid mixing. Fusion was initiated after 30 s with the injection of a concentrated acetic acid/acetate solution to achieve the desired pH. Incubations of 1–2 min did not affect the lipid mixing kinetics. The fluorescence intensity at the maximal lipid dispersion (100% lipid mixing) was determined using $C_{12}E_8$ (Calbichem, CA) detergent lysis. Fusion rate limited data resulted under these conditions. Aggregation rate limited data was collected following the elimination of the preincubation of virus and liposomes in concentrated stocks.

To measure the pH profile of fusion, final concentrations of 0.2 μM viral phospholipid and 10 μM target phospholipid were used. For the temperature-dependent studies, final concentrations were 0.2 μM viral phospholipid and 5, 10, and 20 μM (lipid) glycophorin A liposomes or 5, 10, 20, and 50 μM (lipid) GD1a liposomes. GD1a lipid was corrected as indicated above. Fusion data was recorded for each pre-equilibrated virus/target ratio and repeats were recorded for alternating temperatures. Lipid mixing rates were taken from the initial slopes of the dequenching curves, except in the cases of 10 and 15 mol % GD1a-containing liposomes, where a lag phase was noted at some temperatures. In these cases, maximal rates are reported in order to be consistent with previous papers (Stegmann et al., 1990; Bentz, 1992; Stegmann, 1993). In the absence of a lag phase, initial and maximal rates are identical.

Since the glycophorin liposomes are much larger than the virions, it was necessary to label the virions for the lipid mixing assay using ODR. Dequenching per fusion event would be much greater. With the GD1a LUV, we could have labeled the liposomes in principle, but since we had to add excess GD1a-containing liposomes in order to achieve complete fusion rate limiting lipid mixing kinetics, this was not realistic. Since only one virion can fuse per liposome (Nir et al., 1986), adding excess labeled liposomes would have severely diminished the dynamic range of our experiments. Stegmann (1993) found that ODR-labeled virions fused with unlabeled GD1a-containing liposomes essentially the same as unlabeled virions fusing with GD1a-containing liposomes labeled with NBD/Rh or with NBD/Rh that was altered on the external monolayer, such that only the dequenching of NBD from the inner monolayer was observed. Stegmann et al. (1993) have reported that ODR-labeled and pyrene-labeled semliki forest virus show essentially the same fusion kinetics *in vitro*. In comparable cases, our lipid mixing kinetics are sufficiently similar to those previously reported using these other assays (Stegmann et al., 1985, 1990; Tsurudome et al. 1992; Stegmann, 1993).

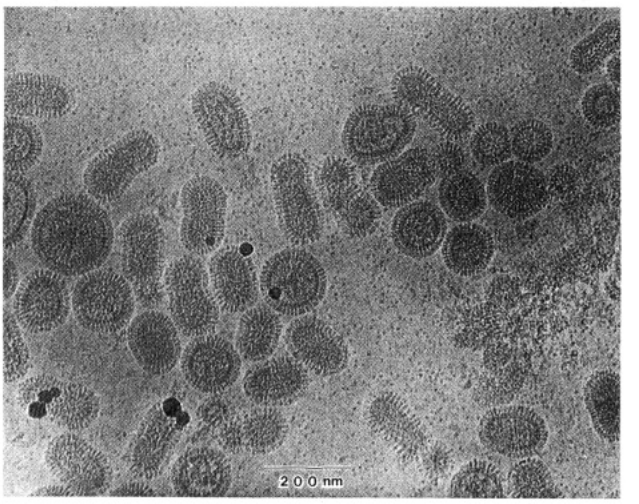

FIGURE 1: Cryoelectron micrographs of the PR/8/34 virions used in this study.

It is of interest that we can obtain 100% dequenching, or lipid mixing, in some cases of the glycoprotein-bearing liposomes, due to their very large size. It had been argued by Wunderli-Allenspach et al. (1993) that the lower dequenching values found in many studies implied some problem with the ODR-labeled virus. Lower dequenching values are likely due simply to the size of the target membrane. With the large dilution of ODR into the target glycophorin-bearing liposomes, it is reasonable to assume that the percent dequenching is equal to the percent of viral lipid which has mixed with target membrane (Düzgünes & Bentz, 1988).

RESULTS

Figures 1, 2, and 3 demonstrate that the virus used in these studies have morphology and fusion behavior consistent with previous literature reports. Figure 1 is a cryoelectron micrograph of the PR/8/34 virions. HA spikes are clearly visible on the surface. Figure 2 shows the lipid mixing, defined as percent dequenching of ODR fluorescence, between the PR8 virions prebound to DOPC/DOPE (2:1)/glycophorin-bearing liposomes as a function of pH. With the large dilution of ODR into the glycophorin-bearing target membrane, it is reasonable to assume that the percent dequenching is equal to the percent of viral lipid which has mixed with target membrane (Düzgünes & Bentz, 1988). Figure 3 shows the pH dependence of the lipid mixing rates between the PR8 virions and glycophorin-bearing liposomes composed of either DOPC/DOPE (2:1) or DOPE-Me + 5% DOG, taken as the initial slope of data curves like those shown in Figure 2. Both compositions show reasonable pH dependencies, as compared with previous studies using other influenza strains (Stegmann et al., 1985, 1987). There are some significant differences between the fusion characteristics of influenza strains (Tsurudome et al., 1992), and even mutants within a strain can show variations in pH dependence (Weis et al., 1990). These data show that pH dependence can also depend on the target membrane.

In order to evaluate the effect of target membrane lipid composition and temperature on the virion–liposome fusion step, per se, it is necessary and sufficient to demonstrate that

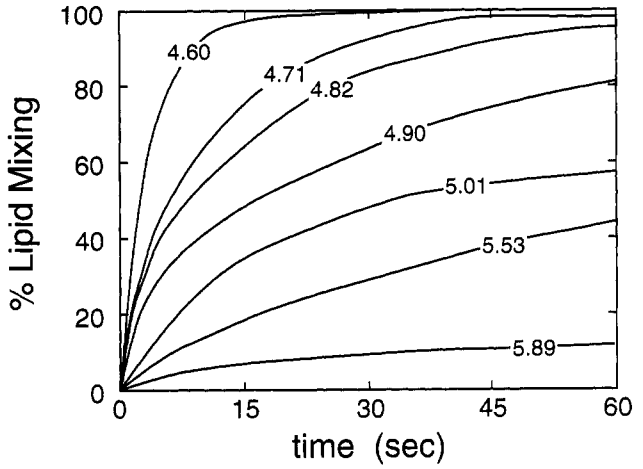

FIGURE 2: Lipid mixing between the PR8 virions and the DOPC/DOPE (2:1) glycophorin-bearing liposomes is shown as a function of pH. Virions and liposomes are prebound at high concentration (20–25-fold greater than the final concentration in the cuvette) at 4 °C for at least 30 min prior to dilution into the 33 °C pH 7.4 buffer in the cuvette. After 30 s, the pH was lowered. The final concentrations in the cuvette were 0.2 μM viral phospholipid and 10 μM liposome phospholipid. In all cases, virus concentration was fixed at 0.2 μM viral phospholipid. All subsequent prebinding experiments were done this way, except for variation in target membrane concentration, as noted.

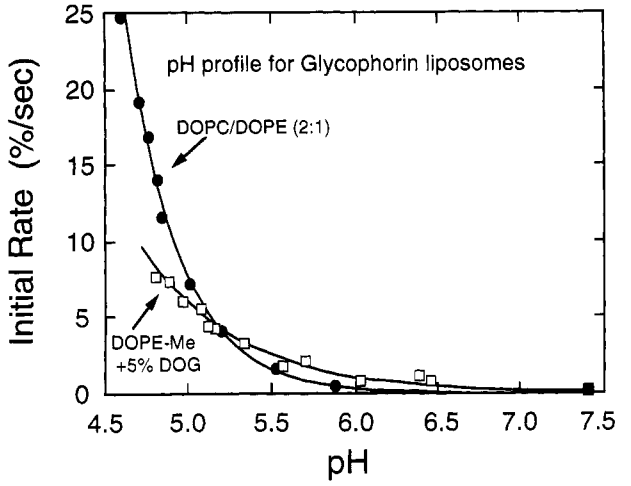

FIGURE 3: pH dependence of the lipid mixing rates between the PR8 virions and glycophorin-bearing liposomes composed of either DOPC/DOPE (2:1) or DOPE-Me + 5% DOG at 33 °C. This rate is defined by the initial slope of data curves like that shown in Figure 2.

the rate of lipid mixing, and not the rate of virion–liposome aggregation, is limiting to the overall reaction. This criterion is rigorously satisfied when it can be demonstrated that the fluorescence dequenching rates do not depend on target liposome concentration (Bentz et al., 1988). Without such proof, it is not known which step is rate-limiting. We preincubate the virions and the glycophorin-bearing liposomes at 4 °C at pH 7.4 for at least 30 min, which is sufficient to eliminate the contribution of virus–liposome aggregation to the lipid mixing rate. Elimination of this preincubation step resulted in aggregation rate limiting lipid mixing kinetics (see Figure 12).

Figure 4 shows the liposome (DOPC/glycophorin) concentration dependence of the lipid mixing kinetics at 33 °C. The lipid mixing rates are identical, proving that the lipid mixing rates are being monitored directly. We did not measure the fraction of the virions bound to the glycophorin-bearing liposomes after the pH is lowered, since this is not relevant to the question of lipid mixing rates. It is adequate to know

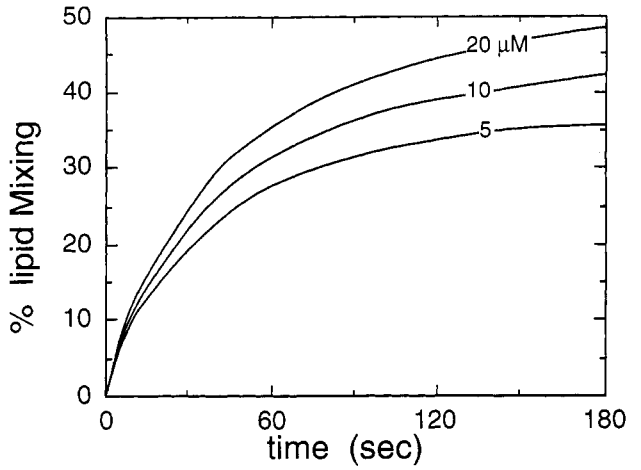

FIGURE 4: Liposome (DOPC/glycophorin) concentration dependence of the lipid mixing kinetics, shown as 5, 10, and 20 μM phospholipid. The initial lipid mixing rates are identical, implying that the membrane destabilization rates are being monitored directly.

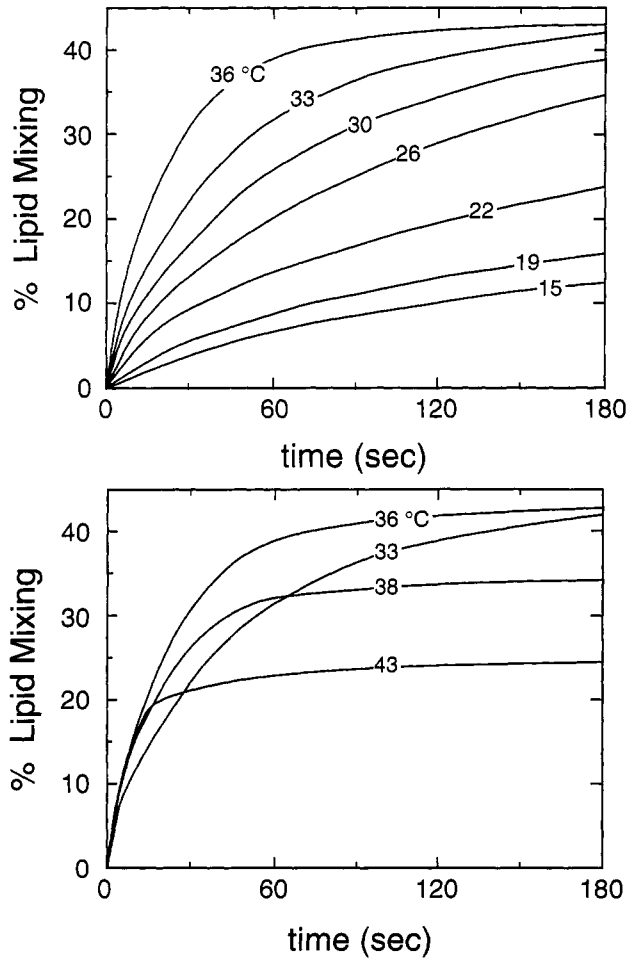

FIGURE 5: Temperature dependence of lipid mixing between PR8 virions and DOPC/glycophorin-bearing liposomes for temperatures between 15 and 36 °C (top) and between 33 and 43 °C (bottom). While the initial rates monotonically increase with temperature, it is clear that above 33 °C viral inactivation is becoming significant.

that if there are any unbound virions, they do not bind and fuse within the initial time period. Note that our data preclude the possibility that prebound virions dissociate first, due to warming and/or low pH, and then bind and fuse with the liposomes.

The temperature dependence of lipid mixing between PR8 virions and DOPC/glycophorin liposomes is shown in Figure

Table 1: Lamellar-Inverted Hexagonal Phase Transition Temperature T_H (°C) versus Lipid Composition

lipid composition	T_H (pH 4.5 and 7.4)	comments	ref
DOPE	5–15 °C		Gagne et al. (1985), Epand (1985)
EPE	40–45 °C		Ellens et al. (1986a)
DOPE-Me	64–66 °C		Gagne et al. (1985), Ellens et al. (1989)
DOPE-Me + 5 mol % DOG	<40 °C	extrapolated from 1–2 mol % DOG; perhaps as low as 10–20 °C	Siegel et al. (1989)
DOPC/DOPE (2:1)	≫100 °C	extrapolated from 1:4, 1:3, and 1:2 DOPC/DOPE mixtures	Ellens et al. (1989)
DOPC ± 5 mol % DOG	≫100 °C	extrapolated	Ellens et al. (1989)

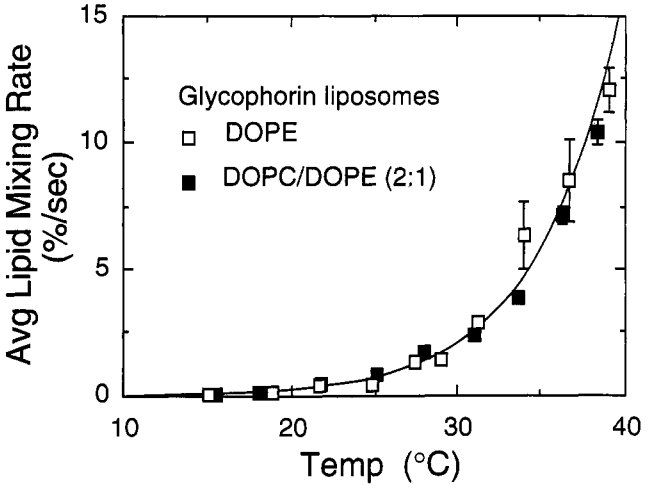

FIGURE 6: Average initial lipid mixing rates from glycophorin-bearing liposomes composed of either DOPE, whose T_H is about 10 °C, or DOPC/DOPE (2:1), which remains lamellar up to at least 100 °C (Gagne et al., 1985; Ellens et al., 1989). These data points represent the average and standard deviation over liposome concentrations, from 5 to 20 μM phospholipid, with $n \geq 3$. The curve is arbitrarily fitted to eq 1, with $A = 7.2 \times 10^{-3}$ (%/s), $\alpha = 0.19$/°C, and the correlation coefficient $R^2 = 0.996$.

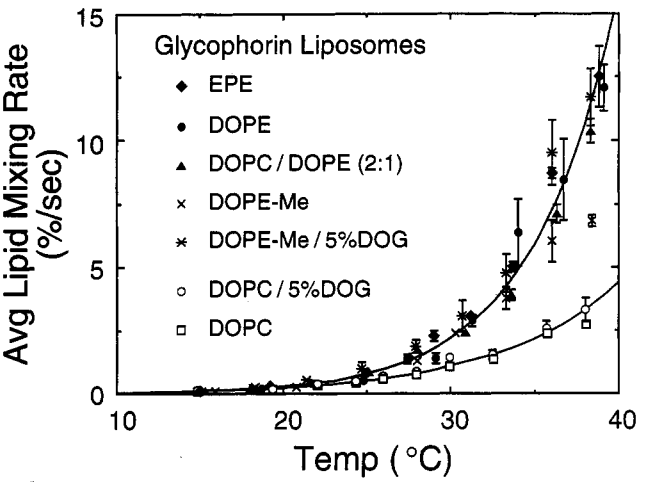

FIGURE 7: Average initial rates of lipid mixing are shown with standard deviations as a function of the temperature for several different bilayer compositions of the glycophorin-bearing liposomes. The curve is eq 1. For the PE-containing liposomes, which is the upper curve, $A = 5.8 \times 10^{-3}$ (%/s), $\alpha = 0.20$/°C, and the correlation coefficeint $R^2 = 0.985$. For the PC-containing liposomes, which is the lower curve, $A = 1.6 \times 10^{-2}$ (%/s), $\alpha = 0.14$/°C, and the correlation coefficient $R^2 = 0.982$.

5 (top) for temperatures between 15 and 36 °C and in Figure 5 (bottom) for temperatures between 33 and 43 °C. While the rates increase with temperature, the decreasing extents of lipid mixing above 33 °C show that viral inactivation is becoming significant. As we will discuss below, when GD1a-containing liposomes are used as the target membrane, it is likely that inactivation is more rapid when the virion is not bound to the target membrane.

We next wanted to investigate whether having a target membrane capable of initiating a transition to the nonlamellar (H_{II}) phase would affect the kinetics of HA-mediated membrane fusion. In Bentz et al. (1993), we showed that the rates of lipid mixing between PR8 virions and glycophorin-bearing liposomes made from either DOPE or EPE were essentially identical from 10 to 40 °C. Here, in Figure 6, we find the same average lipid mixing rates from glycophorin-bearing liposomes composed of either DOPE, whose T_H is about 10 °C, or DOPC/DOPE (2:1), which remains lamellar up to at least 100 °C (Gagne et al., 1985; Ellens et al., 1989). The data points represent the average lipid mixing rate and its standard deviation over liposome concentration, at 5, 10, and 20 μM phospholipid with some replicates, so that $n \geq 3$ in all cases. Standard deviations are shown or are smaller than the symbols used. Clearly, the temperatures at which the underlying bilayers can undergo nonlamellar phase transitions are irrelevant to the temperature dependence of the HA-mediated membrane fusion of these liposomes. The data are arbitrarily fitted to the equation

$$\text{rate (\%/s)} = A \exp\{\alpha T\,(°C)\} \qquad (1)$$

and the fitting parameters are given in the figure legends.

The irrelevance of T_H to the temperature dependence of lipid mixing was more generally demonstrated in Figure 7, where the average rates of lipid mixing are shown as a function of the temperature for several different bilayer compositions of the glycophorin-bearing liposomes. The data points represent the average lipid mixing rate and its standard deviation over liposome concentration, at 5, 10, and 20 μM phospholipid (with some replicates, so $n \geq 3$). Table 1 shows the estimated values of T_H for the pure lipids. Remarkably, all of the PE or PE-containing membranes show essentially the same rate of lipid mixing, while the DOPC membranes show smaller rates. As indicated in the figure legend, the temperature coefficients α derived from fitting these data by eq 1 are quite different for the PE-containing membranes and the PC membranes.

Figure 8 shows the lipid mixing at 33 °C between the PR8 virions and DOPC liposomes which contain different amounts of the ganglioside GD1a. The virions and liposomes were preaggregated, following the same protocol as was used for the glycophorin-bearing liposomes. Except as specifically noted below for the case of 15 mol % GD1a, the lipid mixing rates for these experiments were statistically independent of lipid concentration from 5 to 50 μM phospholipid of the GD1a-containing liposomes over the temperature range of 15–37 °C.

Two general trends can be observed. First, lipid mixing rates fall into two groups, depending upon whether there is 0–5 mol % GD1a or 10–15 mol % GD1a in the membranes. Second, the extent of lipid mixing is reduced with the higher

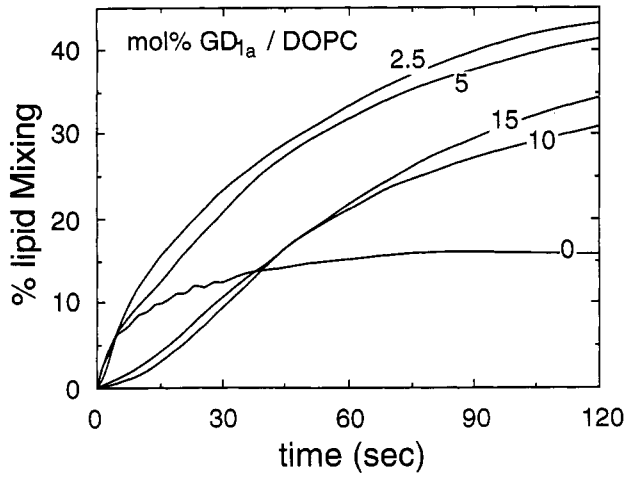

FIGURE 8: Lipid mixing between the PR8 virions and DOPC liposomes which contain different amounts of the ganglioside GD1a, from 0 to 15 mol % and 10 μM lipid. The virions and liposomes are preaggregated, following the same protocol as was used for the glycophorin-bearing liposomes. Except for the case of 15 mol % GD1a, which is discussed in Figure 10 below, the lipid mixing rates for these experiments were statistically independent of lipid concentration from 5 to 50 μM lipid of the GD1a-containing liposomes over the temperature range of 15–37 °C. These data were taken at 33 °C.

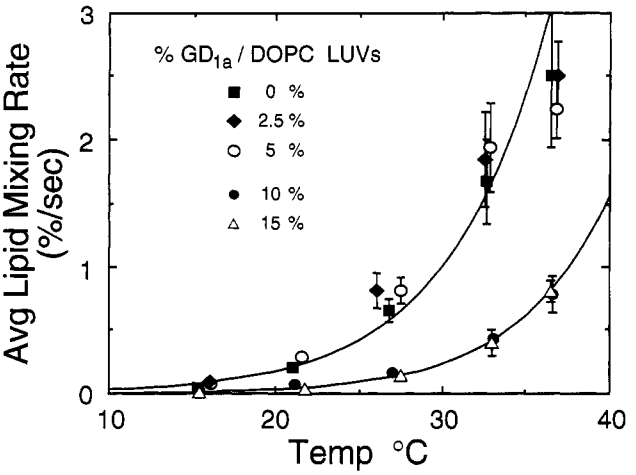

FIGURE 9: Average lipid mixing rates, averaged over all lipid concentrations observed (5–50 μM, $n \geq 4$), as a function of temperature. Vertical bars show standard deviations. The curves are arbitrarily fitted to by eq 1. For the 0–5 mol % GD1a-containing liposomes, $A = 6.0 \times 10^{-3}$ (%/s), $\alpha = 0.17$/°C, and the correlation coefficient $R^2 = 0.958$. For the 10–15 mol % GD1a-containing liposomes, $A = 8.6 \times 10^{-4}$ (%/s), $\alpha = 0.19$/°C, and the correlation coefficient $R^2 = 0.962$.

GD1a concentrations, not including the case of 0 mol % GD1a, i.e., pure DOPC liposomes, where the effect of inactivation is greater.

The first trend is examined in greater detail in Figure 9, where we show the average lipid mixing rates as a function of temperature. Recall that these rates are the maximal lipid mixing rates, which are identical to the initial lipid mixing rates for 0–5 mol % GD1a (see Materials and Methods). The data points represent the average lipid mixing rate and its standard deviation over liposome concentration, at 5, 10, 20, and 50 μM lipid with some replicates, so that $n \geq 4$ in all cases. Vertical bars show standard deviations. We believe that above 33 °C, especially for liposomes containing 5 mol % or less GD1a, the decline in rates relative to this curve reflects viral inactivation; see also Figure 11 below.

The second trend is examined in greater detail in Figure 10. The lipid mixing rates for 15 mol % GD1a-containing liposomes

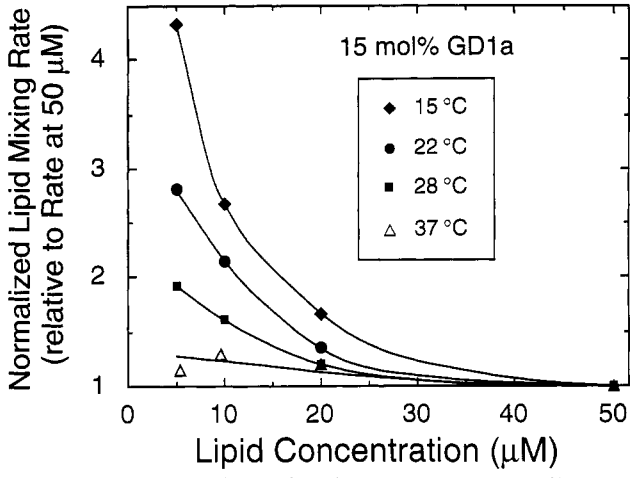

FIGURE 10: Normalized rate of lipid mixing for the 15 mol % GD1a-containing liposomes as a function of total lipid concentration. The normalized rate is defined as the ratio of each rate to the rate at 50 μM lipid at the given temperature. By definition, then, the normalized rate at 50 μM lipid was 1 for each temperature. The curves are interpolated.

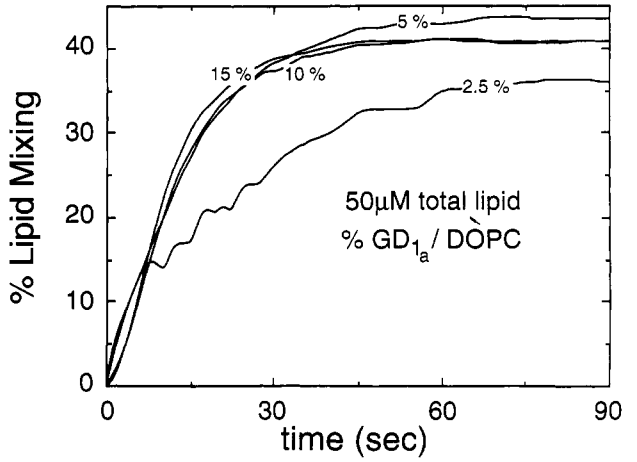

FIGURE 11: High-temperature-induced inactivation of the virus can be minimized by using high concentration (50 μM phospholipid) of GD1a-containing liposomes. At 43 °C, the lipid mixing to 0 mol % liposomes is essentially zero, while the signal for 2.5 mol % GD1a-containing liposomes is very erratic. However, with 5 mol % GD1a or greater, the lipid mixing signals are essentially identical.

at temperatures of 33 °C and below showed a consistent decrease in the rate of lipid mixing as the liposome concentration was increased. Such an effect implies that fusion is inhibited due to the presence of more bound liposomes. The data of Stegmann et al. (1990) suggested that virus (X31 strain) binding to the ganglioside liposomes is very weak, despite the potential multivalent attachments between the virus and the GD1a liposomes.[2] Our data imply that more than one GD1a-containing liposome can bind per virion, which would significantly reduce the surface density of free HAs.

In order to highlight this effect, we plot the rate data normalized to the rate at 50 μM lipid, i.e., at each temperature

[2] The viral envelope has roughly 1.5×10^4 HA/μm^2 (500 HA/0.1-μm-diameter virion; Murphy & Webster, 1990), the glycophorin-bearing liposomes have roughly 1×10^4 glycophorins/μm^2 (1 glycophorin/115–150 phospholipids, see Materials and Methods), and the GD1a surface density is roughly (1.4×10^4) (mol % GD1a)/μm^2 (70 Å^2/DOPC and 70–90 Å^2/GD1a; Marsh, 1990). Thus, in the area of membrane contact, the glycophorin/HA ratio is about 0.7 and the GD1a/HA ratio is essentially equal to the mol % of GD1a. The 15 mol% GD1a containing liposomes have about 16 GD1a per HA in the area of contact. Presumably, not all HAs are bound to sialic acid moieties. Clearly any potential "clustering" of HAs bound to sialic acid moieties is ignored.

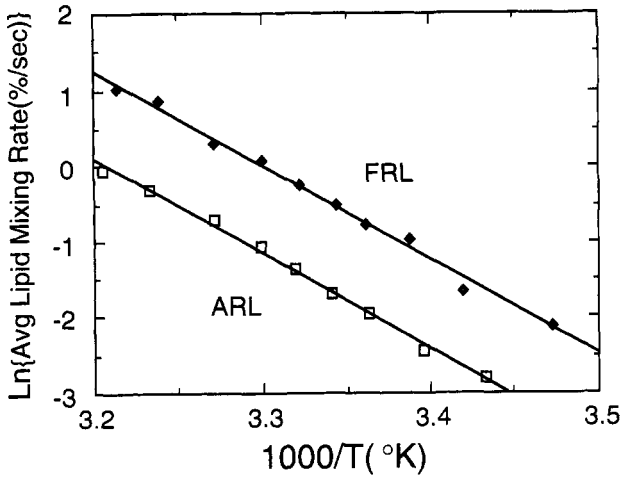

FIGURE 12: Ln{initial rate of lipid mixing} versus $1/T$ (K) for DOPC/glycophorin-bearing liposomes under fusion rate limiting kinetics (FRL, upper curve) and under aggregation rate limiting kinetics (ARL, lower curve).

we plot [initial rate/initial rate(50 μM lipid)] versus lipid concentration; see Figure 10. Obviously, for each temperature, the normalized rate at 50 μM is identically 1. In the case of 37 °C, these normalized rates are about 1 for each lipid concentration; i.e., the rates are independent of lipid concentration. However, as the temperature decreases, the lipid mixing rate at lower lipid concentrations becomes progressively greater than the rate with 50 μM lipid. For example, at 15 °C with 5 μM lipid, the lipid mixing rate is 4.5 times greater than the respective rate at 50 μM lipid. The data for 33 °C are omitted for clarity, although they follow the same trend in that the rate with 5 μM lipid is about 1.6 times that with 50 μM lipid. Not surprisingly from the shape of these curves, the average lipid mixing rates (averaged over lipid concentration), as shown in Figure 9, lie between the individual values for 10 and 20 μM lipid.

Figure 11 shows that the high-temperature-induced inactivation of the virus can be minimized by using a high concentration (50 μM lipid) of GD1a-containing liposomes. At 43 °C, the lipid mixing to 0 mol % GD1a liposomes is essentially zero, while the signal for 2.5 mol % GD1a-containing liposomes is very erratic. However, with 5 mol % GD1a or greater, the lipid mixing signals are essentially identical. Notice that the data reported in Figure 9 does not include temperatures above 37 °C. Clearly, at the higher liposome concentrations and GD1a surface densities, more of the virions remain attached long enough to fuse. Unbound virions in the medium must inactivate more rapidly than bound virions. This agrees with Ramalho-Santos et al. (1993), who looked at the fusion of PR8 virions with PC-12 suspension cells.

Our preliminary experiments with the DOPC/glycophorin-bearing liposomes did not use extensive preaggregation of the virions to the target membranes. In fact, a 30-s incubation prior to acidification showed lipid mixing rates which were purely aggregation rate limiting, i.e., first order in liposome concentration (data not shown). Aggregation rate limiting fusion implies that the time between successful virion–liposome encounters is long compared with the time required for the bound complex to fuse (Bentz et al., 1988). Figure 12 shows the plot of ln {initial rate of lipid mixing} versus $1/T$ (K) for these data, denoted ARL. On the same figure, we have plotted the data for the DOPC/glycophorin-bearing liposomes preaggregated (for 0.5–2 h at pH 7.4 and 4 °C) with the virions. As expected, the absolute rates of lipid mixing were faster with the prebound virions (FRL) than when the virions were mixed with the liposomes at low pH (ARL).

Remarkably, in both kinetic regimes, the slope is nearly identical. Since the rate-limiting steps cannot be the same, there are several possible interpretations, e.g., it is coincidental or it signifies that some single molecular event controls the rate of both processes (e.g., removal of water). It is impossible to know which is the case from these data, largely because dequenching rates depend simultaneously on aggregation rate constants and fusion rate constants. If inadequate preincubation conditions had been chosen, such that both aggregation and fusion contributed to the overall kinetics of lipid mixing, that Arrhenius plot would still have looked linear, but the slope would probably be different (Bentz, 1992). These data show that Arrhenius plots of overall rates for multistep pathways can be easily overinterpreted.

DISCUSSION

Our knowledge of how influenza hemagglutinin induces membrane fusion has reached an advanced stage, especially when compared with what is known about other enveloped animal viruses [see the reviews in Bentz (1993)]. What remains elusive is the architecture of the fusion site, i.e., the arrangement of the fusion proteins at the site and how these proteins induce bilayer destabilization and fusion. Given that only a few HAs out of those on the virion will be involved in fusion sites, with a small fraction of the total numbers of lipids in both bilayers, and for only a short period of time, it is not surprising that unambiguous answers to the fusion site architecture have been elusive.

In this work, we have focused on the role which the target membrane may play in the fusion event. In doing so, we recognize that the target membrane consists of two components, i.e., the sialic acid target on either a glycoprotein or glycolipid (Pritchett et al., 1987) and the underlying lipid bilayer. We wanted to address two general questions: first, what physical properties of the underlying lipid bilayer affect the fusion kinetics, and second, are the HAs bound to the sialic acid moieties part of the fusion site.

Role of Bilayer Lipids. With respect to the role of the lipid bilayer in the HA-mediated fusion event, one outstanding question is whether lipids which are competent to form inverted phases can enhance the protein-mediated fusion mechanism, as has been speculated by many [see Knoll et al. (1988), Ellens et al. (1990), White (1990), Bentz (1991), and Gallaher *et al.* (1992) for recent discussions]. While influenza virus can fuse with liposomes composed of lipids incapable of forming inverted phases on their own (White et al., 1982; Stegmann et al., 1985) or even with lipids initially in L_β gel phase (Stegmann, 1993), these data cannot exclude the possibility that the HAs at the fusion site promote a local environment which permits formation of intermediates of the H_{II} phase transition, especially if the "fusion peptide" concentration at the site were 15–30 mol % with respect to lipid, as speculated in Stegmann (1993).

This question was addressed with the glycophorin-bearing liposomes, since the gangliosides are clearly part of the lipid bilayer and these lipids sharply increase the L_α–H_{II} phase transition temperature, T_H. Using liposomes made of inverted phase forming lipids without glycophorin, e.g., pure DOPE liposomes, is largely irrelevant for two reasons. The first reason is that the aggregation of the liposomes with each other is more rapid than their aggregation with the virions (data not shown). This aggregation of the liposomes produces inverted

phase precursors (Bentz et al., 1987; Ellens et al., 1986a,b, 1989), which would provide a target site for viral membrane destabilization not found in cells. The second reason is that membrane–virion binding which is not mediated by HA1 binding to sialic acid need not reflect the physiological situation.

Our experiments using glycophorin-bearing liposomes with various underlying lipid compositions conclusively show that a change in the potential of the target membrane to form inverted phase intermediates does not change the fusion rate; see Figures 6 and 7 and Table 1. The fact that the viral envelope is composed of other, non-inverted phase forming lipids, does not compromise this conclusion since we have shown previously 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). Verifying that the rate-limiting step for fluorescence dequenching was subsequent to virion–liposome aggregation was essential to reach this conclusion.

The HA-mediated fusion mechanism involves at least four separate steps after binding of the virion to the target membrane, e.g., HA aggregation on the viral envelope, destabilization of outer monolayers, initial pore formation, and expansion of the fusion pore [see the reviews in Bentz (1993)]. However, it has been shown that if any of these rate constants changed dramatically, this change would be observed in the overall rate of lipid mixing, regardless of which step (if any) were rate-limiting (Bentz, 1992). Thus, if the H_{II} phase competence of the underlying lipids of the glycophorin-bearing liposomes could accelerate lipid mixing, that acceleration would have been observed. Furthermore, we must be observing a process sensitive to lipid headgroup, since we observe a clear difference between PE- and PC-containing membranes.

In fact, by looking at a wide range of underlying lipid bilayer compositions we have uncovered a very fundamental property of HA-mediated fusion. There was a very clear distinction between the lipid mixing kinetics of DOPC bilayers (with or without 5 mol % DOG) and bilayers containing PE. The PE-containing bilayers fused faster above 20 °C. This had been speculated previously (Stegmann et al., 1985), albeit from kinetic data which were probably rate-limited by the aggregation of virions with liposomes.

In fact, a bilayer with as little as 33 mol % DOPE in 67 mol % DOPC fused just as fast as a pure DOPE bilayer. There are many differences between PE and PC which have been proposed as being important for liposome–liposome fusion [reviewed in Bentz and Ellens (1988) and Siegel (1993b)]. However, we are not aware of a bilayer or lipid physical property (including the hydration force; Rand et al., 1988) which so clearly separates pure DOPE and DOPC/DOPE (2:1) from pure DOPC.

For example, the relative stability of lamellar L_α, cubic Q_{II}, and hexagonal H_{II} lipid phases (Gruner, 1989; Keller et al., 1993), as well as the free energy associated with any putative lipidic intermediate of liposome–liposome fusion (Siegel, 1993b), can be roughly estimated by summing the curvature elastic energy of the monolayers and the free energy due to stabilization of hydrophobic interstices. In addition to the actual geometry of the phases or fusion intermediates, the fundamental parameters of this theory are the spontaneous curvature of monolayers and the interstice volume, which must be "filled" by stretching of the acyl chains (Keller et al., 1993). To a first approximation, DOPC, DOPC/DOPE (2:1), and pure DOPE would have equal interstice stabilization energies, since they have the same acyl chains. If HA-mediated fusion of these three membranes is assumed to have the same intermediate structures (which is prudent until proven wrong), then this model would predict either that all three initial fusion rates would be the same, in the case that interstice stabilization is more important, or that pure PE membranes will fuse faster than pure PC membranes, in the case that monolayer spontaneous curvature is more important (Siegel, 1993b). Obviously, the latter prediction is consistent with most of the data. The spontaneous curvatures of the various pure PEs shown in Figure 7 are roughly similar, at this level of approximation, and so it is acceptable, with respect to this theory, that these rates are essentially the same.

However, on the basis of calculations done for pure lipid systems (Siegel, 1993b), one would not predict that DOPC/DOPE (2:1) membranes fuse at the same rate as pure DOPE membranes. To a first approximation, Keller et al. (1993) have found that the spontaneous curvature of mixed monolayers equals the mole fraction weighted sum of the curvatures of the pure components. Thus, to this first approximation, DOPC/DOPE (2:1) should show fusion rates more like those of pure DOPC than those of pure DOPE, which clearly does not happen.

There are two straightforward possible explanations for this apparent anomaly. First, if monolayer curvature and lipid interstice stabilization really are the dominant contributions to the HA-mediated fusion mechanism, then the DOPC/DOPE (2:1) membranes must become highly enriched in DOPE at the fusion site. Whether HA could mediate such a phase segregation is unknown.

The other possible explanation is simply that specific lipid–protein interactions dominate the HA-mediated fusion mechanism. The model for HA-mediated fusion proposed in Bentz et al. (1990, 1993) speculated that the initial destabilization involved a "wetting" of the HA by the lipids. If such an event occurs, its dependence on lipid composition remains to be elucidated. It is very interesting, in this regard, that our data do imply that no substantial bilayer–bilayer contact occurs at the HA-mediated fusion site. This is a corollary to the data in Figure 7, which shows no correlation between T_H and lipid mixing rates. Liposomes containing PEs show a substantial increase in lipid mixing kinetics as the temperature of the system approaches that of the inverted hexagonal (H_{II}) phase transition temperature, T_H (Bentz et al., 1985; Ellens et al., 1986a,b, 1989; Siegel et al., 1989). This was true even if only the outer monolayer of one of the liposome of H_{II} phase competent lipids (Bentz et al., 1987).

Both Figures 6 and 7 show fitted curves to the data in the form of an arbitrary curve given in eq 1. This has been done to provide a simple quantitative means to reproduce the basic elements of the data. While such data are often graphed in the form of a Arrhenius plot, i.e., ln {initial rate} vs $1/T$ (K), we have argued that since HA-mediated fusion is a multistep first-order process, it is potentially very misleading to use such plots without independent evidence that the rate-limiting step to fusion is the same over the entire temperature range (Bentz, 1992). The linearity of such a plot is not evidence that there is a single rate-limiting step or even that there is a single pathway. To date, no such evidence exists. Nevertheless, we note that an "Arrhenius plot" of the data shown in Figure 7 would yield "apparent activation energies" of about 25 kcal/mol for the PC bilayers and about 35 kcal/mol for the PE bilayers. An "Arrhenius plot" interpretation has the ironic implications that, while fusing faster, the PE membranes have a greater apparent activation energy, which implies a greater frequency factor.

Role of HAs Bound to Sialic Acid Moieties in the Target Membrane. Since the physiological target of influenza virus could be glycoproteins and/or glycolipids, it is important to examine both receptor systems to determine whether there are major differences between them. In Ellens et al. (1990), we monitored the fusion between HA-expressing fibroblasts and glycophorin-bearing liposomes. Our data proved that the fusion site was not composed solely of HAs bound to glycophorin and strongly suggested that the fusion was mediated solely by HAs not bound to glycophorin. With the GD1a-containing liposomes used here, the data likewise suggest strongly that the HAs bound to GD1a do not participate in fusion. It is germane to note that initially in the area of contact there are 0.7 glycophorin/HA, while the GD1a/HA ratio essentially equals the mol % of GD1a, e.g., 10 mol % GD1a has about 10 GD1a/HA. Of course, each glycophorin should have 16 sialic acid moieties.[2]

Following the same preincubation routine as was used for the glycoprotein-bearing liposomes, we found that the lipid mixing rates were independent of target liposome concentration; *except* for the case of 15 mol % GD1a, which at lower temperatures showed a *decrease* in rate with increased lipid concentration. This case is discussed in detail below. Nevertheless, in all cases, the lipid mixing rates did not depend upon virion–liposome aggregation kinetics. Establishing this is essential in order to unambiguously monitor the lipid mixing kinetics of virions specifically bound to sialic acid moieties. Merely noting that a fraction of the virions are bound to liposomes prior to lowering pH does not constitute proof that lipid mixing proceeds from those bound virions. Without evidence to the contrary, the lipid mixing could be due to the liposomes binding after low pH treatment.

Figure 9 shows that the lipid mixing rates, averaged over all lipid concentrations, depend on GD1a surface density in a very interesting fashion. Within experimental error, the rates were the same for low GD1a surface densities (0, 2.5, and 5 mol %) and they were smaller for two higher GD1a surface densities (10 and 15 mol %). In addition, Figure 8 shows that the extent of lipid mixing decreases at the higher GD1a surface densities, and this trend is preserved throughout the temperature range of 15–43 °C. This result is the opposite of the dependence found by Kawasaki and Ohnishi (1992) using DMPC/GD1a-containing liposomes with PR8 virus. The reason for this is not known but can probably be ascribed to the fact that their ESR-monitored lipid mixing was done with 3 orders of magnitude higher viral lipid concentration, 2–3 orders of magnitude higher liposomal lipid concentrations, and 1–2 orders of magnitude lower ganglioside surface densities.

The only two parameters likely to change as a function of GD1a surface density are the composition of the lipid within the fusion site and the fraction of HAs bound to sialic acid moieties. While the specific effect of GD1a concentration on lipid intermediates within the fusion site is entirely unknown, part of the effect we observe can be attributed to the fraction of HAs bound to GD1a.

Figure 10 shows that with 15 mol % GD1a there is a consistent decrease in the lipid mixing rate with increasing lipid concentration and this decrease is greatest at the lowest temperature. The number of HA–sialic acid binding contacts can be expected to increase with increasing target liposome concentration, as one virion binds to more than one liposome. If the fusion site were composed of HAs bound to GD1a, then fusion should either increase or remain constant as the liposome concentration increases, depending upon which steps are rate-limiting. Since the opposite effect is observed, it is evident that the fusion site diminishes with increasing HA–GD1a contacts. This strongly suggests that we have reached an extent of HA–GD1a binding which sufficiently diminishes the free HA surface density to a level where the number of fusion-competent HA aggregates is reduced enough to inhibit the lipid mixing rate.

The temperature dependence observed in Figure 10 supports this interpretation. Clearly, the absolute rate of lipid mixing decreases with decreasing temperature, as seen in Figures 7 and 9. However, the HA–GD1a binding reaction should become less reversible as temperature decreases. Thus, GD1a–HA contacts remain bound for longer times. So, when the lipid mixing rate is limited by low HA surface density, due to high HA–GD1a binding, one would expect the greatest increase in lipid mixing due to reducing the GD1a-containing liposome concentration (from 50 to 5 μM) to occur at the lowest temperature, as is observed in Figure 10. At higher temperatures, the greater reversibility of the HA–GD1a contact permits a larger pool of HAs the opportunity to participate at fusion sites on the time scale of fusion.

Our suggestion that the HAs bound to sialic acid-bearing receptors do not participate in fusion differs from the claims of Niles and Cohen (1993) and Melikyan et al. (1993), who were measuring the fusion of ODR-labeled PR8 virions or influenza-infected MDCK cells, respectively, with planar membranes. However, those studies used only one ganglioside concentration, i.e., 0 and 10 mol % ganglioside. Our kinetic data for these two cases agree well enough with theirs. It was the comparison of several ganglioside and target liposome concentrations which led to our finding.

While it seems evident that HAs bound to GD1a do not participate at the fusion site, just as is the case with HAs bound to glycophorin, the reason is unknown. It could be that the HAs bound simultaneously to both closely apposed membranes simply lack the lateral mobility to participate in many HA aggregates. Estimating this effect will be difficult, since lateral motion will depend upon both the correlation of Brownian motion in the two membranes and fluctuations in membrane separation. Intuitively, both factors should significantly reduce lateral mobility relative to that found for HA in isolated membranes (Ellens et al., 1990). Henis (1993) and Gutman et al. (1993) have discussed the evident necessity of viral fusion protein mobility in membrane fusion.

Virion Inactivation. Influenza virus inactivation at low pH has been long noted (White et al., 1982; Sato et al., 1983; Junankar & Cherry, 1986; Stegmann et al., 1986, 1990; Puri et al., 1989; Stegmann & Helenius, 1993) and recently studied in detail by Nir and colleagues (Nir et al., 1988, 1990, 1993; Stegmann et al., 1989b; Duzgunes et al., 1992; Pedroso de Lima, 1992; Ramalho-Santos et al., 1993). Detailed kinetic analyses of a variety of data have shown that inactivation occurs at all temperatures and it is simply the case that at higher temperatures the inactivation rate becomes similar to the fusion rate (Nir et al., 1990, 1993; Bentz, 1992; Ramalho-Santos et al., 1993). Whether inactivation is completely irreversible or not may depend on the target membrane (Duzgunes et al., 1992; Ramalho-Santos et al., 1993). No mechanism for this inactivation has been established, but our data contribute some interesting elements.

We have found that while inactivation was significant at 37 °C, the lipid mixing rates with the 10 and 15 mol % GD1a-containing liposomes showed less inactivation of the virus. This suggested that being bound to the target membrane decreased the rate of inactivation, as had been proposed

previously (Stegmann et al., 1986; Nir et al., 1993; Ramalho-Santos et al., 1993). This was consistent with our finding that the extent of lipid mixing with the DOPC/glycophorin-bearing liposomes was greater with higher liposome concentrations, despite the fact that the rates were the same, Figure 4. A virion which dissociates from the glycophorin will find another target membrane more quickly with the higher lipid concentrations and, hence, will be more likely to fuse before inactivation.

We tested this hypothesis with the data shown in Figure 11. At 43 °C with the highest lipid concentration, 50 μM, where the greatest fraction of virus would be bound, the lipid mixing curves are the same with 5, 10 and 15 mol % GD1a. With 2.5 mol % GD1a some inactivation is seen, and with 0 mol % GD1a there was no lipid mixing, i.e., the virus was completely inactivated before fusion with the liposomes could occur. Clearly, the virions rapidly dissociated from the 0 and 2.5 mol % GD1a liposomes. The extent of lipid mixing and, for all practical purposes, the lipid mixing rates will depend upon the fraction of virions which inactivate rather than fuse. Thus, while the 5 mol % GD1a-containing liposomes fuse faster on average than the 10–15 mol % GD1a-containing liposomes below 40 °C, as shown in Figure 9, at 43 °C these relative fractions are the same for 5–15 mol % GD1a. Our results qualitatively agree with those of Ramalho-Santos et al. (1993), who report that the rate constant of PR/8 inactivation is reduced by about half when the virions are prebound to suspension cells.

On the basis of kinetic reasoning, Ramalho-Santos et al. (1993) have suggested that a common mechanism yields both inactivation and fusion. Typical examples can be modeled as two pathways emanating from a common intermediate (Bentz, 1992). However, we have shown here that fusion depends on HAs not bound to sialic acid moieties and inactivation is reduced when HAs are bound to sialic acid moieties.

At first glance, this may seem contradictory, but the resolution lies in not confusing the rate of HA aggregation with the rates of formation of the subsequent intermediates to fusion. While it has been suggested that inactivation is due to clustering of conformationally altered HAs (Junankar & Cherry, 1986) and it is generally accepted that HA aggregation is required to assemble a fusion site [see the influenza chapters in Bentz (1993)], there is no evidence, even at low temperature, that the rate-limiting step for fusion is the formation of this aggregate (Bentz, 1992). Currently, the most general description of the process is that HA aggregates randomly form and fall apart rapidly in the viral envelope within the area of close membrane apposition. Each aggregate has a small probability of successfully accomplishing the three or more subsequent steps of bilayer destabilization which culminate in membrane fusion before falling apart (Bentz, 1992). Increased HA surface density will increase the surface density of HA aggregates, the surface density of fusion competent aggregates, and so, the likelihood that fusion will happen sooner rather than later. Such a process is consistent with all current data, including the reported HA surface density dependence of delay times before the onset of fusion (Clague et al., 1991, 1993). The fusion rate will increase with increased HA surface density, regardless of which step in the process is rate-limiting. Therefore, the data in Figure 10 do not imply that inhibition of lipid mixing is due to inhibition of HA aggregation kinetics, only that the total number of HA aggregates is diminished.

If it is assumed that inactivation proceeds from the HA aggregate, then it appears that HA–receptor binding reduces viral inactivation because the HAs bound to sialic acid moieties are prevented from aggregating into potential fusion sites, where they can be inactivated. If it is assumed that inactivation proceeds from individual HAs, then it appears that HA–receptor binding inhibits the conformational change of HA to the inactive state. In either case, the release of HA from the sialic acid moiety, as part of binding reversibility, is a source of new HAs capable of aggregating with other HAs and of mediating fusion.

ACKNOWLEDGMENT

We thank Dr. David P. Siegel for performing the cryo-electron microscopy and for illuminating discussions.

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