Influenza Hemagglutinin-Mediated Membrane Fusion: Influence of Receptor Binding on the Lag Phase Preceding Fusion†

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ABSTRACT: Fusion of influenza virus with liposomes is triggered by low pH, resulting in a conformational change in the fusion protein (HA) and the insertion of fusion peptides from HA into the liposomal membrane. Fusion does not take place immediately after insertion but is preceded by a lag phase, the duration of which, as we have found previously, depends on the presence of ganglioside receptors in the liposomal membrane [Stegmann, T., White, J. M., & Helenius, A. (1990) *EMBO J. 9,* 4231−4241]. Here we have investigated why that is the case. Surprisingly, the 2−4-fold shorter lag phase observed with phosphatidylcholine (PC)/phosphatidylethanolamine (PE)/ganglioside liposomes was not due to slower or more readily reversible binding of the virus to PC/PE liposomes lacking receptors. Nevertheless, using liposomes with various glycolipids as targets, it was found that specific HA−receptor interactions were required for a shorter lag, and not just the negative charge of the gangliosides, or the presence of ceramide lipid tails in the liposomal membrane. Receptor binding also did not facilitate the conformational change in HA. Surprisingly, however, it was found that after an incubation of the virus at low pH in the absence of target membranes at 0 °C for several minutes, the binding and fusion activity of virus using PC/PE liposomes, but not PC/PE/ganglioside liposomes as targets, was decreased. The population of virus that did still bind to and fuse with the PC/PE liposomes after low pH preincubation did so after a significantly increased lag time. Binding of virus to liposomes without receptors is solely due to insertion of viral fusion peptides into the liposomal membrane, suggesting that the availability of fusion peptides is decreased after low pH preincubation. In accordance with this suggestion, if the liposomal lipid bilayers were in the gel phase, binding of virus to PC liposomes but not to PC/ganglioside liposomes was strongly inhibited, and the lag phase was about 9 times shorter for liposomes with receptors. Therefore, these results suggest that ganglioside receptors shorten the lag phase because they facilitate insertion of fusion peptides into the target membrane.

Membrane fusion mediated by the influenza virus hemagglutinin (HA)[1] is triggered by acidification. Low pH induces a conformational change in the protein which results in the exposure of a "fusion peptide", the N-terminus of the HA2 subunit of HA (Skehel et al., 1982; Doms & Helenius, 1986; White & Wilson, 1987; Stegmann et al., 1990). Subsequently, the peptide is inserted into the target membrane for fusion (Stegmann et al., 1991; Tsurudome et al., 1992). Fusion does not occur immediately after insertion, but after a delay or lag phase. The lag time depends on the system studied, the pH, and the temperature. In the case of X-31 virus fusing with liposomes at pH 5.1, 0 °C, the conformational change and insertion were found to take place within about 15 s (Stegmann et al., 1991; Tsurudome et al., 1992), whereas the lag phase took several minutes (Stegmann et al., 1990). In this system, the lag time equals the time required, for those virus−liposome complexes that are the first to fuse, to undergo all the changes that are necessary for fusion. Although these changes are irreversible, the virus−liposome complexes need to be at low pH for the entire length of the lag phase to initiate fusion (Stegmann et al., 1990). When fusion of HA-expressing cells with other cells is studied, there also is a clear lag phase, but in this case the cells need to be at low pH only part of the time, indicating that other events can contribute to the lag phase as well (Morris et al., 1989; Sarkar et al., 1989).

Lag phases were also found to precede the fusion of a number of other viruses, like vesicular stomatitis virus (Clague et al., 1990), rabies virus (Gaudin et al., 1991), and Semliki Forest virus (SFV) (Bron et al., 1993), indicating that they may represent a common step in virus-induced fusion. Studying fusion of liposomes with cells expressing HA on their cell surface, Ellens et al. (1990) have shown that the amount of fusion depends on the density of HA, and that there is a 4.4-fold increase in the amount of fusion for a 1.9-fold increase in density. Because of this cooperativity and on the basis of theoretical considerations (Siegel, 1993) and a number of other observations (Doms & Helenius, 1986; Morris et al., 1989; Sarkar et al., 1989), it is thought that multiple HAs rearrange to form a "fusion complex", and at least part of the lag phase could be accounted for by the time such a fusion complex takes to form. In the case of SFV, there is clear evidence that, early during the lag, homotrimers of the fusion protein are formed, which

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; HA, hemagglutinin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(4-morpholino)ethanesulfonic acid; *N*-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PC, egg phosphatidylcholine; PE, egg phosphatidylethanolamine; SFV, Semliki Forest virus.

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represent the fusion-active conformation of the protein (Bron et al., 1993).

We have found previously that the lag time also depends on the presence of a receptor in the target membrane. During the course of influenza virus entry into its host cell in vivo, HA first binds to sialic acid containing receptors at the cell surface. The virus is then taken up by endocytosis and fuses with the endosomal membrane. Thus, at least some of the viral HAs interact with receptors during fusion. Fusion of virus with liposomes does not require receptors (White et al., 1982; Stegmann et al., 1985, 1989). In the absence of receptors, influenza can bind zwitterionic liposomes at low pH through insertion of the fusion peptides of HA into the liposomal membranes (Stegmann et al., 1987, 1991). However, fusion with ganglioside-containing zwitterionic liposomes was found to occur after a much shorter lag than fusion with zwitterionic liposomes lacking these receptors (Stegmann et al., 1990). Since virus can bind to receptor-containing liposomes via inserted fusion peptides and through HA1−receptor interaction, the simplest explanation for this phenomenon would be that virus binds liposomes that contain a receptor much faster. Alternatively, the gangliosides might have an effect besides receptor binding, e.g.: (1) The presence of their ceramide tails in the lipid bilayer of the liposomes could change the properties of the target membrane and thus facilitate fusion. This was recently demonstrated for SFV (Nieva et al., 1994; Wilschut et al. 1994). (2) The negative charge of the ganglioside headgroup could result in a lower pH close to the membrane than for liposomes lacking gangliosides, shortening the lag phase. (3) The conformational change could be slower if HA is not bound to a receptor. In this paper, we present data showing that the difference in lag time involved specific HA1−receptor recognition, but not because binding of virions to liposomes was faster or more efficient. Instead, the reason for the different lag time appears to be that the fusion peptides of HA2 are much more efficiently inserted into liposomal target membranes if bound to a receptor.

MATERIALS AND METHODS

Liposomes and Virus. Multilamellar vesicles were produced by resuspension of dry lipid films in 145 mM NaCl and 2.5 mM HEPES, pH 7.4. Subsequently, the suspensions were frozen and thawed at least five times, and large unilamellar vesicles were made from the multilamellar vesicles by extrusion through 0.1 μm defined-pore polycarbonate filters (Nucleopore, Pleasanton, CA) (Mayer et al., 1986). After extrusion, remaining multilamellar liposomes were removed by centrifugation. Liposomes were stored under argon at 4 °C, except for dimyristoylphosphatidylcholine (DMPC) containing vesicles. DMPC containing liposomes were extruded and kept at 37 °C. Phospholipid phosphate was determined according to Böttcher et al. (1961). Phospholipids were from Avanti Polar Lipids (Birmingham, AL) except for DMPC (Fluka, Buchs, St. Gallen, Switzerland). Bovine brain gangliosides (type III, estimated molecular weight 1500) and galactocerebrosides (type I) were from Sigma (St. Louis, MO).

The X-31 recombinant strain of influenza A virus [from plaque C-22 (Doms et al., 1986)] was grown for us by the Schweizerische Serum und -Impfstoffinstitut (Bern, Switzerland) in the allantoic cavity of embryonated eggs and purified, handled, and stored essentially as described before (Stegmann et al., 1985). Viral phospholipid was extracted according to Folch (1957), and phosphate was determined according to Böttcher et al. (1961).

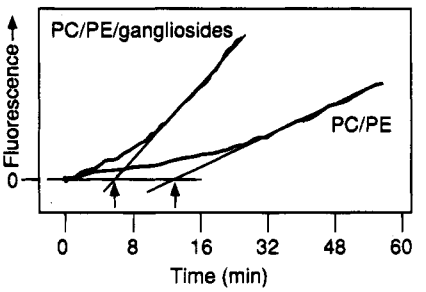

FIGURE 1: Fusion of influenza virus with liposomes at pH 5.1, 0 °C. Fusion was initiated by injecting virus into a cuvette containing PC/PE/ganglioside (6:3:1) or PC/PE (2:1) liposomes at pH 5.1, 0 °C, at time 0 and measured by the resonance energy transfer assay (Struck et al., 1981). The arrow indicates the lag time, read at the point where the extrapolated baseline fluorescence and the tangent to the steepest part of the fusion curve cross. In this case, the lag phases would be read as 5.9 and 13.0 min, respectively. The rate of fusion was calculated from the slope of the tangent. The final concentration of liposomes and virus was 5 μM of phospholipid phosphate each.

Fusion Measurements. Fusion between labeled liposomes and unlabeled influenza virus was measured by the resonance energy transfer (RET) assay (Struck et al., 1981). Labeled liposomes contained 0.6 mol% each of *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (*N*-Rh-PE) and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE). Fluorescence was recorded at excitation and emission wavelengths of 465 and 530 nm, respectively, with a 515 nm long-pass filter between cuvette and emission monochromator (Stegmann et al., 1985) on a Schoeffel RRS 1000 fluorimeter with continuous stirring in a thermostated cuvette holder. For calibration of the fluorescence scale, the initial residual level of fluorescence was considered to be 0% fluorescence and the fluorescence at infinite probe dilution to be 100%. The latter value was obtained by lysis of the liposomes with Triton X-100 (0.5% v/v) and subsequently corrected for dilution and the effect of Triton on the quantum yield of N-NBD-PE (Struck et al., 1981). Fusion was measured in a buffer containing 135 mM NaCl, 15 mM sodium citrate, 10 mM MES, and 5 mM HEPES at pH 5.1, essentially as described in Stegmann et al. (1985).

Determination of Lag Phases and Rates of Fusion. In a previous paper (Stegmann et al., 1990), we have defined the lag time as the time where the fluorescence first rises above background. Here, the delay time was estimated by a more objective and mathematically precise method, as proposed by Bentz (1992, 1993), which is illustrated in Figure 1. The delay time is thus defined as the intercept with the time axis of the tangent to the steepest part of the fusion curves. The slope of this line was taken as the rate of fluorescence increase.

Modification of N-Acetylneuraminic Acid. Conversion of the sialic acid of gangliosides incorporated into liposomes was carried out essentially according Suttajit and Winzler (1971) with modifications as described before (Stegmann et al., 1989). Briefly, liposomes were incubated for 2 h with a 40-fold molar excess of sodium (meta) periodate in the dark. A 3-fold excess of glycerol was added to stop the

reaction. After removal of the glycerol by gel filtration, Na_2CO_3 and NaCl were added to final concentrations of 0.1 and 0.15 M, respectively. The solution was added to dry $NaBH_4$ to a final borohydride concentration of 0.13 M. After 2 h at 0 °C, the reaction was terminated by the dropwise addition of glacial acetic acid, and the liposomes were dialyzed against 145 mM NaCl and 2.5 mM HEPES. Sialic acid was determined according to Warren (1959) or Hammond and Papermaster (1976).

Binding Assay and Calculation. Binding of liposomes to virus was determined by mixing virus and liposomes in low pH buffer. The samples were then neutralized and centrifuged for 20 min at 16000*g*. Pellet and supernatant were carefully separated, and the fluorescence of *N*-NBD-PE in the samples was determined after the addition of Triton X-100. Binding was calculated as follows:

$$\% \text{ binding} = 100 \times [F_{\text{pellet}} - A(F_{\text{pellet}} + F_{\text{supernatant}})]/(F_{\text{pellet}} + F_{\text{supernatant}})$$

where *A* stands for the percent of liposomes brought down in control incubations in the absence of virus (generally 3−8%), and *F* stands for fluorescence.

Measurement of the Amount of Conformational Change in HA. Virus (10 nmol of phospholipid phosphate) was incubated at various pHs for 45 min at 0 °C or for various amounts of time at pH 5.1, 0 °C, in the absence or presence of a 25- or 100-fold molar excess with respect to HA of *N*-acetylneuraminlactose (type I from Sigma, St. Louis, MO, approximately 85% α2−6 glycosidic linkages of the neuraminic acid group, from human milk), neutralized, and then digested with 0.1 or 1 mg/mL of proteinase K for 1 h at 37 °C. The samples were loaded on a discontinuous sucrose gradient, consisting of 0.6 mL 60% (w/v) sucrose in 145 mM NaCl, 2.5 mM HEPES, pH 7.4, and 2.8 mL of 20% sucrose, in the same buffer, and spun for 1 h at 160000*g*. The bands at the interface were collected and analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis followed by silver stain. The silver stained gels were scanned on a Molecular Dynamics gel scanner, and the amount of HA was determined relative to the amount of matrix protein, which was unaffected by the proteolytic treatment and served as an internal control for the amount of virus loaded.

RESULTS

The Longer Lag before Fusion with Liposomes Lacking Gangliosides Is Not due to Slower or More Reversible Virus Binding. Fusion of influenza virus with large unilamellar liposomes containing PC/PE (2:1) or PC/PE/gangliosides (6:3:1) was measured by the resonance energy transfer assay (Struck et al., 1981), which assays mixing of labeled liposomal with unlabeled viral membrane lipids. As shown in Figure 1, upon addition of the virus to liposomes at pH 5.1, 0 °C, the fluorescence first remained stable during several minutes (lag phase), and then there was an increase in fluorescence. It was previously shown that the increase in fluorescence is caused by membrane fusion (Stegmann et al., 1990). At pH 5.1, the lag observed with PC/PE liposomes, measured as described in Materials and Methods and indicated by the arrows in Figure 1, was about 14 min, and with PC/PE/ganglioside liposomes it was about 6 min.[2] The absolute length of the lag phase and the rate of fusion varied for different preparations of the virus, but the lag phase was always 2−4 times longer for liposomes without gangliosides, confirming previous observations (Stegmann et al., 1990).

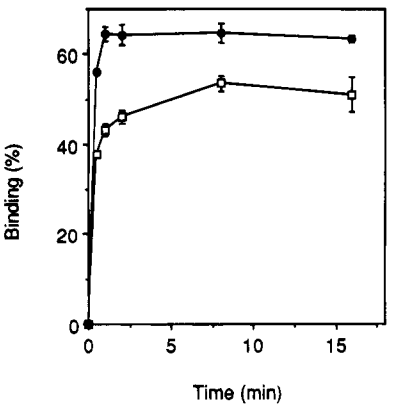

FIGURE 2: Binding of liposomes to influenza virus at pH 5.1, 0 °C. Virus was added to liposomes in the low pH buffer, mixed rapidly, and neutralized after the amount of time indicated. Duplicate determinations of binding were carried out as described in Results; percent binding was calculated as described in Materials and Methods. Open squares, PC/PE; closed circles, PC/PE/gangliosides. Error bars are one standard deviation. Where no error bars are shown, they are smaller than the drawn data point symbols. The final concentration of liposomes and virus was 10 μM of phospholipid phosphate each, which means that the virus−liposome particle ratio is about 1:1.

Since fusion can only take place after binding of the virus to liposomes, and gangliosides can serve as receptors for the virus, the most obvious explanation for the longer lag would be that binding to PC/PE liposomes is slower. To measure the rate of binding, virus was added to fluorescently labeled liposomes in pH 5.1, 0 °C buffer, and the mixture was neutralized after various periods of time. Immediately thereafter, the virus plus bound liposomes were pelleted by centrifugation, and the fluorescence in pellet and supernatant was measured. With both types of liposomes, binding was more than 80% complete within 30 s after acidification (Figure 2). The total amount of binding to PC/PE liposomes was somewhat lower. Thus, the longer lag phase observed with liposomes lacking gangliosides is not due to slower binding.

It could also be that virus bound to PC/PE liposomes more readily dissociates from these than from ganglioside containing liposomes and then has to reassociate with another liposome before it can fuse, explaining the longer lag phase. To investigate this possibility, virus was incubated with either type of fluorescently labeled liposomes for various amounts of time, and then a 10-fold excess of unlabeled liposomes was added for 4 min, after which the samples were neutralized. Then binding of the fluorescent liposomes was measured as described above. It was not possible to use a larger excess of liposomes because the light scattering of the liposome−virus aggregates interfered too much with fluorescence detection. It was found that, after 2 min at low pH, only 20% of the bound liposomes could still be competed

[2] In a previous paper (Stegmann et al., 1990), the lag phase was measured in a different fashion, leading to somewhat different estimates of the lag especially for PC/PE liposomes. This does not affect the conclusions.

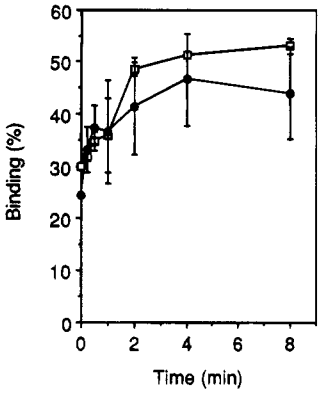

FIGURE 3: Binding of labeled liposomes to influenza virus after the addition of unlabeled liposomes at pH 5.1, 0 °C. Virus and fluorescently labeled liposomes (5 μM of phospholipid phosphate each) were incubated for the times indicated at pH 5.1, 0 °C, and then a 10-fold molar excess of unlabeled liposomes was added. After 4 min, the samples were neutralized, and binding was measured in duplicate as described for Figure 2. Open squares, PC/PE; closed circles, PC/PE/gangliosides.

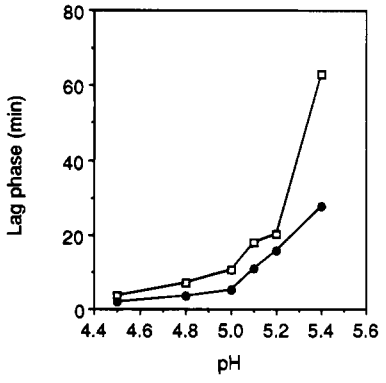

FIGURE 4: pH dependence of the lag phase: lag time preceding fusion of influenza virus with liposomes at 0 °C. Lags were measured as described in the legend to Figure 1. Open squares, PC/PE; closed circles, PC/PE/gangliosides. Error bars are one standard deviation. The final concentration of liposomes and virus was 12 μM of phospholipid phosphate each. The results presented in this figure were obtained using a different batch of virus than throughout the rest of the paper, explaining the slightly longer lag phase.

off (Figure 3; see also Figure 2), and this affected ganglioside containing liposomes slightly more than PC/PE liposomes. Therefore, the longer lag phase preceding fusion with liposomes lacking receptors is not due to more rapid and/or extensive dissociation of virus from PC/PE liposomes.

Head or Tails? Besides functioning as a receptor, gangliosides could affect fusion in other ways. For example, the interaction of their ceramide tails with the lipids of the liposomes could modulate the properties of the liposomal membranes, and thus affect fusion, as found recently for SFV induced membrane fusion (Nieva et al., 1994; Wilschut et al., 1994). Another possibility is suggested by the data presented in Figure 4. The lag time was found to decrease continuously with decreasing pH, irrespective of whether PC/PE or PC/PE/ganglioside liposomes were used as target membranes (Figure 4). Thus it could be that the negative charge of the neuraminic acid-containing headgroups of the gangliosides resulted in a lower local pH close to the membrane than for PC/PE liposomes, giving rise to a shorter lag phase. To investigate the influence of headgroup charge and ceramide tail of the gangliosides, we measured fusion with liposomes containing PC and PE in a 2:1 ratio and one

Table 1: Lags Preceding Fusion with Various Target Membranes

liposome composition	lag time (min)[a]
PC/PE/gangliosides (6:3:1)	5.8 ± 0.1
PC/PE/gangliosides (12:6:1)	6.2 ± 0.5
PC/PE/modified gangliosides (6:3:1)	8.5 ± 0.5
PC/PE/sphingomyelin (6:3:1)	14.1 ± 1.0
PC/PE/galactosylceramides (6:3:1)	13.8 ± 1.8
PC/PE/phosphatidylserine (6:3:1)	16.0 ± 2.5
PC/PE (2:1)	14.0 ± 0.3

[a] Fusion was measured as indicated in the legend to Figure 1. Measurements were made in triplicate and are presented ± one standard deviation. The molar ratio of the lipids is in parentheses.

of several different lipids with ceramide tails, or the negatively charged phospholipid phosphatidylserine (Table 1). Fusion with liposomes containing 5 mol % gangliosides showed a lag phase which was not significantly different from that of liposomes containing 10 mol % gangliosides, which bear twice the charge. Incorporation of 10 mol % of phosphatidylserine in PC/PE liposomes did not result in a shorter lag phase than found for PC/PE liposomes. If liposomes containing 10 mol % gangliosides were modified as described in Materials and Methods, resulting in approximately 75% of the ganglioside molecules having their *N*-acetylneuraminic acids shortened by one or two carbon atoms (Suttajit & Winzler, 1971; Stegmann et al., 1989), the lag phase observed using these modified liposomes was intermediate in length between that of liposomes with and without gangliosides. Neuraminic acids modified in this fashion can no longer function as receptors for the virus, but their overall charge is unchanged (Suttajit & Winzler, 1971; Stegmann et al., 1989). The resulting lag was most likely shorter than that observed for PC/PE liposomes because of the remaining 25% of the gangliosides that remained unmodified by the procedure. With liposomes containing 10 mol % of sphingomyelin or galactocerebrosides, which have ceramide tails like gangliosides, but do no contain neuraminic acid or possess a net charge, the lag phases were about as long as for PC/PE liposomes (Table 1). Taken together, these data indicate that neither the net negative charge of liposomes nor the ceramide tail of the gangliosides causes the lag phase to be shorter than for PC/PE liposomes, and therefore specific HA1−receptor interactions must be involved.

HA Conformational Change and Inactivation. The rate of the conformational change in HA had previously been determined using a monoclonal antibody against the fusion peptide (Stegmann et al., 1990). Alternatively, it can be inferred from the rate of binding of the virus to zwitterionic liposomes (Stegmann et al., 1990) (cf. Figure 2). As estimated by these methods, maximal exposure of the fusion peptide was found to take place within a minute at pH 5.1, 0 °C. However, neither of these methods gives a quantitative estimate of the number of HA molecules per virion that convert to the acid form, because the monoclonal only recognizes around 10% of the fusion peptide maximally, and the number of fusion peptides necessary for the binding of a virion to a zwitterionic liposome is unknown. Therefore, two experiments were performed to determine if more HA molecules converted to their acid form upon acidification if they were bound to a receptor.

As shown in Figure 4, the pH dependence of the lag phase is generally similar for ganglioside containing and PC/PE

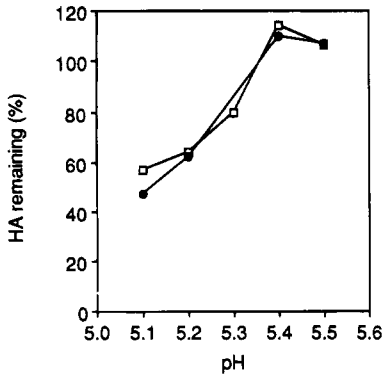

FIGURE 5: Conformational change in the absence and presence of bound ligand vs pH. Virus was incubated at the pH indicated for 45 min, in the absence or presence of a 25-fold excess of the ligand *N*-acetylneuramin lactose, and then neutralized. Subsequently, the virus was digested with proteinase K for 1 h at 0 °C and purified, and the viral proteins were analyzed as described in Materials and Methods. The amount of HA remaining after incubation at neutral pH was set to 100%.

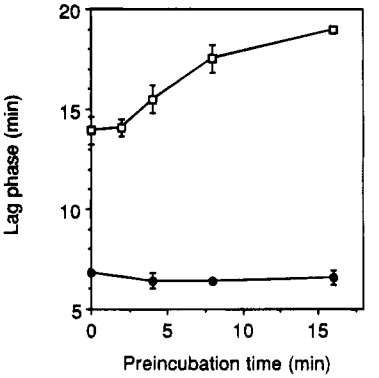

FIGURE 6: Lag time preceding fusion of acid-preincubated influenza virus with liposomes at pH 5.1, 0 °C. Virus was preincubated at pH 5.1 for the time indicated, and then liposomes were added. Lags were measured as indicated in Figure 1. Open squares, PC/PE; closed circles, PC/PE/gangliosides. Results are expressed relative to virus that had not been incubated at low pH. Error bars and concentrations are as described in the legend to Figure 2.

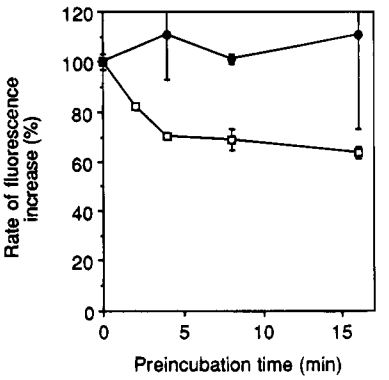

FIGURE 7: Rate of fluorescence increase upon fusion of acid-preincubated influenza virus with liposomes at pH 5.1, 0 °C. Virus was preincubated at pH 5.1 for the time indicated, and then liposomes were added. Open squares, PC/PE; closed circles, PC/PE/gangliosides. Conditions are as described in the legend to Figure 6.

vesicles. However, the lag phase with PC/PE liposomes seems to decrease more from pH 5.4 to 5.2 than for ganglioside containing liposomes. To investigate whether this could reflect a different number of HA molecules undergoing a conformational change at a certain pH in the absence or presence of receptors, virus was incubated with or without a 25-fold molar excess over HA of *N*-acetylneuramin lactose (with the type of glycosidic linkage that X-31 prefers; see Materials and Methods), acidified to different pHs for 45 min, neutralized, and digested with proteinase K. This protease digests the acid, but not the neutral form of HA (Doms & Helenius, 1986; Stegmann et al., 1987). Virus and protease were separated on sucrose gradients, and the viral proteins were analyzed by nonreducing SDS−PAGE, as described in Materials and Methods. The gels were silver-stained, and the stain in the bands was quantitated by means of a scanning densitometer. Care was taken to ensure that the stained bands were within the linear range of the densitometer. The errors in such measurements were found to be around 5−10% of the HA density in pilot experiments. The results are shown in Figure 5. Clearly, there is no significant difference in the amount of conformational change at pHs where significant fusion takes place, although no significant conformational change can be detected in either case by this method at pH 5.4. Moreover, at pH 5.1, 0 °C, it was found that about 37% of the total HA converted to the acid form after 1 min in the presence of (α2−6)*N*-acetylneuramin lactose and 33% in the absence of ligand. Longer protease digestion or higher concentrations of the protease did not lead to further digestion in the above cases. Therefore, there was no significant difference in the extent or the rate of the conformational change in the absence or presence of bound ligand.

It was not possible to perform this experiment using liposomes rather than soluble *N*-acetylneuramin lactose as a ligand, because they would tend to form large aggregates with the virus, leading to great variability in the results of the digestion experiment. Since it was possible that binding of HA to receptors on a liposome, rather than to a soluble receptor was cooperative, or otherwise different in nature, we sought to confirm these results by a second, independent experiment.

In this experiment, the conformational change was initiated in the absence of target membranes, by incubating the virus at low pH, after which liposomes were added to the acid-activated virus. If the lag phase preceding fusion with PC/PE liposomes would be longer because the conformational change was slower for HA not bound to gangliosides, one would expect the lag phase to decrease after preincubation of the virus at low pH. Using PC/PE/ganglioside liposomes as target membranes, the lag remained constant for 0−16 min of preincubation, confirming earlier observations (Stegmann et al., 1990). Surprisingly, however, for PC/PE liposomes, it was found that the lag time *increased* with the time of preincubation (Figure 6). There was a corresponding decrease in the rate of fusion for PC/PE, but not for PC/PE/ganglioside liposomes (Figure 7).

Previously, we had found that the viral fusion capacity toward PC/PE/ganglioside liposomes is rapidly inactivated by treatment of the virus alone at low pH at 37 °C, but not at 0 °C (Stegmann et al., 1987). Inactivation is caused by lateral aggregation of HA molecules (Gutman, et al., 1993), probably mediated by hydrophobic interactions between the fusion peptides of HA (Stegmann et al., 1987). It now appears that inactivation also occurs at 0 °C, but at such a low rate that it is barely perceptible using PC/PE/ganglioside liposomes as targets. These findings prompted us to

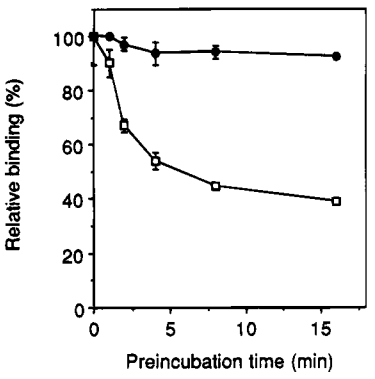

FIGURE 8: Binding of liposomes to influenza virus after preincubation of virus at pH 5.1, 0 °C. Open squares, PC/PE; closed circles, PC/PE/gangliosides. After the preincubation time indicated, virus and liposomes were allowed to bind for 4 min, and then the mixture was neutralized and binding was measured in duplicate as described in Materials and Methods. Error bars are one standard deviation; concentrations are as in Figure 2.

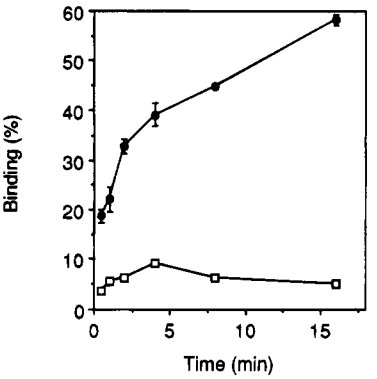

FIGURE 9: Binding of gel phase liposomes to influenza virus at pH 5.1, 0 °C. Liposomes were kept at 37 °C until just before the experiment, diluted, and taken through the phase transition quickly in low pH buffer at 0 °C. Virus was then mixed rapidly, and the mixture was neutralized after the amount of time indicated. The binding assay was as described for Figure 2. Open squares, DMPC; closed circles, DMPC/gangliosides. Error bars are one standard deviation; where none are shown they are smaller than the data point symbols. The final concentration of liposomes and virus was 10 μM of phospholipid phosphate each.

reinvestigate if some rate of inactivation could be established for ganglioside containing liposomes at 0 °C also. We found that after 2 h of preincubation of the virus at pH 5.1, 0 °C, the lag phase as measured with ganglioside containing liposomes had increased 2-fold, and the rate of fusion was found to be halved. Thus inactivation takes place at 0 °C, but not on a time scale relevant for fusion using PC/PE/ganglioside liposomes as targets, consistent with recent observations by Ramalho-Santos et al. (1993). We have previously shown that extensive dissociation of the tips of the HA trimers is necessary for inactivation, and that such dissociation does not take place after an incubation for 1 h at pH 5.1, 0 °C, as opposed to 37 °C (Stegmann et al., 1990). Therefore, the new data suggest that inactivation can also take place with HA whose tip domains are not dissociated, but this inactivation is very slow; Ramalho-Santos et al. (1993) estimate it is about 1000 times slower.

To investigate whether inactivation at 0 °C involves the fusion peptides, binding to PC/PE or ganglioside containing liposomes was measured after preincubation of the virus at pH 5.1, 0 °C (Figure 8). Influenza virus does not bind PC/PE liposomes at neutral pH, but at low pH it acquires the ability to bind to these liposomes, as a result of insertion of the fusion peptides into the target membrane (Stegmann et al., 1987, 1991). Preincubated virus was allowed to bind to liposomes for 4 min, after which the mixture was neutralized and binding measured. It was found that binding of preincubated virus to PC/PE liposomes was affected. Binding to ganglioside containing liposomes, which involves HA1–sialic acid interactions, was not affected, confirming earlier results with a related strain of influenza virus (Stegmann et al., 1987). Therefore, the prolonged lag phase before fusion of preincubated virus with PC/PE liposomes most likely resulted from a decreased availability of fusion peptides, affecting the binding to PC/PE liposomes. Importantly, the data presented in Figures 5 and 6 indicate that after a preincubation of 16 min at pH 5.1, 0 °C, about 40% of the virus can still bind to PC/PE liposomes (Figure 8) within 4 min, but fusion of these virus-liposome complexes takes place after a considerably increased lag phase (Figure 6).

What is the relevance of these observations for the difference in lag phase between PC/PE and PC/PE/ganglioside liposomes? Previously, it was found that inactivation does not occur if virus is already bound to ganglioside-containing liposomes before lowering the pH (Stegmann et al., 1987). If this is different for PC/PE liposomes, inactivation could compete with fusion, continuously removing virions that could have otherwise fused with PC/PE liposomes from the population. In that case, one would expect the final level of fusion of influenza virus with PC/PE liposomes to be lower than that with ganglioside containing liposomes. However, it was found that after overnight incubation at pH 5.1, 0 °C, the final level of fusion with PC/PE liposomes (39%) was similar to that with PC/PE/ganglioside liposomes (38%), suggesting that inactivation of HA on virus already bound to PC/PE liposomes either does not occur or does not affect the final level of fusion.

Therefore, these data suggest that ganglioside receptors facilitate the insertion of fusion peptides into the target membrane. In an earlier study, we have measured insertion of the fusion peptides directly using photoactivatable probes present in the liposomal membrane (Stegmann et al., 1991). In view of the very low labeling achieved under extreme conditions (Tsurudome et al., 1992), and the background signal that is always present (Stegmann et al., 1991; Tsurudome et al., 1992), we felt that it was not possible, using such techniques, to determine subtle differences between the number of inserted fusion peptides in the particularly difficult cases, such as with partially inactivated virus or gel phase liposomes, that were studied herein. Therefore, a more indirect approach was chosen. To investigate insertion, the binding of virus to liposomes composed of either dimyristoylphosphatidylcholine (DMPC) or DMPC and gangliosides was investigated. We have previously shown that at 10 °C, where the membranes of these liposomes are in the gel phase, fusion is still possible (Stegmann, 1993). However, low pH induced binding of HA to these rigid gel phase membranes is impaired (Doms et al., 1985). Accordingly, at 0 °C, binding of the virus to DMPC liposomes was not significant during the first 16 min after incubation, whereas binding to DMPC/ganglioside liposomes was slower than to PC/PE/ganglioside liposomes but still 50% complete within the first 2 min (Figure 9). These data suggest that insertion of the fusion peptides into

target membranes that are in the gel phase was severely impaired. To see how the impaired insertion affects fusion, we attempted to measure fusion at 0 °C but found that it was difficult to measure a precise lag phase for fusion with DMPC liposomes at 0 °C because fusion was extremely slow. Previously we have shown that, at 10 °C, fusion with DMPC liposomes has a lag of about 64 min, whereas fusion with DMPC/ganglioside liposomes occurred after a lag of about 7 min (Stegmann, 1993). This 9-fold difference between liposomes with and without gangliosides is much larger than the 2−4-fold difference found with membranes that are in the liquid crystalline phase (Figure 1). Taken together, the inactivation data and the results with the gel phase membranes suggest that the length of the lag phase with PC/PE liposomes is determined by the number of fusion peptides inserted into the target membrane.

DISCUSSION

Membrane fusion mediated by the influenza virus HA glycoprotein is preceded by a lag phase. Fusion of influenza virus with zwitterionic liposomes takes place after a longer lag phase than fusion with liposomes that also contain a receptor. The data presented in this paper suggest that the presence of a receptor on the target membrane shortens the lag phase not because virus particles bind faster to liposomes but because, as a result of specific HA1−receptor recognition, receptors facilitate the insertion of fusion peptides into the target membrane, thus increasing the concentration of fusion peptides at the site of fusion.

Lag phases are generally thought to result from the formation of "fusion complexes", higher order oligomers of fusion proteins, at the site of fusion. For HA, the most direct evidence for the formation of a fusion complex comes from experiments in which the amount of fusion of liposomes with HA-expressing cells was found to depend on the density of HA in a cooperative fashion (Ellens et al., 1990). The lag phase preceding influenza virus−cell fusion depended on the density of HA, but not in a cooperative fashion (Clague et al., 1991), although the validity of the latter assessment has been questioned (Bentz, 1993). In the case of SFV fusion, the lag time was found to correspond in part to the time required for the formation of a homotrimer of the E1 protein, which is the fusion active conformation of the protein (Bron et al., 1993), but most of the lag phase occurred after this step. Formation of a fusion complex is most likely important for membrane fusion because it leads to a high concentration of fusion peptides in the membrane at the site of fusion, which leads to the membrane perturbation that is necessary for fusion (Siegel, 1993). It has been estimated that the fusion peptides can comprise 15−30% of the surface area at the fusion site (Siegel, 1993). If gangliosides facilitate the insertion of fusion peptides into the target membrane, the concentration of fusion peptides necessary for fusion is more readily reached.

Our interpretation of the lag phase as measured here and shown previously (Stegmann et al., 1990, 1991) is that the lag phase represents the time needed, for those virus−liposome complexes which are the first to fuse, to undergo all the changes that are necessary for fusion. For ganglioside containing liposomes, initial virus−liposome binding takes place via HA1−receptor interactions. With the membranes already in contact, all of the fusion peptides that can insert into the membrane will insert within 10 s after lowering the pH (Stegmann et al., 1991; Tsurudome et al., 1992). HA−target membrane binding at the site of virus−liposome contact is irreversible, and further diffusion of more HA into the fusion site is most likely sterically inhibited, explaining why the number of fusion peptides that are inserted into the target membrane remains constant throughout the lag, as was shown previously (Stegmann et al., 1991; Tsurudome et al., 1992). During the rest of the lag phase, the HAs that have their fusion peptides inserted then form one or more fusion complexes.

For liposomes without receptors, immediately after lowering the pH some fusion peptides insert into the target membrane. Binding is concomitant with exposure of the fusion peptides, and therefore it takes place on approximately the same time scale as binding to receptor containing liposomes (Figure 2). The number of fusion peptides inserted is sufficient to bind virus irreversibly to the liposomes (Figure 3). However, at a given virus concentration, the number of liposomes without receptors that binds to the virus is lower than for liposomes with receptors (Figure 2), indicating that there is a population of virions that cannot expose sufficient fusion peptides for binding. Furthermore, binding to and fusion with PC/PE liposomes are readily inactivated by a low pH preincubation at low temperature that hardly affects fusion with ganglioside containing liposomes (Figures 6−8). Therefore, the number of inserted fusion peptides is probably just sufficient for binding. If virus is subjected to low pH inactivation to the extent that only about 40% of the virions can still bind to PC/PE liposomes (after 4 min: Figure 7), then 60% of the virus population can no longer bind to and fuse with PC/PE liposomes, but the remaining 40% of the virus which does bind to liposomes is also affected. Fusion of these virus−liposome complexes takes place after a considerably increased lag phase (Figure 6). Since inactivation decreases the availability of fusion peptides for interactions with the target membrane (Figure 8), these data suggest that additional fusion-active HA molecules need to reach the fusion site, and insert their fusion peptides, for fusion to begin. Diffusion of additional HA molecules over the surface of the virion to the fusion site is slow at 0 °C because of the limited mobility of HA at that temperature (Junankar & Cherry, 1986), and while some of their fusion peptides eventually insert into the target membrane, others may be inactivated *en route*. Mobility of HA is a prerequisite for fusion, and inactivation leads to a reduced mobility of HA (Junankar & Cherry, 1986; Gutman et al., 1993). Only after a sufficient number of HA molecules has assembled into a fusion complex, and therefore a sufficient local concentration of fusion peptides is reached, can fusion begin.

In this respect, fusion of X-31 virus with PC/PE liposomes as shown above resembles fusion of the A/PR/8/34 strain of influenza virus with ganglioside containing liposomes at low temperatures (Tsurudome et al., 1992). In the latter case, it was found that for this strain of virus only limited insertion of fusion peptides occurred at low temperatures, which led to irreversible virus−liposome binding but not fusion. Fusion only occurred after raising the temperature, which was found to result in massive additional insertion of fusion peptides (Tsurudome et al., 1992). If insertion is difficult, such as for DMPC vesicles in the gel phase (Figure 9), the difference in lag between target membranes with and without receptors is therefore extreme [9-fold (Stegmann, 1993)].

Thus the main difference between fusion with target membranes with and without receptors must be that, in the presence of receptors, initially many more fusion peptides are inserted than are necessary for the formation of a fusion complex, because of HA1−receptor interactions. Therefore, there was almost no effect of inactivation during the first 16 min at low pH on fusion with ganglioside-containing liposomes (Figures 6 and 7). In the absence of receptors, additional fusion peptides still need to be inserted for fusion.

Recent data by Bullough et al. (1994) are in good agreement with the above results showing that the insertion of fusion peptides into the target membrane is facilitated by receptor binding. They subjected the bromelain-released ectodomain of HA to low pH and subsequently digested the low-pH form with proteases. X-ray crystallography of a resulting fragment showed that, as a result of the low pH induced conformational change, the fusion peptides could be translocated almost 100 Å in the direction of the target membrane and end up close to the receptor-binding site (Bullough et al., 1994). Although it remains to be determined whether the structure they have found is representative of the fusion-active form of HA, in this conformation the fusion peptides would be directed to insert into the target membrane if their HAs were bound a receptor.

It has been argued that those HA molecules that are bound to gangliosides are not capable of initiating lipid mixing in the fusion site (Bentz et al., 1993; Alford et al., 1994). On the other hand, it was shown for fusion of virus with planar lipid bilayers that binding of HA to a receptor molecule fundamentally alters the route of fusion and in fact thereby facilitates fusion (Niles & Cohen, 1993). The data presented in this paper do not address this controversy directly. However, although HA can clearly induce fusion in the absence of receptors, and fusion complex formation may involve HA molecules that are not bound to receptors themselves, it does seem not very likely that the HA molecules that were recruited into the fusion complex by receptors would detach from them before inducing lipid mixing.

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