Membrane penetration of Sendai virus glycoproteins during the early stages of fusion with liposomes as determined by hydrophobic photoaffinity labeling

(membrane fusion/virus infection)

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ABSTRACT The hydrophobic photoaffinity label 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine was used to label Sendai virus proteins during fusion with cardiolipin and phosphatidylserine liposomes. Preferential labeling of the viral fusion protein during the initial stages of fusion demonstrated that this protein interacts with the hydrophobic core of the target membrane as an initiating event of virus–liposome fusion. Labeling showed time, temperature, and pH dependence consistent with earlier fluorescent measurements of fusion kinetics. The present method provides conclusive evidence supporting the hypothesis that hydrophobic interaction of the fusion protein with the target bilayer is an initial event in the fusion mechanism of viral membranes.

The infectious entry of enveloped viruses is accomplished by a mechanism involving membrane fusion (1–3). Sendai virus, a paramyxovirus, enters host cells by fusion of the viral envelope with the cell's plasma membrane, mediated by the two Sendai envelope glycoproteins (1, 3). The hemagglutinin/neuraminidase (HN) mediates viral attachment to sialic acid-containing cell surface receptors, while the fusion (F) protein, which consists of two disulfide-linked subunits, F_1 and F_2 (4), triggers the actual fusion reaction. It has been proposed that fusion is initiated as a result of the insertion of the hydrophobic F_1 NH_2 terminus, consisting of about 20 amino acids, into the target membrane (1, 3, 5, 6).

Hydrophobic protein–lipid interactions (7–10) and some proteins that cause membrane fusion (11, 12) have been investigated by using photoaffinity labels. Such studies typically involve labeling of both protein and lipid after an incubation period, allowing identification of the transmembrane segments of proteins or a distinction between subunits potentially interacting with membranes. Protein-induced fusion involves an initial local interaction between fusogen and apposed membranes, rapidly followed by randomization of membrane components in the lateral plane of the newly formed (i.e., fused) membrane. By focusing on the very early events at the onset of fusion—i.e., those prior to membrane randomization—the proteins penetrating the target membrane as fusion initiators can be selectively labeled. In the case of Sendai virus fusion, such an experiment would allow analysis of the hypothesis that fusion is initiated by insertion of the hydrophobic F_1 NH_2 terminus into the target membrane. In order to examine exclusively these initial interactions, photolabeling must be done for limited periods of time—i.e., while fusion is in progress, before the proteins have reoriented in the fused membrane. Obviously, this requires a detailed knowledge of the kinetics of the fusion reaction.

Although circumstantial evidence has been collected (1, 3, 5, 13, 14), no chemical evidence of the penetration of viral glycoproteins into target membranes during the initial moments of fusion has been reported thus far. The present study shows preferential labeling of the Sendai virus F protein at the initiation of the fusion reaction with negatively charged liposomes containing 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine (TID). Although the fusion between a virus and a liposome may not resemble in every respect the fusion that occurs under biological conditions (14), it is our contention that this approach provides a unique opportunity to identify the fusion-initiating proteins and permits greater insight into the mechanisms of viral entry and membrane fusion.

METHODS

Virus. Sendai virus (Z strain) was grown for 72 hr in the allantoic compartment of 10-day-old fertilized chicken eggs. The virus was purified by differential ultracentrifugation and stored in 150 mM NaCl/5 mM Hepes, pH 7.4, at −70°C (15). Viral protein concentration was determined by the Peterson modification of the Lowry method (16).

Liposomes. Large unilamellar vesicles (sized through 0.1-μm polycarbonate membranes) were prepared from bovine heart cardiolipin or bovine brain phosphatidylserine (Avanti Polar Lipids) by the reverse-phase evaporation method (14). Lipid phosphorus concentration was determined by the modification of the Bartlett assay described by Böttcher *et al.* (17).

Fusion and Photolabeling. A 1-mCi/ml solution of TID (Amersham, 10 Ci/mmol, 90% radiochemical purity, 1 Ci = 37 GBq) in 10% ethanol (10 μl) was added to 200 nmol of liposomes of appropriate composition in 940 μl of buffer. The mixture was mixed on a Vortex and incubated for 1 hr on ice in the dark. Additional details are provided in *Results*. Sendai virus (80 μg) was added at the specified temperature and the mixture (final volume, 1 ml) was stirred continuously. The sample was irradiated for 30 sec by an Osram HBO 100W/2 super-pressure mercury lamp at 10 cm, with a Schott Glass Technology WG-360 high-pass cutoff filter (9). The reaction was stopped by immersing the sample in ice in the dark. The kinetics of fusion between Sendai virus and liposomes under the described conditions were monitored continuously by the R_{18} (octadecylrhodamine B chloride) fusion assay, as described elsewhere (18).

Analysis of Labeled Viral Proteins. Proteins were precipitated by addition of 75 μl of cold 72% (wt/vol) trichloroacetic acid, resuspended in reducing electrophoresis sample buffer (5% sodium dodecyl sulfate/5% 2-mercaptoethanol/8 M

Abbreviations: HN, hemagglutinin/neuraminidase; F protein, fusion protein; TID, 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine.
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urea/62.5 mM Tris·HCl, pH 6.8/0.01% bromophenol blue), and denatured and separated by PAGE (19). Protein bands were stained with 0.2% Coomassie brilliant blue R-250. Dried gels were autoradiographed at −70°C with Kodak XAR-5 film and a calcium tungstate intensifying screen. Scintillation counting was conducted after solubilizing 2- to 4-mm gel slices in 0.5 ml of Lumasolve (Lumac, Landgraaf, The Netherlands) and adding 10 ml of Hydrocount scintillation fluid (Baker) per sample.

RESULTS

TID Incorporation into Liposomes. TID incorporation, assayed by liquid scintillation counting, was 80 ± 2% of added activity in all cases (data not shown) with the exception of addition to phosphatidylserine vesicles at pH 7.4. In that case only 59 ± 2% of added activity was incorporated, perhaps because the smaller inter-head-group distances in phosphatidylserine compared to cardiolipin (20) hamper the ability of the probe to penetrate and insert into the hydrophobic core of the lipid bilayer (see ref. 14). In the course of these experiments we noted that unbound probe sticks very efficiently to the plastic Eppendorf tubes used, so that it was not necessary to routinely chromatograph each sample prior to use. However, the sticking of probe to the tubes may have contributed to the 20% loss in the other cases.

Preferential Association of F Protein with Cardiolipin Vesicles. Sendai virus fuses readily with negatively charged cardiolipin or phosphatidylserine vesicles (14). At neutral pH the fusion event is dependent on the F protein, as trypsinization of the virus, which removes specifically the F protein, inhibits fusion by about 80% (14). The hydrophobic interaction of the F protein with cardiolipin vesicles during early stages of fusion is preferential (Fig. 1). Nearly 80% of all labeling immediately after addition of virus is of the F protein. As the reaction continues, labeling of other proteins increases, with a concomitant decrease in F labeling, presumably due to later interactions of these proteins with the target membrane during membrane mixing and protein reorientation. Hence, the transmembrane parts of both the F and HN peptide chains will also become labeled. Typically, a protein-labeling efficiency of 0.1–0.3% was obtained. Although the 30-sec photolysis was the minimum period necessary for

sufficient labeling with the light source used, shorter photolysis periods can be used if the photon flux is increased (data not shown). Approximately 80% of the TID is photolyzed during the 30-sec period (9). The presence of larger amounts of TID in the membrane did not significantly increase the amount of labeling, indicating that the amount of probe is not limiting (ref. 9 and data not shown).

Carbenes formed by photolysis of diazirines are more reactive and less selective than nitrenes (8, 21, 22), but it appeared that protein was preferentially labeled over lipid. This gave the appearance of a "competition" among proteins for label, such that if fusion and F labeling were impeded, labeling of other viral proteins increased without any change in the magnitude of lipid labeling. This is explained by the preferential addition of singlet carbenes formed upon diazirine photolysis to the commonly occurring double bonds and heteroatomic single bonds of the proteins, rather than insertion into the C—H bonds in the lipid core (21, 22).

F-Protein Labeling During Fusion Between Sendai Virus and Cardiolipin Vesicles. In addition to the time-dependent preference in F labeling relative to labeling of the other proteins, the incorporation of label into the F protein showed a time dependence with striking similarity to the kinetics of virus–cardiolipin vesicle fusion (14). When fusion was allowed to proceed at 37°C before photolabeling, the percentage of radioactivity in F decreased sharply (Fig. 2). This corresponds to a hydrophobic interaction of the F protein with the target membrane as an initiating event in protein-mediated membrane fusion, followed by randomization of viral and target membranes, causing the labeling of viral proteins other than the F protein.

F_1 **vs.** F_2 **Labeling.** The F_1 subunit's hydrophobic NH_2 terminus has been proposed to be the fusion-initiating peptide (5), whereas the F_2 subunit, located outside the viral membrane (23), does not participate in fusion. To ascertain the exclusive involvement of F_1 in viral fusion, TID labeling was conducted as described earlier. Proteins were separated by gel electrophoresis and analyzed for label incorporation. Label was concentrated in the F_1 subunit, with little or no detectable label showing up with the F_2 subunit (Fig. 3).

Effect of Temperature. Sendai virus fusion with both biological and artificial membranes is temperature-dependent (14, 24). Similarly, F-protein labeling showed a strong temperature dependence (Fig. 4), consistent with earlier reports that fusion at neutral pH is related to a temperature-
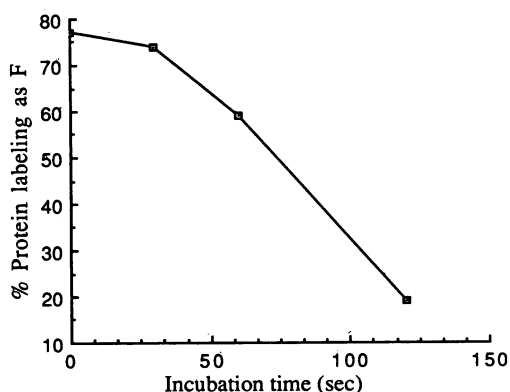

FIG. 1. Preferential association of F protein with cardiolipin vesicles during initial membrane interactions. Sendai virus (80 μg of viral protein) was added to 200 nmol of cardiolipin vesicles, containing TID, in a total volume of 1 ml. The mixture was incubated at 37°C in 150 mM NaCl/5 mM Hepes, pH 7.4, with a magnetic stirrer. After various incubation times, the samples were photolyzed for 30 sec. Protein label incorporation was determined by liquid scintillation counting of 2-mm gel slices after sodium dodecyl sulfate/PAGE. The labeling of F protein, as a percentage of total protein labeled, was calculated and plotted as a function of the incubation time before photolabeling.
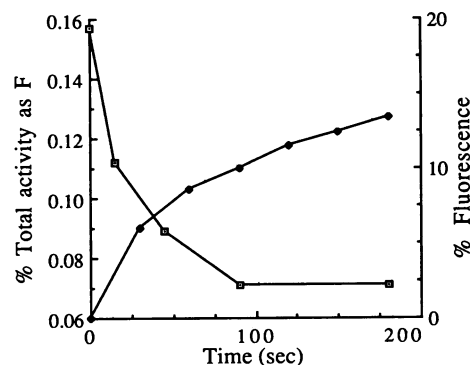

FIG. 2. Hydrophobic interaction of F protein with cardiolipin vesicles as a function of time. TID labeling was conducted during fusion of Sendai virus (80 μg of protein) and 200 nmol of cardiolipin vesicles at 37°C. Labeling of F protein as a percentage of total sample activity (□) was calculated and plotted as a function of time before photolabeling. Data are compared with the kinetics of fusion (% fluorescence) of Sendai virus and cardiolipin vesicles (●) as determined by the R_{18} lipid-mixing assay (18) for fusion. The kinetics measurements were carried out in a parallel experiment under otherwise identical conditions as the photolabeling experiments.
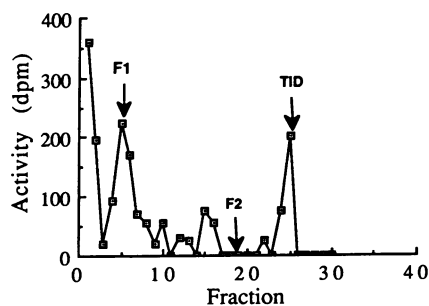
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FIG. 3. F_1 vs. F_2 labeling. Sendai virus and cardiolipin vesicles were mixed under conditions as described in the legend to Fig. 2. Photolysis was conducted as described in *Methods*. Proteins were separated by PAGE and label incorporation was determined by γ counting of gel slices.

dependent increase in rotational mobility of the F protein (24, 25). The temperature dependence of F labeling corresponds closely to the initial rates of fusion (14). An interesting and perhaps significant observation is that under conditions in which fusion does not occur (2°C), substantial labeling of the viral binding protein, HN, was observed (Fig. 4). This suggests that in addition to electrostatic interactions, hydrophobic interaction between HN and the target membrane may be important in viral attachment. HN labeling decreased with increasing temperature, as F protein–liposome hydrophobic interactions increased. The increase in F labeling and the concomitant increase in the initial fusion rate with temperature (Fig. 4 *Inset*) further support the view that hydrophobic interaction of F with the target membrane represents the ultimate trigger of viral fusion activity.

At present, we assume that after the initial penetration of the hydrophobic F_1 NH_2 terminus, subsequent randomization of viral and TID-labeled liposomal membranes during fusion causes redistribution of the label. This also causes TID

to hydrophobically interact with viral proteins other than the membrane glycoproteins. These other proteins are not believed to play critical roles in membrane fusion, but chemical evidence has been found for close interaction of nucleocapsid and matrix proteins with the viral membrane glycoproteins (26).

Effect of Lipid Composition of Target Vesicles and pH. Labeling of F protein during fusion of Sendai virus with cardiolipin vesicles was consistently higher than that observed during fusion with phosphatidylserine vesicles (data not shown). This observation is entirely consistent with the higher kinetics and extent of virus fusion with cardiolipin vesicles (14). Labeling of both F and HN showed similar temperature dependence for both vesicle types, with F labeling increasing and HN labeling decreasing with increasing temperature, corresponding to the amount of fusion occurring (Fig. 4). The F/HN labeling ratios give some indication, then, of the type of interaction occurring, in terms of fusion vs. attachment. With both cardiolipin and phosphatidylserine vesicles, F/HN labeling ratios were similar, indicating the same relative amounts of hydrophobic interaction of F and HN in the early stages of fusion. This is also suggestive of a common fusion mechanism, in spite of differences in the kinetics and extent of fusion (14).

In addition, F and HN labeling during fusion of Sendai virus with both vesicle types under various conditions of pH showed similar labeling ratios, further supporting a common fusion mechanism. The ratio of F/HN labeling at pH 7.4 was much higher than that seen at pH 5.0 for either vesicle type, in agreement with the earlier finding that fusion at low pH is mediated to a large extent by HN (14).

DISCUSSION

The experiments in the present study demonstrate the use of a hydrophobic photoaffinity probe for covalent labeling of
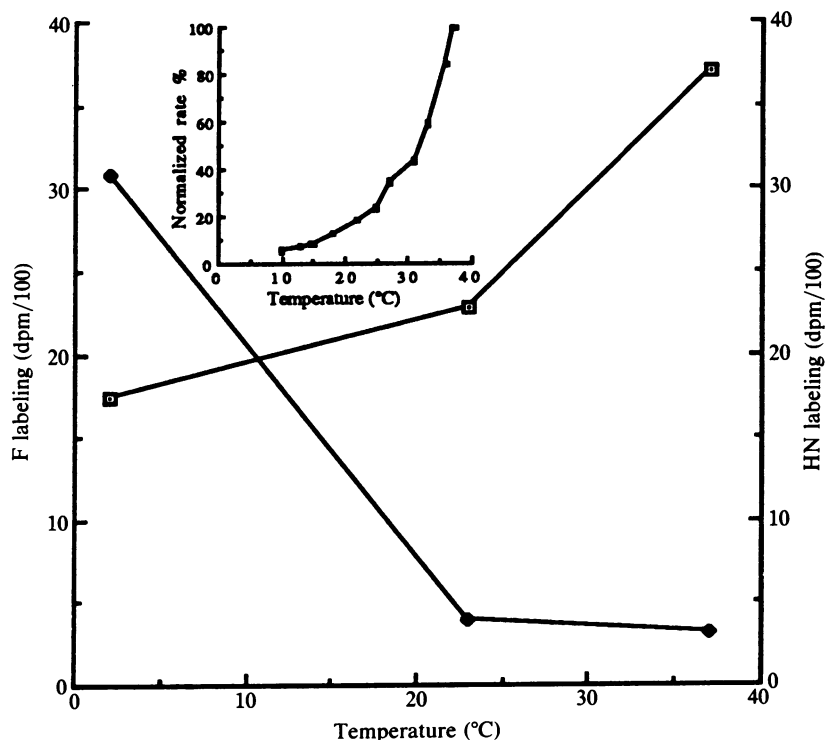

FIG. 4. Temperature dependence of F and HN labeling and initial rates of Sendai virus–cardiolipin vesicle fusion. TID labeling during fusion of Sendai virus (80 μg of protein) and 200 nmol of cardiolipin vesicles was conducted as described for Fig. 2. Labeling of F (□) and HN (●) were determined and plotted as a function of temperature. The temperature dependence of the initial rates of fusion is also shown (*Inset*). Initial fusion rates at various temperatures [$V_i(t)$] are normalized to the corresponding initial rate at 37°C [$V_i(37°C)$].

viral proteins that interact with the hydrophobic core of target membranes at the onset of membrane fusion. By limiting the time of irradiation and by commencing photolysis simultaneously with virus addition to liposomes, labeling during initial interactions can be isolated from that which might occur during subsequent events. The results provide strong, direct support for the hypothesis that the Sendai virus F_1 peptide mediates fusion at neutral pH by hydrophobic penetration into the target membrane (5, 6).

Although this method is clearly useful as a probe of the fusion mechanism, practical considerations dictate that the results obtained from this type of experiment be treated carefully. Hydrophobic probes such as TID partition with great preference into the inner core of membranes (9) but may diffuse out and bind to hydrophobic domains of proteins in the aqueous phase (23). This limitation imposes restrictions on the interpretation of absolute amounts of label incorporated, but relative labeling patterns of different proteins under a given set of conditions yield a profile of protein–lipid interactions consistent with existing models. To eliminate the possibility of viral protein labeling due to diffusion of probe outside the bilayer, an experiment in the presence of reducing agents such as glutathione and dithiothreitol would have been desirable (27). These agents inhibit labeling of the proteins by probe diffusion through the aqueous phase. Unfortunately, at the concentrations required, both compounds immediately inhibit the fusion activity of the virus (15). As a control, we therefore examined the extent of labeling of Sendai virus proteins upon incubation of the virus with "free" TID. The results showed a labeling pattern entirely different from that seen when the virus had interacted with membrane-inserted TID [in the former case, 28% of the label was associated with F, 14% with HN, and 57% (± 2%) with other viral proteins, independent of conditions]. Hence, in conjunction with the results in Fig. 1, showing an almost exclusive labeling of F under appropriate conditions, and those in Fig. 4, demonstrating a remarkably distinct labeling pattern as a function of temperature, we exclude the possibility that (at least during the early interactions), a significant contribution of the labeling occurred as a result of processes other than the penetration of viral proteins into the target membrane.

The present approach cannot provide insight into the depth of protein penetration into the target membrane (23). Such information could be obtained by using photoaffinity probes that are lipid-bound.

The strong preference of F labeling at early times during fusion (Fig. 1) provides conclusive evidence that the hydrophobic interaction between the F protein and the target membrane occurs prior to other interactions during fusion at neutral pH with cardiolipin vesicles and that this interaction constitutes the initiating event in fusion. This result thus provides direct chemical evidence supporting the hypothesis that viral fusion is initiated by hydrophobic interaction with the target membrane (5). The time dependence of F labeling during Sendai virus–cardiolipin vesicle fusion at neutral pH closely parallels the kinetics of fusion (Fig. 2 and ref. 14). This result lends further support for the occurrence of a hydrophobic penetration of F protein into the target membrane as a key event in the triggering of viral fusion activity. Although the exact location of the probe in the F protein remains to be identified, the result that TID labels the F_1 polypeptide specifically (Fig. 3) is highly suggestive for labeling of the hydrophobic NH_2 terminus. Such hydrophobic sequences have been found in a variety of virus families, leading to the proposal that penetration of these peptides into the target membrane may represent the universal trigger of viral fusion (3, 11). In fact, penetration of hydrophobic peptides into membranes at neutral or low pH may be a common theme in protein-induced fusion.

The temperature dependence of F labeling (Fig. 4) is also in agreement with requirements for increased protein rotational mobility during fusion at neutral pH (24, 25) and mirrors the temperature-dependent initial rates of fusion (ref. 14 and Fig. 4 *Inset*). The strong temperature dependence of HN labeling at neutral pH (Fig. 4) indicates that hydrophobic interactions, in addition to electrostatic interactions, may play a role in viral attachment by HN. We cannot at this point rigorously exclude the possibility that HN labeling at low temperature might be due to the transfer of probe to bound virus. However, the lack of HN labeling at elevated temperatures makes this seem an unlikely possibility.

The extrapolation of mechanistic studies of fusion with liposomes to biological membranes must be done cautiously, since evidence suggesting that liposomal membranes may not be suitable models for physiological membrane fusion has been obtained (14). Liposomal models are useful, however, for creating well-defined membranes in which particular structural elements may be isolated for study. With both cardiolipin and phosphatidylserine vesicles it has been demonstrated that at neutral pH, fusion of Sendai virus with such vesicles is largely dependent on F protein. The almost exclusive labeling of F during initial fusion events with these vesicles is consistent with this notion.

Labeling during fusion of virus with cardiolipin vesicles was higher than that seen during fusion with phosphatidylserine vesicles, consistent with the higher extent and faster kinetics of fusion observed for cardiolipin (14). The very similar F/HN labeling ratios observed under various conditions of temperature and pH suggest a common fusion mechanism for both vesicle types. Furthermore, the higher HN labeling relative to F observed at low pH supports the hypothesis that HN mediates fusion at pH 5.0 by a low-pH-induced conformational change allowing hydrophobic interaction with the target membrane (14, 28), and is consistent with the model for fusion mediated by water-soluble proteins at low pH (12, 29, 30).

These studies provide a means of obtaining direct chemical evidence leading to a structural and mechanistic understanding of the protein–lipid interactions that lead to membrane fusion. In addition to studies of fusion initiated by other viral and cellular proteins, continuing studies are underway to identify the fragments of fusion-initiating proteins which are labeled. Having set up the principle of the approach in a semi-artificial system, the following challenge is to evaluate this approach in a pure biological system. It is our contention that such studies will help in gaining an understanding of the molecular mechanism of viral fusion activity, as well as of peptide–lipid interactions in other biological systems that initiate fusion.

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