Probe Transfer with and without Membrane Fusion in a Fluorescence Fusion Assay†

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Received August 13, 1997; Revised Manuscript Received March 13, 1998

ABSTRACT: An analysis of the R_{18} fusion assay was made during the fusion of the Sendai virus with erythrocyte ghosts. The increase in R_{18} fluorescence, reflecting the interaction process, was evaluated in terms of the different processes that in principle may contribute to this increase, that is, monomeric probe transfer, hemifusion, and complete fusion. To this end, the kinetics of the R_{18}-labeled lipid mixing were compared to those obtained with an assay in which the fusion-monitoring probe, eosin-maleimide, was attached to the viral surface proteins. The experiments relied on the use of native and fusion-inactive viruses and studies involving viral and target membranes that were modified by the incorporation of the lysophospholipid. The total dequenching signal detected in the R_{18} assay consists of components from probe transferred without fusion and from fusion itself. At 37 °C, the initial rate of dequenching (within two minutes) was predominately from the probe diluted by fusion with little contribution from transfer. The dequenching signal due to the probe transfer without fusion occurred at temperatures as low as 10 °C and increased linearly with time. Complete fusion started at about 20−25 °C and increased sharply at 30 °C. The extent of hemifusion was deduced from the total R_{18} dequenching data and those of the eosin-maleimide labeled protein dilution method for the limiting cases; the analysis indicates that hemifusion started at about 15 °C and increased over the range 20−25 °C. The initial rate of dequenching of the R_{18} assay measured within 2 min gives an accurate measure of membrane fusion above 30 °C.

There are two major pathways by which enveloped viruses introduce their genome into target cells; (a) via exoplasmic fusion between the viral envelope and the target cell plasma membranes, and (b) via receptor-mediated endocytosis of virions and, subsequently, fusion of the viral envelope with the endosomal membrane (*1*, *2*). Sendai virus (SV) is known to use the former pathway. Fluorescence assays for studies of virus−cell fusion have made it possible to observe the dynamic behavior of the events in a quantitative manner. One of the most frequently used of these assays is the R_{18} dequenching assay (*3*). In this method, the fluorescence probe, octadecyl rhodamine B chloride (R_{18}), is incorporated into viral envelopes at self-quenching concentrations. Upon fusion of the viral envelopes with target cell membranes, the probe in the viral envelope is transferred to the target cell membrane. The resulting dilution of the probe leads to an increase in the emission signal (dequenching). By measuring this dequenching signal, one can calculate the rate and extent of fusion. This method is widely used because it is easy to incorporate the probe into biological membranes and the emission itself is stable and gives reproducible data. Therefore, many fusion studies dealing with biological membranes have used the R_{18} fusion assay (*3*−*8*).

However, it has been found that there is more than one molecular process mediating probe transfer between interacting membranes. There is transfer without changing the morphology of the interacting membranes (without membrane fusion), transfer due to hemifusion (joining of only the two outer monolayers of the interacting membranes), and transfer due to fusion (two membranes join together to form one membrane resulting in a complete morphological alteration). In fluorescence fusion assays using labeled lipid molecules, probe transfer has been detected with and without fusion (*9*−*12*). The first evidence of hemifusion was provided by experiments on virus−cell fusion (*13*), and further studies using a virus-planar lipid bilayer and lipid vesicle-planar lipid bilayer systems (*14*, *15*) have provided detailed molecular insight into the relevance of hemifusion in the overall process of membrane merging. In the R_{18} fusion assay, like in any assay based upon lipid mixing, all of these events will contribute to the overall signal that results in the occurrence of membrane mixing.

In this paper, we examined how much of the dequenching signal in the R_{18} fusion assay is due to simple probe transfer, to hemifusion, and to the complete fusion process. We intend to assess the validity of the assay and to establish criteria for its proper utilization. For the studies, we used the Sendai virus-erythrocyte ghost fusion system employing two fluorophores, the R_{18} probe incorporated into the viral lipid envelope, and an eosin-5-maleimide probe attached to the viral surface proteins, with and without modification of virus and target cell membranes. We also used a lipid vesicle

† This work was partly supported by a grant (T.D.F. and S.O.) of Multi-disciplinary project program from SUNY at Buffalo.

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S0006-2960(97)02016-3 CCC: $15.00
Published on Web 05/02/1998

fusion system with a content mixing fluorescence assay in order to confirm the results obtained with the probe-dilution fusion assays.

EXPERIMENTAL METHODS AND PROCEDURES

Chemicals. Fluorescence probes, R_{18} and eosin-5-maleimide (EML), were obtained from Molecular Probes Inc., and terbium chloride hexahydrate ($TbCl_3 \cdot 6H_2O$) was purchased from Alfa Chemical Co. Dipicolinic acid (pyridine-2,6-dicarboxylic acid (DPA)), trypsin (from bovine pancreas), trypsin inhibitor (from soybean), and tris(hydroxymethyl)-aminomethane (Tris) were purchased from Sigma Chemical Co. Hepes (N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid) was from Calbiochem. Co. Sephadex G-75 and Sepharose CL-2B were purchased from Pharmacia Fine Chemicals. Both phosphatidylserine (PS) (bovine) and lysophosphatidylcholine (1-stearoyl-2-hydroxy glycerol-3-phosphocholine (SLPC)) were obtained from Avanti Polar Lipids, Inc. Bio-Rad protein assay dye and gamma-globulin used as a protein standard were from Bio-Rad Co. All other chemicals were of reagent grade from Fisher Chemical Co.

Virus. Sendai virus (SV) (Cantell strain) was grown for 72 h in 10-day-old embryonated chicken eggs. The virus was purified from allantoic fluid by differential centrifugation according to published procedures (*16*, *17*) and stored at −70 °C in Tris-buffered saline (0.15 M NaCl/10 mM Tris/1 mM EDTA, pH 7.4, NTE). The concentration of the virus was normally about 1 mg/mL, which was determined by a dye-binding protein assay (*18*). The hemagglutinating titer (HA) of the preparations was usually 5000−10000 (hemagglutinating units/0.1 mL).

Erythrocyte Ghosts (EG). Human red blood cell (type A^+) ghosts from a single donor were prepared immediately after the collection of blood according to published procedures (*19*, *20*). EG were suspended in 130 mM KCl/20 mM NaCl/10 mM phosphate buffer, pH 8.0 (KNP) at 2 mg/mL and the ghost stock suspension was stored (4 °C) until used.

Incorporation of R_{18} into the Viral Membrane. An ethanolic solution of R_{18} was injected, during vortexing, into 1 mL of virus suspension (1 mg/mL of 0.15 M NaCl/10 mM phosphate/pH 7.4 (NPB)). The final concentrations of the probe and ethanol were 5 μM and 0.5% (v/v), respectively. However, when a smaller volume of virus suspension was used, a smaller volume of the ethanolic solution was added to keep the respective ratios of the probe to virus and the volume of ethanol to the suspension the same as above. The suspension was left at room temperature for 2 h. Unbound probe was removed by chromatography on a Sephadex G-75 column. The rest of the procedure was the same as previously described (*4*). These labeled preparations were designated SV(R_{18}).

Labeling of Virus Proteins with Eosin-5-maleimide (EML). Labeling of Sendai virus proteins with EML was done essentially as described by Hoekstra et al. (*21*). Probe, one tenth of the weight of the protein, was added to the virus suspension in NPB, and the mixture was incubated for 2 h at room temperature. The unbound probe was removed by chromatography on Sephadex G-75. These viruses were designated SV(EML). It should be noted that the hemolytic and hemagglutination activity of the EML-labeled virus did not differ from that of the unlabeled virus (not shown, cf. ref *21*), thus excluding artifacts resulting from the labeling procedure.

Trypsin-Treated Virus. Trypsin treatment was carried out by incubating 0.5 mg of virus with 20 μg of trypsin (4% (w/w) with respect to viral protein) for 20 min at 37 °C. The reaction was terminated by adding a 2-fold excess of trypsin inhibitor (from soybean, Sigma Chemical Co.). Then the virus suspension was diluted 4-fold with NTB and centrifuged at 15000*g* for 30 min, and the pellet was resuspended in 0.5 mL of NTB. The results of the trypsin treatment were evaluated by SDS−PAGE with silver stain (*22*). For the trypsin-treated virus, the HN band appeared unchanged but the F band was considerably reduced. The HA titer of trypsin-treated virus was reduced about 1- or 2-fold (e.g., from 5120 to 2560) while the fusion activity was abolished. These virus preparations were designated Try-SV.

Lysophospholipid (LPC) Incorporation into Virus and Erythrocyte Ghost Membranes. A 0.5% (w/w) aqueous dispersion of stearoyl-lysophosphatidylcholine (SLPC) was vortexed intensively until clear. A small amount of the dispersed LPC was added to 1.0 mL of virus suspension (0.5 mg/mL) or to an erythrocyte ghost suspension (0.5 mg/mL), respectively, so that the concentration of LPC was 40 μM. The suspensions were incubated for 10 min at room temperature, and the unbound LPC was removed by centrifugation, 15000*g* for 30 min for the virus and 8000*g* for 10 min for the erythrocyte ghosts. The pellets were resuspended in 0.5 mL of NTB. The dequenching of R_{18} brought about by the incorporation of LPC into the virus increased from 7% (that of LPC-unincorporated virus) to 12.5%. These virus and erythrocyte ghost preparations were designated LPC-SV and LPC-EG, respectively. LPC incorporation into lipid vesicles was approximately the same as that for the virus preparation.

Binding of Virus to Erythrocyte Ghosts. Aliquots of R_{18}-labeled Sendai virus (20 μg) and EG (80 μg) were suspended in 0.1 mL of NPB at 4 °C and incubated for 5 min. Then, the suspension was centrifuged at 8000*g* for 10 min at 4 °C. The ratio of bound to unbound virus was determined by measuring the respective dequenching of fluorescence by the addition of Triton X-100 to both the supernatant and the pellet suspensions of equal volume.

R_{18} Fusion Assay. The fusion of the Sendai virus with erythrocyte ghosts was monitored by the R_{18} dequenching assay (*3*). The assay is based on the relief of self-quenching of the probe which, when diluted into the target membrane as a result of fusion and/or probe transfer, gives rise to an increase in the fluorescence emission signal. R_{18} fluorescence in labeled virus, SV(R_{18}), was about 93% quenched. In the assay, an aliquot of the R_{18}-labeled virus (10 μg of virus protein) and an aliquot of erythrocyte ghosts (40 μg of protein) were suspended in 2.0 mL of NPB prewarmed to the reaction temperature (10−40 °C). The increase in the fluorescence signal at $\lambda_{emission} = 585$ nm ($\lambda_{excitation} = 550$ nm) resulting from dequenching was continuously recorded on a strip chart recorder with a spectrofluorimeter (Perkin-Elmer, LS-5). To quantify the probe dilution during virus−cell interaction, the initial fluorescence signal of R_{18}-labeled I_0 was measured ($\lambda_{excitation} = 550$ nm, $\lambda_{emission} = 585$ nm). Then the time-dependent dequenching fluorescence signal (I) was

read similarly at 585 nm, 10 min after starting the experiment. At the end of each experiment, Triton X-100 (0.5% final concentration) was added to the reaction mixture, and the maximum value (I_{tri}) of the dequenched fluorophore signal was obtained. This value, corrected for probe dilution, was considered as 100% fusion and/or probe transfer from the virus to the target cells. The extent of fusion and/or probe transfer is proportional to percent fluorescence dequenching and can thus be defined as

$$F_D = 100(I - I_0)/(I_{tri} - I_0) \quad (1)$$

EML (Eosin-5-maleimide) Fusion Assay. The dequenching signal of EML bound to virus proteins was measured at 548 nm with the spectrofluorimeter by exciting the probe at 505 nm. Ten micrograms of EML-labeled virus (SV(EML)) and 40 μg of EG were mixed in 2 mL of NPB prewarmed to a specified temperature (10−40 °C). The dequenching signal was then continuously measured over time, in a manner similar to that described above for the R_{18} dequenching assay. On the basis of the observed dequenching signals, the extent of fusion was analyzed in two different ways. In one approach, the percent of dequenching of the EML probe was calculated from the ratio of fluorescence measured before and after the addition of Triton X-100, identical to the procedure used for the R_{18} fusion assay (cf Figure 2 and Figure 4d). In a second approach, we correlated the percent dequenching in the EML assay to the percent dequenching obtained for the R_{18} assay corrected for the simple transfer of probe due to nonfusion. With the former calibration, one can obtain the underestimated values for the extent of fusion, and the latter calibration would give the upper limit of the fusion extent.

Lipid Vesicles. Large unilamellar phosphatidylserine vesicles (PS-LUV), which contained either Tb or DPA, were prepared according to published methods (*23*). The vesicles were passed through a Sepharose BL-2C column to fractionate them into different sizes. The size of vesicles in the eluted fractions was determined with a submicron particle analyzer (Coulter N4). Tb/PS vesicles with an average diameter of 150 nm and DPA/PS vesicles with an average diameter of 180 nm were used in the experiments. The incorporation of SLPC into these two types of vesicles was done in a way similar to that for virus and erythrocyte ghosts: LPC was added to 1.0 mL of vesicle suspension (0.5 mg of phospholipid/mL) at a final LPC concentration of 20 μM and 40 μM, respectively. After an incubation for 10 min at room temperature, the vesicles were passed over a Sephadex G-75 column to remove unincorporated LPC. The lipid concentration of the vesicles was determined by a phosphorus assay (*24*).

Lipid Vesicle Fusion. Fusion of lipid vesicles induced by divalent cations was followed by the Tb/DPA content mixing fusion assay (*25, 26*). Briefly, equal amounts of Tb- and DPA-containing lipid vesicles were suspended in 0.1 M NaCl/10 mM Hepes/0.1 mM EDTA, pH 7.4 at room temperature. As the concentration of Ca^{2+} was raised to about 2 mM, fusion of the vesicles occurred (*23, 27*). The fluorescence intensity increase, due to mixing of Tb^{3+} and DPA^{2-} upon the fusion of the two different vesicle populations, was monitored with a fluorimeter (LS5, Perkin-Elmer) at 495 nm, while exciting the fluorophore at 279 nm. The

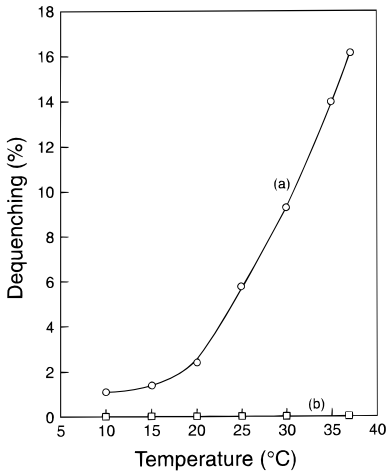

FIGURE 1: The extent of fluorescence dequenching of R_{18} incorporated into the Sendai virus ($SV(R_{18})$) envelope in the cases where the virus is incubated with and without erythrocyte ghosts (EG) in NPB solution at various temperatures. The percent of dequenching was calculated with use of eq 1 for the increases in fluorescence dequenching during 10 min after suspending the samples in NPB, prewarmed at a specified temperature. Experimental error was within 10%: (a, ○), $SV(R_{18})$ (10 μg) + EG (40 μg) in 2.0 mL of NPB; (b, □), $SV(R_{18})$ (10 μg) alone in 2.0 mL of NPB.

value for 100% fusion was determined in the presence of 30 μM dipicolinic acid, after the release of the contents from an amount (in lipid) of Tb-PS vesicles equal to that used in the fusion experiment, with 0.5% (w/w) sodium cholate. The vesicle fusion experiments were performed at room temperature.

EXPERIMENTAL RESULTS

When R_{18}-labeled Sendai virus and erythrocyte ghosts are suspended in a buffer, the R_{18} fluorescence signal gradually increases as a function of time, the rate of which depends on the incubation temperature. Figure 1 shows the % dequenching, plotted as a function of temperature, as obtained after an incubation period of virus and ghosts of 10 min. The percent of fusion/dequenching was calculated, using eq 1. A characteristic feature of the temperature-dependent probe dequenching, occurring upon Sendai virus-ghost interaction, is that, at the lower temperature range, a relatively low dequenching and only a small increase in its rate is seen, while the magnitude of the change in dequenching substantially increases above a certain threshold temperature (Figure 1a). Indeed, only at around 20 °C did the dequenching rate increase rapidly with increasing temperature. This rapid increase has been considered a consequence of dilution of the probe as a result of fusion between the viral envelope and the target cell membrane. Note that the control of R_{18}-labeled virus alone (i.e., no target cells) shows no increase in the dequenching signal (Figure 1b).

Figure 2 shows the results of a similar experiment in which, at otherwise identical conditions, fusion of the virus was determined by probe dequenching that relied on the dilution of the fluorophore EML that had been attached to the viral membrane proteins (*21*). In this case also, the dequenching signal of the probe was observed at all temperatures tested and increased steadily with time (data not shown). In Figure 2, the increase in fluorescence dequenching measured at 10 min after SV(EML) and EG or

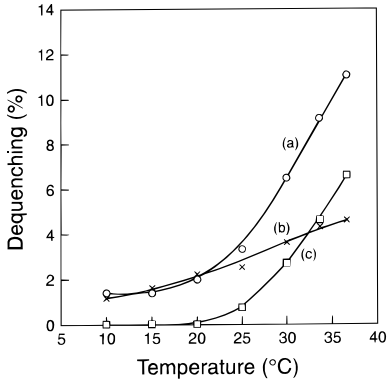

FIGURE 2: The extent of dequenching of eosin-5-maleimide (EML) attached to the Sendai virus surface proteins (SV(EML)) in the case where the virus is incubated with or without EG in NPB solution at various temperatures. The percent of dequenching was calculated in the same manner as with the R_{18} assay, using eq 1, for the increases in fluorescence dequenching during 10 min after these samples were suspended in NPB prewarmed at a specified temperature. Experimental error was within 10%: (a, ○), SV(EML) (10 μg) + EG (40 μg) in 2.0 mL of NPB; (b, ×), SV(EML) (10 μg) in 2.0 mL of NPB; (c, □), a − b.

SV(EML) alone were suspended in buffer at a specified temperature is plotted as a function of temperature. The change in the fluorescence signal increased in magnitude in relation to the temperature for the labeled virus both alone (Figure 2b) and when mixed with target cells (Figure 2a). The subtracted value plot (Figure 2c) resembles the increase in the R_{18} assay, except that the rate increases at a higher temperature, that is, the threshold temperature has shifted to a higher temperature. Since viral membrane proteins (HN and F) do not likely transfer to the target cell membranes without complete fusion, the dequenching signal arising from the probe transfer from the viral envelope to the EG membranes is a consequence of membrane fusion. The observation of the fluorescence signal increase in the case of the EML-labeled virus alone (Figure 2c) was different from that observed for the case of the R_{18}-labeled virus alone (Figure 1a). In the latter case, no increase in dequenching was observed at any temperature. A similar observation with the EML-labeled Sendai virus alone was made by Hoekstra et al. (*21*). They interpreted the dequenching to occur as a result of the disaggregation of virus surface proteins with increasing temperature.

Lipid dilution, as monitored by the R_{18} assay, obtained for LPC-SV(R_{18}) and Try-SV(R_{18}) with EG as target membranes, is shown in Figure 3. The data show a significant reduction of dequenching when compared to the control (Figure 3a). The signals increased with time as well as temperature, but in both cases (Figure 3b, c), the treated viral particles displayed very similar activities, as a function of both time and temperature, and the increase in dequenching was about 4% of the maximum dequenching over the initial 10 min at 37 °C. Since virus treated with LPC has been shown not to fuse with target cells (*28* and see below), the observed dequenching signal is considered the result of the transfer of the probe without fusion. It is relevant to note here that the LPC-incorporated virus had the same HA titer as that of the native virus, but the trypsin (4% (w/w)) treated virus had an HA titer that was 1−2-fold lower (2500−5000). The LPC-treated virus had approximately the same binding ability as the native virus with erythrocyte

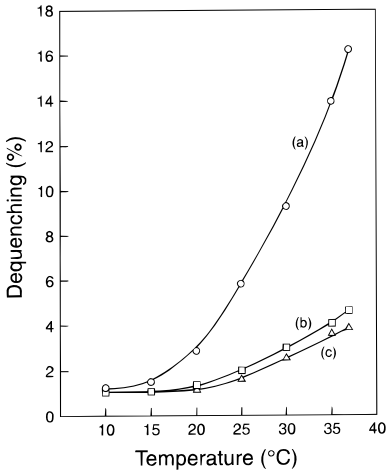

FIGURE 3: The extent of dequenching of the R_{18}-incorporated Sendai virus SV(R_{18}) or the modified SV(R_{18}) upon interaction of the virus with EG or LPC-EG in NPB at various temperatures. The extent of dequenching plotted represents the increase in dequenching signals during 10 min after suspending the samples in NPB at each temperature. Modified viruses were the trypsin-treated Sendai virus (Try-SV(R_{18})) or the LPC-incorporated Sendai virus (LPC-SV(R_{18})). Experimental error was within 10%: (a, ○), SV(R_{18}) (10 μg) + EG (40 μg) in 2.0 mL of NPB; (b, □), LPC-SV(R_{18}) (10 μg) + LPC-EG (40 μg) in 2.0 mL of NPB; (c, △), Try-SV(R_{18}) (10 μg) + EG (40 μg) in 2.0 mL of NPB.

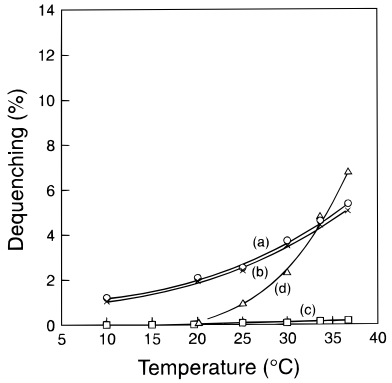

FIGURE 4: The extent of dequenching of the EML-labeled Sendai virus, with or without the incorporation of LPC, upon interaction of the virus with LPC-incorporated erythrocyte ghosts (LPC-EG) in NPB at various temperatures. The percent of dequenching plotted was calculated in the same way as that in Figure 2 for the increase in dequenching fluorescence during 10 min after the samples were suspended in NPB at each temperature. Experimental error was within 10%: (a, ○), LPC-SV(EML) (10 μg) + LPC-EG (40 μg) in 2.0 mL of NPB; (b, ×), LPC-SV(EML) (10 μg) in 2.0 mL of NPB; (c, □), a − b; (d, △), SV(EML) (10 μg) + EG (40 μg) in 2.0 mL of NPB (same as Figure 2c).

ghosts; the trypsin-treated virus showed only a slightly lower binding capacity (approximately 10%) than the native one.

The results with the EML fusion assay using LPC-SV(EML) and LPC-EG are shown in Figure 4. Figure 4a shows the fluorescence dequenching reached, at 10 min after LPC-EG (40 μg/mL) and LPC-SV(EML) (10 μg/mL) were suspended in NPB, prewarmed at the specified temperature. Also in this case, the dequenching signal increased with time at a given temperature (data not shown) and similarly increased as a function of temperature per se. Experiments with LPC-SV(EML) alone are shown in Figure 4b. There was practically no difference in the dequenching signal between the target cell and the virus, and the virus alone. This suggests that no transfer of the probe occurred from

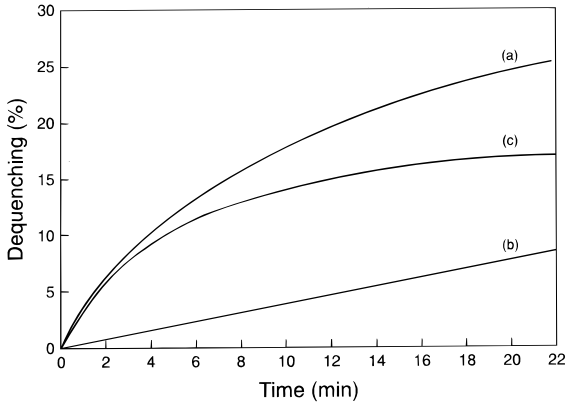

FIGURE 5: The time courses of R_{18} dequenching upon interaction of (a) the R_{18}-labeled native Sendai virus and (b) the R_{18}-labeled LPC virus with erythrocyte ghosts at 37 °C. Experimental error was within 10%: (a), SV(R_{18}) (10 μg) + EG (40 μG) in 2.0 mL of NPB; (b), LPC-SV(R_{18}) (10 μg) + LPC-EG (40 μg) in 2.0 mL of NPB; (c), a − b.

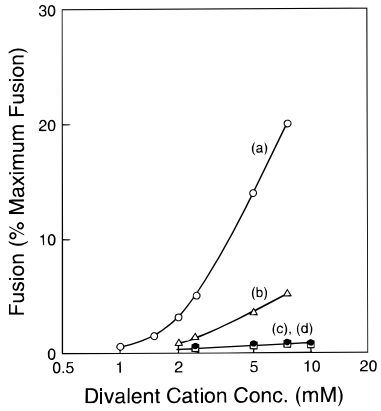

FIGURE 6: Fusion of the large unilamellar phosphatidylserine vesicle (PS-LUV) as a function of divalent cation concentrations: (a and d), two kinds of PS-LUVs (0.05 μmol lipid of 150-nm diameter vesicles which contained 5 mM Tb/100 mM NaCl and 0.05 μmol of lipid of 180-nm diameter vesicles, in which 80 mM DPA was encapsulated) were suspended in 2 mL of 0.1 M NaCl/10 mM Hepes (NHB), pH 7.3; (b), the above-mentioned LUVs, containing SLPC (vesicles were incubated in NHB containing 20 μM SLPC); (c), the same as in b but incubated in NHB containing 40 μM SLPC. Fusion, induced upon the addition of the divalent cations ((a, b, and c), Ca^{2+}; (d), Mg^{2+}), was monitored by the Tb/DPA assay as described in Experimental Methods and Procedures. The extent of vesicle fusion (F) was calculated, and plotted versus the cation concentration, using the following formula: $F = 100(I - I_0)/(I_{tot} - I_0)$ (eq 2), where I is the Tb fluorescence measured at 2 min after the addition of the divalent cation into the vesicle suspension, I_0 is the initial signal of the vesicle suspension before the addition of the divalent ion, and I_{tot} is the total fluorescence signal after the addition of Na-cholate into the vesicle suspension.

virus to erythrocyte ghost membranes, and therefore, there was no fusion in this system. Curve c in Figure 4 represents the difference in dequenching signals between curves a and b in this figure. Figure 4d shows the positive control of SV(EML) with erythrocyte ghosts (same as Figure 2c).

Figure 5 illustrates the time course of lipid mixing, as obtained with SV(R_{18}) and EG (a), and with LPC-SV(R_{18}) and LPC-EG (b), respectively, suspended in NPB at 37 °C. The SV(R_{18}) interacting with EG showed an initial sharp increase in signal and the rate of increase declined with time (Figure 5a), while the interaction of the lysoPC-treated virus with lysoPC-treated erythrocyte ghosts shows approximately a linear increase with time for a longer period (e.g., up to 20 min), as shown in Figure 5b. Although less steeply, the increase continued for several hours (data not shown). Subtraction of these values (Figure 5b) from those obtained for mixing at control conditions (Figure 5a) gives Figure 5c, which should correspond to the time course of dequenching due to the transfer of the probe by hemifusion and/or by complete fusion. The dequenching signal sharply increases with time within the first few minutes and approaches nearly to a limiting value within 20 min.

To verify the effectiveness of LPC in inhibiting fusion under the present conditions, its effect on a vesicular system (PS vesicles) was examined, using a content-mixing assay. Figure 6 shows cation-induced fusion between PS-LUVs and between LPC-incorporated PS-LUVs as a function of the Ca^{2+} and Mg^{2+} concentrations. The extent of fusion of the vesicles reached two minutes after the addition of the divalent cations to the vesicle suspension is plotted versus the concentration of the divalent cation. The extent of fusion of PS-LUV increased sharply at approximately 2 mM Ca^{2+} (a) but no vesicle fusion was observed up to 10 mM Mg^{2+} (d). These results correspond to the results obtained by earlier workers (*23*, *27*). For the PS vesicles incubated with 20 μM LPC, the extent of vesicle fusion was suppressed considerably at comparable cation concentrations, leaving a residual activity of approximately 25%. However, the threshold for fusion was about the same, that is 2 mM Ca^{2+} (Figure 5b). For the PS vesicles incubated with 40 μM LPC, vesicle fusion was almost completely suppressed, as shown in Figure 5c. Note that the latter conditions were used when examining the effect of LPC on virus-erythrocyte ghost fusion, as described above (Figures 4 and 5).

DISCUSSION

The present work revealed that, by monitoring the fusion of the Sendai virus with erythrocyte membranes by lipid mixing and by (viral) protein dilution, in conjunction with modification of the fusion membranes with lysoPC, detailed insight can be obtained on the relative contribution of lipid transfer, hemifusion, and complete fusion in the overall merging event. The results obtained with the EML fusion assay for the Sendai virus-erythrocyte ghost fusion system, as shown in Figure 4, indicate that no fusion occurred between LPC-SV and LPC-EG, since the same dequenching signals were obtained for LPC-SV(EML) alone, and for the mixture of LPC-SV(EML) and LPC-EG(EML). The increase in the dequenching signal with temperature for LPC-SV(EML) alone is presumably due to a temperature-mediated dissociation of clustered viral surface proteins as suggested by Hoekstra et al. (*21*). The fluorescence curve originating from the difference in dequenching signals observed by the EML fusion assay, when comparing SV(EML) interacting with EG (Figure 2a) and SV(EML) alone (Figure 2b), should then correspond to the fusion of the virus with erythrocyte ghosts, since viral envelope proteins do not likely transfer without complete membrane fusion. It is seen from Figure 2c that significant fusion of SV with EG does not occur below 20 °C and starts to increase at about 25 °C. Fusion sharply increases above 30 °C. It should be noted that although the relative kinetics of fusion are appropriately reflected by EML dilution, an exact estimation of the extent

of fusion is difficult to determine from the observed signal, since the viral proteins may not completely dissociate after they are transferred to the target cell membrane. As demonstrated before (*29*), after integration into the target membrane, the lateral diffusion rate of fluorescently tagged Sendai virus proteins, prepared in a very similar manner as in the present work, is of the same order of magnitude as the diffusion rate of protein monomers, while the recovery after photobleaching is essentially complete. Accordingly, at these conditions, the lateral mobility of the potentially clustered proteins appears not to be significantly different from that of the monomers. Thus, the rate of protein dilution is not rate-limiting in revealing the fusion of the protein-labeled virus. However, at the same time, this notion concerning potential protein clustering in the target membrane could imply that the extent of fusion may be underestimated. Yet, as described below, the results can be readily discussed in terms of upper and lower limits of the extent of fusion.

It has been shown that the insertion of lysophospholipid into artificial or biological membranes inhibits membrane fusion (*30*−*33*) including hemifusion (*15*). In Figure 6, it is also shown that fusion in a well-established system (PS vesicle fusion induced by Ca^{2+}) was indeed inhibited by the insertion of lysophospholipid into the membranes. Similar experimental conditions were used for LPC incorporation into the virus and the erythrocyte ghost membranes (Figures 4 and 5). Furthermore, in a variety of other studies, including our own, an inhibition of Sendai virus fusion by more than 90% is usually observed upon trypsin treatment (*4*, *30*). Similarly, more than 90% inhibition of fusion has been reported for viral systems (other than Sendai virus), after LPC treatment, using the R_{18} assay (*28*). Hence, it is very reasonable to assume that also under the present conditions of the incorporation of LPC into the membranes, virus fusion was effectively inhibited, that is, without LPC per se causing significant monomeric transfer (cf. *28*, *32*). Thus, we conclude that the dequenching signals shown in Figure 3b are due to probe transfer from the virus to the erythrocyte ghosts without fusion. A similar situation is seen where trypsin-treated virus was used (Figure 3c). The dequenching rate for Try-SV with EG was slightly less than that of LPC-SV interacting with EG. This may be due to a reduced ability for Try-SV to bind to EG, as mentioned before.

Here, we propose an analysis of the total dequenching signal obtained 10 min after mixing SV and EG, described in terms of the contribution of the probe transfer without fusion (Figure 3b), that of complete membrane fusion (Figure 2c), and that of hemifusion. The analysis with the use of the lower limiting value for the extent of fusion is the following: subtract the probe transfer without fusion (Figure 3b or Figure 7A(c)) and that due to complete membrane fusion (Figure 2c or Figure 7A(b)) from the total dequenching signal (Figure 1a or Figure 7A(a)). This will lead to the determination of the extent of probe transfer, reflecting hemifusion; thus, [probe transfer due to hemifusion] = [total dequenching signal]−[probe transfer without fusion]−[probe transfer due to complete membrane fusion]. Hence, the resultant difference shown as Figure 7A(d) should be due to the signal resulting from the probe transferred due to the hemifusion process. In this analysis, the extent of complete fusion is *underestimated*, because it may well be that not all

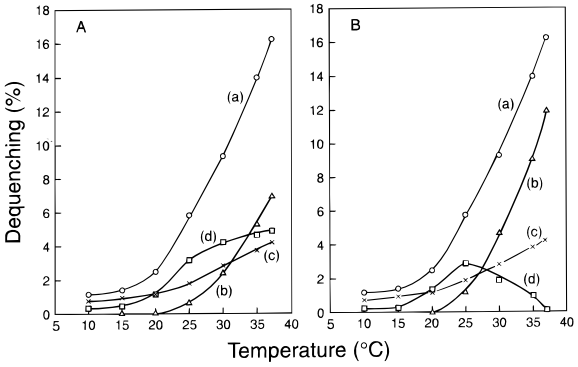

FIGURE 7: Part A: an analysis of the total R_{18} dequenching signal (a) for the Sendai virus/erythrocyte ghost interacting system in terms of signals contributed by probe transfer upon complete fusion (b), those from nonfusion (c), and those from hemifusion (d) [curve 7A(d) = curve 7A(a) − curve 7A(c) − curve 7A(b)]. Here, complete fusion was calculated using eq 1, as shown in Figure 3. Note that the extent of complete fusion is underestimated (see text). Part B: an alternative analysis of the same total dequenching signal (a) in terms of signals contributed by probe transfer as a result of complete fusion (b), by nonfusion (c), and by hemifusion (d) [curve 7B(d) = curve 7B(a) − curve 7B(c) − curve 7B(b)]. Here, the dequenching signal due to complete fusion as shown in Figure 2 was recalibrated by assuming that there was no hemifusion signal contributed by the R_{18} dequenching signals obtained at 37 °C; that is, a contribution due to complete fusion and probe transfer without fusion. The extent of complete fusion shown in part B(b) represents, therefore, the upper limiting value (see text).

the viral membrane proteins are completely dispersed after their transfer to the target cell by fusion. An alternative analysis assumes that the difference between the total dequenching signal and that due to nonfusion processes observed at 37 °C corresponds to probe transfer, which is exclusively due to complete fusion. This assumption may be reasonable for the experiment done at a higher temperature, such as 37 °C, since the complete fusion process is expected to be dominant over the hemifusion process in this temperature range. The use of such a calibration gives an *upper limit* of the fusion extent. The extent of complete fusion derived from such a calibration for the EML assay as given in Figure 2c, is shown in Figure 7B(b). In this case, the dequenching signal due to hemifusion becomes that shown in Figure 7B(d). From these analyses, the following conclusion can be reached regarding the R_{18} fluorescence fusion assay. When the virus is able to bind to the target membrane, probe transfer without fusion may occur, the extent of which will depend on the temperature and the incubation time. At low temperatures, there is less transfer than at higher temperatures. The kinetics of probe transfer increases approximately linearly with time (within an hour). Such transfer results in approximately 4% of maximal dequenching at 37 °C in 10 min.

The threshold temperature for the fusion of the virus with erythrocyte membranes is centered around 25 °C and fusion increases sharply at about 30 °C. Still, membrane lipid transfer due to hemifusion seems to occur at temperatures as low as the temperature where virus binds with the target cells. However, the magnitude is small. The hemifusion (i.e., fusion of the outer lipid monolayers) increases in particular in the temperature range 20−25 °C. A decrease of its contribution is seen above 30 °C, when complete membrane merging becomes dominant. Importantly, this general tendency is held for the two extreme cases of lower

and upper limits of the extent of fusion. The present analysis shows that under the conditions where most experiments were done (i.e., 10 min at 37 °C), approximately 50% of the dequenching signal was due to complete fusion between the virus and the target cells, 25% of the signal was due to probe transfer without fusion, and 25% was attributed to the signal of the hemifusion process, with use of the lower limit of complete fusion. When applying the upper limit approach, the signal originates for 75% from complete fusion, 25% from transfer of the probe, and none from hemifusion. The actual percentages for complete fusion and hemifusion lie somewhere between these two extreme cases.

The analysis as presented in the present work is important because many results which show about 4−5% dequenching of R_{18} with respect to the total dequenching could be due to simple probe transfer and not to membrane fusion. In addition to LPC treatment and trypsin treatment, there are several other situations in which the Sendai virus fusion is effectively blocked, yet probe transfer can be observed. (1) The inactive virus (high HA activity yet noninfectious) showed approximately 4% dequenching after 10 min at 37 °C. (2) Similarly, when the active virus was tested against 0.5% (w/w) glutaraldehyde-treated erythrocyte ghosts, there was approximately 4% dequenching observed after 10 min at 37 °C. However, it should be noted that the present analysis was made by determining the dequenching signal over the initial 10-min incubation period, after the mixing of the samples. As shown in Figure 5, the fusion events take place relatively rapidly, compared with probe transfer. From the analysis of the time courses for dequenching due to fusion (Figure 5c) and that due to probe transfer (Figure 5b), the relaxation time of the transfer process is about 10-fold greater for simple probe transfer ($k_2 = 40$ min) than that ($k_1 = 4$ min) for fusion. We can derive the relaxation time for each process by fitting two exponential curves, Figure 5, parts b and c, to eq 2 (see Appendix). Therefore, if the initial rate of the dequenching signal observed within a shorter time (e.g., 2 min) is analyzed, such analysis gives a good measure for the extent of fusion, since, over this early time interval, the predominant portion of the dequenching signal arises from the process of fusion and the contribution from simple probe transfer will be small. This conclusion is also supported by experiments in which the fusion of the influenza virus with erythrocyte ghosts was monitored with two different fluorescence fusion assays using R_{18} on one hand and a resonance energy-transfer method on the other hand (*5*). However, the rate of the dequenching signal in the R_{18} assay measured at a later time (e.g., several minutes) for a fusion system similar to that above was slightly greater than that obtained by the fluorescence energy-transfer method (*34*). While the process of fusion may saturate within a short time, simple probe transfer continues and increases with time. Therefore, depending on the time when the measurement is made, the contributing factors from each process are different. Cobaleda et al. (*12*) analyzed the dequenching curve of the R_{18} assay for the Newcastle virus-erythrocyte system and found that the total dequenching curve can be fitted well with two exponential curves, one displaying a shorter relaxation time and the other a longer one. They interpreted the former due to fusion and the latter due to probe transfer. The overall trend reported in that study compares well with the present results.

However, when carrying out a similar analysis of the data for the case in which the magnitudes of dequenching signals are small, for example, at low temperature in the range 10−25 °C, the foregoing conclusion is not warranted. First of all, at such conditions, it is difficult to distinguish between the signals due to simple transfer and fusion because, as indicated, the two signals are comparable and small. Moreover, it is difficult to determine the initial rate of each process. Another complication is due to the lag time, that is, the time between mixing and the actual initiation of fusion, which depends on temperature. The lower the temperature, the longer the lag time (*21*). Since the EML fluorescence signal is rather small, it may be difficult to discern quantitatively the contributions of these signals arising from hemifusion and those from complete fusion, particularly at low temperature. Many investigators have followed a procedure which includes preincubation of the mixture of the labeled virus and target cells at low temperature with a subsequent increase of temperature to the desired experimental range. It is evident that under such conditions the probe may transfer without fusion.

In summary, we have applied a novel approach, based on viral membrane-protein dilution, to monitor the fusion of a virus. A comparison of its kinetics to those obtained by a lipid mixing assay allowed us to determine the relative contribution of probe transfer, hemifusion, and genuine fusion to the overall dilution seen in the latter assay. The results revealed that hemifusion started around 20−25 °C, whereas at 37 °C, the *initial* rate of lipid mixing most closely matches the actual rate of viral membrane fusion. The observation that lysoPC inhibits the fusion of Sendai virus may bear relevance to its molecular mechanism of membrane merging (*32*) and merits further investigation.

APPENDIX

The time course F_D of the total dequenching signal of the R_{18} assay as shown in Figure 5a can be composed of two curves, Figure 5, parts b and c, as described in the Experimental Results section. One is due to probe transfer with fusion and the other is due to that without fusion. We assume that these curves can be described by two exponential curves, and denote them $F^1{}_D$ and $F^2{}_D$, respectively. F_D is then expressed as:

$$F_D = F^1{}_D + F^2{}_D = f^1(1 - \exp(-t/k_1)) + f^2(1 - \exp(-t/k_2)) \quad (2)$$

where f^1 and f^2 are equilibrium values of the percent dequenching, and k_1 and k_2 are the relaxation times for respective dequenching processes. The value of f^1 was 18%, representing the percent dequenching value of curve c at 1 h (data not shown in the figure), and f^2 was 25%, which was the dequenching value after 24 h for a LPC-SV and LPC-EG interacting system, respectively, under the conditions used in our experiments. Then, by fitting two experimental curves to eq 2, we obtain relaxation times of approximately 4 and 40 min for the respective probe-transfer processes. More precisely, curve c in Figure 5 seems to be fitted by two other exponential curves. Such analysis provides a means to separate the probe transfer, due to either complete fusion or hemifusion, or both.

ACKNOWLEDGMENT

The authors also wish to express many thanks to the Department of Physiology and Biophysics for generous support for this project.

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BI972016G