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Effects of Fusion Temperature on the Lateral Mobility of Sendai Virus Glycoproteins in Erythrocyte Membranes and on Cell Fusion Indicate That Glycoprotein Mobilization Is Required for Cell Fusion†

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ABSTRACT: In order to investigate the requirement for lateral mobilization of viral envelope glycoproteins on the cell surface in the induction of cell–cell fusion, we employed fluorescence photobleaching recovery to study the effect of the fusion temperature on the lateral mobilization of Sendai virus glycoproteins in the human erythrocyte membrane. As the fusion temperature was reduced below 37 °C (to 31 or 25 °C), the rates of virus–cell fusion, the accompanying hemolysis, and cell–cell fusion were all slowed down. However, the plateau (final level) after the completion of fusion was significantly reduced at lower fusion temperatures only in the case of cell–cell fusion, despite the rather similar final levels of virus–cell fusion. A concomitant decrease as a function of the fusion temperature was observed in the fraction of cell-associated viral glycoproteins that became laterally mobile in the erythrocyte membrane during fusion, and a strict correlation was found between the level of laterally mobile viral glycoproteins in the cell membrane and the final extent of cell–cell fusion. The accompanying reduction in the lateral diffusion coefficients (D) of the viral glycoproteins (1.4-fold at 31 °C and 1.9-fold at 25 °C, as compared to 37 °C) does not appear to determine the final level of cell–cell fusion, since fusing the cells with a higher amount of virions at 25 °C increased the final level of cell–cell fusion while D remained constant. The results demonstrate that lateral mobilization of the viral glycoproteins in the target cell membrane is not an immediate consequence of viral envelope–cell fusion and support the view that it plays an essential role in the induction of cell–cell fusion by native Sendai virions.

The membrane fusion activities of animal enveloped viruses have been shown to be mediated by specific viral envelope glycoproteins (Poste & Pasternak, 1978; Volsky & Loyter, 1978a; Hsu et al., 1979; White et al., 1983; Florkiewicz & Rose, 1984). Among the paramyxoviruses, which fuse with cell membranes at neutral pH values (Poste & Pasternak, 1978; White et al., 1983), the fusogenic activities of Sendai virus have been the most extensively investigated. The envelope of Sendai virions contains two glycoproteins: the fusion protein F,[1] which is required for virus–cell and cell–cell fusion, and the hemagglutinin-neuraminidase protein (HN), which serves to bind the virions to sialic acid containing membrane components (Poste & Pasternak, 1978; Choppin & Scheid, 1980).

The mechanism of virus–cell and cell–cell fusion is still obscure, although an involvement of hydrophobic interactions between viral glycoproteins and target membranes was proposed (Gething et al., 1978; Hsu et al., 1981; White et al., 1983). Employing fluorescence photobleaching recovery (FPR), we have recently demonstrated that the envelope glycoproteins of native Sendai virions become laterally mobile on the surface of human erythrocytes following fusion and that this mobilization is blocked under conditions that eliminate virus–cell and cell–cell fusion. These findings supported the notion that lateral motion of the viral glycoproteins in the cell membrane may be required for the induction of cell–cell fusion (Henis et al., 1985; Volsky & Loyter, 1978b; Kuroda et al., 1980). However, since both virus–cell and cell–cell fusion were blocked, it was not clear whether the lateral mobilization of

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[1] Abbreviations: D, lateral diffusion coefficient; DTT, dithiothreitol; F, fusion protein; FPR, fluorescence photobleaching recovery; HAU, hemagglutinating unit(s); HN, hemagglutinin-neuraminidase protein; PMSF, phenylmethanesulfonyl fluoride; R_f, mobile fraction; RSVE, reconstitute Sendai virus envelopes; R_{18}, octadecylrhodamine B chloride; TMR, tetramethylrhodamine; Tricine, *N*-[tris(hydroxymethyl)methyl]-glycine.

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the viral glycoproteins in the target cell membrane is an essential part of the mechanism leading to cell–cell fusion or whether it is merely a consequence of viral envelope–cell fusion. Some insight into this question was provided by studies with reconstituted Sendai virus envelopes (RSVE), which were as active as the native virions in envelope–cell fusion but induced cell–cell fusion to a much lower degree (Henis & Gutman, 1987). These studies demonstrated a strong reduction in the fraction of laterally mobile viral glycoproteins after fusion with human erythrocytes, in correlation with their lower cell–cell fusion activity. However, the RSVE (prepared from detergent-solubilized virions) clearly differed in their fusogenic properties from native virions, possibly due to altered organization of the viral glycoproteins in the RSVE membrane as compared to that of native virions. Therefore, it is possible that the failure of the viral glycoproteins to diffuse over the cell membrane after RSVE–cell fusion represents a special case and does not apply to native virions.

In order to investigate the requirement for viral glycoprotein mobilization in the induction of cell–cell fusion by native virions, we have studied in the present work the lateral mobility of F and HN following fusion of human erythrocytes by native Sendai virions at suboptimal temperatures. The reduced fusion temperature does not affect the *final* level (although it slows down the rate) of virus–cell fusion but markedly reduces the final extent of cell–cell fusion. This generates conditions where the fusion reaction is inhibited after the stage of virus–cell fusion. Our results show a concomitant reduction in the mobile fraction of the viral glycoproteins and in the level of cell–cell fusion as the temperature is reduced below 37 °C. The results demonstrate that lateral mobilization of the viral glycoproteins in the target cell membrane is not an immediate consequence of virus–cell fusion and support the suggestion that it is an essential step in the induction of cell–cell fusion by native Sendai virions.

Materials and Methods

Reagents. Octadecylrhodamine B chloride (R_{18}) was purchased from Molecular Probes (Junction City, OR). Tetramethylrhodamine (TMR) 5-isothiocyanate was from Research Organics (Cleveland, OH). Trypsin (twice recrystallized) and dithiothreitol (DTT) were from Sigma (St. Louis, MO).

Virus. Sendai virus (Z strain) was grown in the allantoic sac of 10–11 day old embryonated chicken eggs, harvested 48 h after injection, and purified by established procedures (Peretz et al., 1974; Hsu et al., 1982; Henis et al., 1985). The virus was resuspended in 160 mM NaCl/20 mM Tricine, pH 7.4 (solution A), and stored at −70 °C. Viral protein concentration was determined by a modified Lowry procedure (Markwell et al., 1978). Viral hemagglutinating activity (around 13 000 HAU/mg of viral protein) was measured in hemagglutinating units (HAU) as described (Peretz et al., 1974). Treatments of Sendai virions with trypsin or with phenylmethanesulfonyl fluoride (PMSF) under conditions that eliminate their fusogenic activities without interfering with their binding and hemagglutination were performed as described in detail earlier (Maeda et al., 1979; Israel et al., 1983; Aroeti & Henis, 1986a).

Interaction of Sendai Virions with Human Erythrocytes. Fresh human blood (group O, Rh positive) was obtained from a blood bank and stored with sodium citrate up to 7 days at 4 °C. Prior to use, erythrocytes were washed with solution A, diluted to 2% (v/v) with the same buffer, and incubated (15 min, 4 °C) with 400 HAU/mL Sendai virions. The virions were earlier subjected to mild sonication and filtrated through a 0.45-μm Acrodisc filter to eliminate large viral aggregates (Henis et al., 1985). Excess virus was removed by washing twice with cold solution A. The pellet was gently vortexed and resuspended in 1 mL of solution A at the desired fusion temperature (37, 31, or 25 °C). The degree of cell–cell fusion was evaluated by determining the percentage of fused cells among the total cell population, employing phase-contrast microscopy (Henis et al., 1985; Peretz et al., 1974).

Determination of Virus–Cell Fusion by Dequenching of R_{18} Fluorescence. Viral envelope–cell fusion was determined by R_{18} fluorescence dequenching (Hoekstra et al., 1984). R_{18} was incorporated into the envelope of native Sendai virions as described (Hoekstra et al., 1984; Henis & Gutman, 1987), yielding 2–3 mol % R_{18} in the viral envelope with a quenching degree (Q) of 60–80% ($Q = [(F_T - F_0) \times 100]/F_T$, where F_0 and F_T are the fluorescence intensities prior to and after addition of 1% v/v Triton X-100). The residual quenching (RQ) after incubation of the virions with the cells was determined similarly, from the fluorescence ratio before and after the addition of Triton X-100. The percentage of fused viral particles was determined from the dequenching (DQ) of the fluorescence during the incubation with the cells, according to the formula $DQ = 100(Q - RQ)/Q$. The fluorescence was measured with a Perkin-Elmer MPF-4 spectrofluorometer (λ_{ex} = 560 nm; λ_{em} = 590 nm) equipped with a thermostated cuvette holder.

R_{18}-labeled virions were incubated with intact erythrocytes as described in the former section. At the end of the fusion period, the hemoglobin was removed by washing with solution A after lysis. The additional wash after the incubation at the fusion temperature removes the viral particles that detach from the cells during the incubation period (Hoekstra & Klappe, 1986). Thus, this procedure measures the percentage of virions fused with the cells out of the total amount of cell-associated virions (adsorbed or fused) at the end of the incubation period.

Antibodies and Fab' Fragments against Viral Envelope Proteins. The antibodies employed in the present study were those described by us earlier (Henis et al., 1985; Henis & Gutman, 1987). They were raised in rabbits by intracutaneous injections of RSVE or of vesicles containing either F or HN proteins. Monovalent Fab' fragments labeled with TMR isothiocyanate (TMR-Fab') were prepared from the IgG fraction as described (Henis et al., 1985; Henis & Gutman, 1987). They did not cross-react with erythrocyte membrane components and were specific to the viral glycoproteins originally incorporated into each type of vesicle (Henis et al., 1985).

Use of DTT To Remove Adsorbed Virions from Cells. Sendai virions adsorbed to human erythrocytes were detached from the cells in several experiments by incubation with 50 mM DTT and 2 mM ethylenediaminetetraacetate in solution A (15 min at 4 °C, followed by 30 min at 22 °C, with vigorous vortexing every 10 min). This treatment removes adsorbed, but not fused, Sendai virions from human erythrocytes (Chejanovsky et al., 1984; Henis & Gutman, 1987). Detached virions were removed by centrifugation.

Fluorescence Photobleaching Recovery. Lateral diffusion coefficients (D) and mobile fractions (R_f) of the viral glycoproteins in the cell membrane were measured by FPR (Koppel et al., 1976; Axelrod et al., 1976) with a previously described apparatus (Henis & Gutman, 1983). The bleaching conditions in the FPR studies were shown not to alter the lateral mobilities measured (Wolf et al., 1980; Koppel & Sheetz, 1981). Following incubation with the virions, the resulting ghosts [formed by the virally mediated hemolysis, or by hypotonic

Table I: Effect of Fusion Temperature on the Final Levels (after Completion of Fusion) of Virus–Cell Fusion, Hemolysis, and Cell–Cell Fusion[a]

temp (°C)	fraction of fused virions: R_{18} fluorescence dequenching	fraction of fused virions: DTT treatment	fused virions/cell	hemolysis (% of total)	cell–cell fusion (% fused cells)
4	0.08 ± 0.05	0.07 ± 0.06	5 ± 3	4 ± 3	none
37	0.80 ± 0.03	0.77 ± 0.15	50 ± 2	80 ± 7	65 ± 2
31	0.74 ± 0.04	0.75 ± 0.16	46 ± 2	80 ± 10	26 ± 2
25	0.79 ± 0.04	0.80 ± 0.15	39 ± 2	76 ± 8	9 ± 1

[a] Sendai virions (400 HAU/mL) were incubated in solution A with human erythrocytes (2% v/v) at 4 °C (row 1) or at 4 °C followed by the indicated fusion temperature (all other rows). The various fusion parameters were determined after the completion of fusion (60 min at 37 °C, 150 min at 31 °C, and 500 min at 25 °C). The results are mean ± SE of four separate experiments in each case. Measurements of hemolysis, cell–cell fusion (scoring 200–300 cells to determine the percentage of fused cells), and viral envelope–cell fusion by dequenching of R^{18} fluorescence were performed as described under Materials and Methods. DTT treatment was performed as described under Materials and Methods, and the fraction of cell-associated virions which could not be removed by DTT was determined by labeling the cells (either prior to or after the DTT treatment) with anti-RSVE TMR-Fab′ fragments (100 μg/mL, 30 min, 22 °C, in solution A containing 0.2% bovine serum albumin) and measuring the fraction of remaining cell-associated fluorescence after the DTT treatment with the FPR instrumentation under nonbleaching conditions (30 cells scored in each case; Henis & Gutman, 1987). The number of fused virions per cell was determined by multiplying the fraction of fused virions by the total number of cell-associated virions after the completion of fusion. The latter parameter was determined by solubilizing the washed ghosts obtained after fusion with R_{18}-labeled virions with 1% (v/v) Triton X-100 and measuring the fluorescence intensity; the level of R_{18} fluorescence per microgram of viral protein (derived from the fluorescence of a known amount of virions solubilized with 1% Triton X-100) was used for calibration (Hoekstra & Klappe, 1986; Aroeti & Henis, 1987). One microgram of viral protein was assumed to contain 1.3×10^9 viral particles (Hoekstra & Klappe, 1986). At all the fusion temperatures employed, control experiments using trypsin- or PMSF-treated virions yielded results similar to those obtained after incubation at 4 °C only (no cell–cell fusion, below 5% hemolysis, and a fraction of fused virions of 0.10 ± 0.06).

lysis and resealing according to Steck and Kant (1974)] were attached to glass cover slips precoated with polylysine [10-min incubation of the cover slip with a 5 μg/mL solution of poly(L-lysine) in 20 mM phosphate buffer, pH 8]. After labeling of the viral glycoproteins with anti-F or anti-HN TMR-Fab′ fragments (100 μg/mL, 30 min, 22 °C, in solution A containing 0.2% bovine serum albumin), the cover slips with the attached ghosts were wet mounted in solution A on a temperature-controlled microscope stage.

The monitoring laser beam (529.5-nm, 0.5-μW, argon ion laser) was focused through the microscope to a Gaussian radius of 0.93 μm with an 100 × oil-immersion objective. A brief pulse (5 mW, 20 mS) bleached 50–70% of the fluorescence in the illuminated region. The time course of fluorescence recovery was followed by the attenuated monitoring beam. D and R_f were determined from the fluorescence recovery curves (Axelrod et al., 1976) by nonlinear regression analysis (Petersen et al., 1984). Incomplete recovery was interpreted to represent fluorophores which are immobile on the experimental time scale ($D \leq 5 \times 10^{-12}$ cm^2/s). In order to ascertain that the R_f values are determined accurately and to distinguish between low R_f and low D, we have employed experimental scan times that were over 10-fold longer than the half-time of the fluorescence recovery (total scan times of 51.2 s at 37 °C and 102.4 s at 25 or 31 °C, as compared with half-times around 10 and 5 s, respectively). Under such conditions, the fluorescence recovers to over 92% of its final level during the time of the measurement, ensuring an accurate determination of R_f. Moreover, the D and R_f values extracted by fitting the entire set of data points or the first half to the lateral diffusion equation were similar within the experimental error.

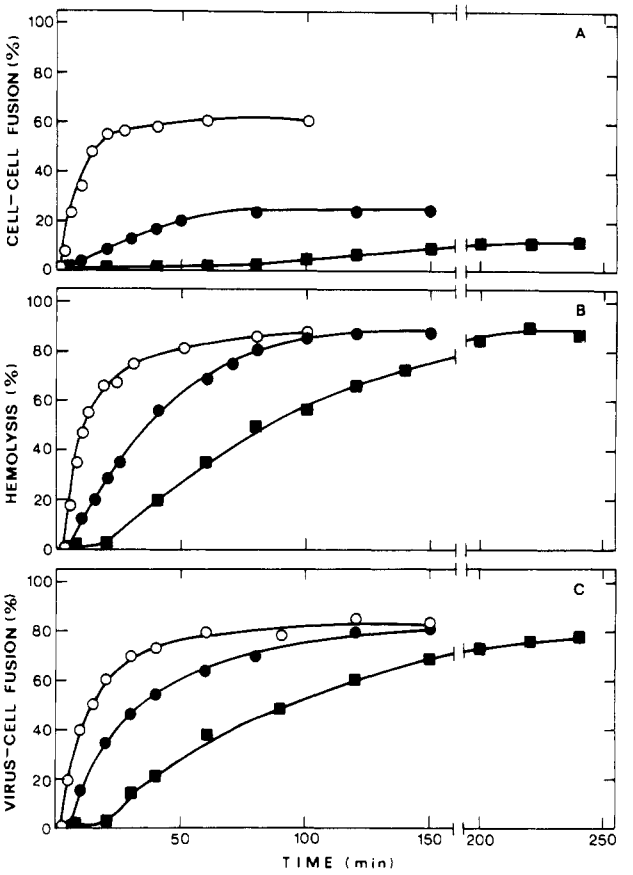

FIGURE 1: Typical curves showing the time course of cell–cell fusion (A), hemolysis (B), and virus–cell fusion (C). The fusion temperatures employed were 37 (○), 31 (●), or 25 °C (■). Erythrocytes were incubated with Sendai virions at 4 °C followed by incubation at the fusion temperature as described under Materials and Methods. Samples were withdrawn after various incubation periods, and the various fusion parameters (cell–cell fusion, hemolysis, and virus–cell fusion measured by R_{18} fluorescence dequenching) were determined as described under Materials and Methods.

RESULTS

Effects of Temperature on the Fusion of Sendai Virions with Human Erythrocytes. In order to determine the time required to reach the final plateau levels of the fusogenic activities of Sendai virions under the conditions employed in the mobility measurements, the time course of virus–cell fusion, hemolysis, and cell–cell fusion at several temperatures (37, 31, and 25 °C) was measured (Figure 1). The rate of the various viral fusogenic activities was reduced with the fusion temperature, in accord with former reports (Lyles & Landsberger, 1979; Hoekstra et al., 1985). After establishment of the time required for the completion of fusion (the time required to reach the plateau levels in Figure 1) at each temperature, fusion was allowed to proceed to completion, and the final levels of the various fusion parameters were determined. The results (Table I) clearly demonstrate that the final, maximal level of cell–cell fusion is drastically reduced with the fusion temperature. In contrast, the final levels of virus–cell fusion (both in terms of the percentage of fused virions out of the total population of cell-associated virions at the end

of the incubation period and in terms of the number of fused virions per cell) and of the accompanying hemolysis remained unaltered upon reduction of the fusion temperature from 37 to 31 °C. At 25 °C, the final percentage of cell-associated virions fused with the cells after the incubation was similar to that obtained at 37 or 31 °C, but there was a small decrease (20–25%) in the number of fused virions per cell after the completion of fusion (Table I). This reduction is most likely due to dissociation of some of the virions adsorbed to the cells at 4 °C during the prolonged incubation at 25 °C—as a result of the viral neuraminidase activity, which decreases with temperature less than the fusion process (Micklem et al., 1985). However, the slight decrease in the number of fused virions per cell at 25 °C is not the reason for the drastic reduction in the final level of cell–cell fusion at this temperature, since incubation of the erythrocytes at 37 °C with a lower virus concentration (300 HAU/mL), which resulted in the same level of fused virions per cell as in the incubation with 400 HAU/mL virus at 25 °C (40 fused virions/cell), induced only a minor reduction in the final level of cell–cell fusion (from 65% to 60%). It should be noted that the final fractions of fused virions (about 0.8) were higher than the fused fractions of 0.4–0.5 observed in former studies (Lyles & Landsberger, 1979; Maeda et al., 1981; Hoekstra et al., 1984) which did not employ an additional washing step after the incubation at the fusion temperature to remove virions detached during the incubation. Thus, those studies measured the fused fraction of the virions associated with the cells prior to the incubation at the fusion temperature, while the current experiments measure the fraction of fused virions out of those which are cell-associated (bound or fused) after the incubation under the fusion-promoting conditions.

In order to verify that lipid exchange processes do not contribute significantly to the measurements of viral envelope–cell fusion by R_{18} fluorescence dequenching described in Figure 1 and in Table I, similar experiments were performed with Sendai virions pretreated with trypsin or PMSF. These treatments, which eliminate the fusogenic activities of Sendai virions (Maeda et al., 1979; Israel et al., 1983), prevented the increase in R_{18} fluorescence at all the fusion temperatures employed (37, 31, and 25 °C) (Table I). Moreover, the final level of virus–cell fusion was also determined by an independent method, based on the use of DTT treatment to remove adsorbed (but not fused) Sendai virions from the erythrocyte membrane (Table I). This method is free of any possible contribution by lipid exchange processes, since the fraction of cell-associated virions that cannot be removed from the cells by DTT treatment is determined by measuring the fluorescence on the cells following labeling with TMR-Fab′ fragments directed against the viral envelope proteins (F and HN), and which do not bind to lipids (Henis & Gutman, 1987). This method yielded results essentially identical with those obtained by R_{18} fluorescence dequenching (Table I).

Dependence of the Lateral Mobility of the Viral Glycoproteins in the Cell Membrane on the Fusion Temperature. The reduction in the final level of cell–cell fusion at fusion temperatures below 37 °C without a parallel decrease in viral envelope–cell fusion generates conditions where the involvement of the lateral mobilization of the viral envelope proteins on the cell surface in the mechanism of cell–cell fusion can be investigated. To explore this question, we studied the lateral motion of the viral F and HN proteins in the erythrocyte membrane following fusion at 37, 31, and 25 °C. In these experiments, the erythrocytes were incubated with the virions at 4 °C to enable viral adsorption and hemagglutination,

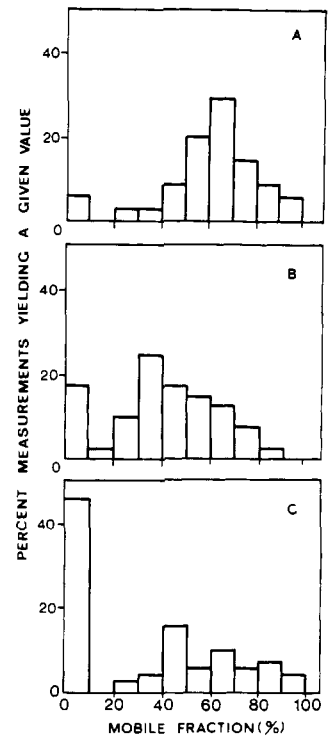

FIGURE 2: Distribution of the mobile fractions of the viral F proteins over the population of human erythrocytes ghosts after fusion at 37 (A), 31 (B), and 25 °C (C). FPR measurements were performed at the appropriate fusion temperature after the completion of fusion at each fusion temperature, as described in Table II and under Materials and Methods. The F proteins were labled with anti-F TMR-Fab′ fragments. A total of 50–60 cells was measured in each case. Similar results were obtained for the viral HN proteins (labeled with anti-HN TMR-Fab′ fragments) after the completion of fusion.

Table II: Lateral Mobility of Sendai Virus Envelope Glycoproteins on Human Erythrocyte Ghosts after Fusion at Various Temperatures[a]

envelope protein labeled	temp (°C)	R_f	D ($cm^2/s \times 10^{10}$)
F	37	0.61 ± 0.04	6.4 ± 0.5
F	31	0.40 ± 0.04	4.4 ± 0.4
F	25	0.32 ± 0.03	3.4 ± 0.2
HN	37	0.60 ± 0.03	6.2 ± 0.6
HN	31	0.42 ± 0.02	4.6 ± 0.3
HN	25	0.30 ± 0.04	3.2 ± 0.3

[a] Human erythrocytes were incubated with Sendai virions until the completion of fusion at each fusion temperature as described in Table I. The ghosts formed were prepared for the FPR experiments as described under Materials and Methods. Anti-F and anti-HN TMR-Fab′ fragments were employed to label the F or HN proteins, respectively (see Materials and Methods). Anti-F and anti-HN TMR-Fab′ fragments were employed to label the F or HN proteins, respectively (see Materials and Methods). The FPR measurements were conducted in solution A at the fusion temperature (37, 31, or 25 °C). The results are mean ± SE of 50–60 measurements in each case.

followed by incubation at the desired fusion temperature for periods sufficient for the completion of fusion. The viral glycoproteins (F or HN) were then labeled by TMR-Fab′ fragments, and their lateral motion in the cell membrane was measured at the fusion temperature by FPR. These measurements (Table II and Figure 2) demonstrated that the fraction of viral glycoproteins that became laterally mobile in the cell membrane following fusion (R_f) was reduced concomitantly with the fusion temperature, although the fraction of cell-associated virions which have fused with the cells was unaltered (Table I). It therefore follows that the fraction of viral glycoproteins which are immobile in the cell membrane on the experimental time scale ($D \leq 5 \times 10^{-12}$ cm^2/s) is not

Table III: Correlation between the Amount of Fused Viral Glycoproteins Laterally Mobile in the Cell Membrane and the Level of Cell–Cell Fusion[a]

temp (°C)	virus concn (HAU/mL)	FV	R_f	FV × R_f	cell–cell fusion (% fused cells)
37	400	50 ± 2	0.61 ± 0.04	30	65 ± 2
31	400	46 ± 2	0.40 ± 0.04	18	26 ± 2
25	400	39 ± 2	0.32 ± 0.03	12	9 ± 1
25 + 37	400	38 ± 2	0.65 ± 0.05	25	60 ± 2
25	1200	58 ± 3	0.36 ± 0.06	21	30 ± 4

[a] Human erythrocytes were incubated with Sendai virions until all the fusion parameters reached their maximal and final levels at each fusion temperature as described in Table I. In row 4, fusion was carried out to completion at 25 °C (500 min), and the sample was then further incubated 60 min at 37 °C. The mobile fractions (R_f) were measured by FPR at the fusion temperature on 50–60 cells in each case, employing anti-F TMR-Fab′ fragments to label the F proteins (similar results were obtained for the HN proteins). The number of fused virions per cell (FV) and cell–cell fusion were determined in four separate experiments in each case, as described in Table I. The amount of fused viral glycoproteins that become laterally mobile on the cell surface to directly proportional to FV × R_f, the product of the number of fused virions per cell and the fraction of laterally mobile viral glycoproteins (R_f of F and HN were similar). All results are mean ± SE.

the result of a higher percentage of adsorbed unfused virions following fusion at the lower temperatures; this conclusion is supported by the finding that DTT treatment to remove adhered, unfused virions after the completion of fusion at 25 °C did not increase the R_f values of F or HN (0.30 ± 0.05 in both cases). The lower levels of viral glycoprotein mobilization in the cell membrane at fusion temperatures below 37 °C are further emphasized in the distributions of the R_f values of F and HN following fusion (Figure 2). Fusion at 37 °C results in a normal distribution of the R_f values around the mean; however, as the fusion temperature is lowered, a skewed distribution with a significant increase in the percentage of cells exhibiting undetectable R_f values of the viral glycoproteins is obtained.

Lower fusion temperatures result not only in reduced R_f values of the viral glycoproteins but also in lower D values as measured at the fusion temperature after the completion of fusion (Table II). However, the reduction in D appears to be simply due to the use of a lower temperature (resulting in lower membrane lipid fluidity) in the FPR measurements. This notion is supported by the finding that when the temperature was reduced to 31 or 25 °C only after the completion of fusion at 37 °C, an identical reduction in the D values (but not in R_f, which remained around 0.60) was observed. Moreover, when all the FPR measurements were performed at the same temperature (22 °C) after the completion of fusion at each specific fusion temperature, identical D values [(3.1–3.2) × 10^{-10} cm^2/s] were obtained for F and HN, regardless of the original fusion temperature. This contrasts with the reduction in the R_f values of F and HN at lower fusion temperatures (Table II) which depended on the fusion temperature but not on the temperature of the FPR measurements. This suggests that, once released, the lateral mobility of the viral glycoproteins that undergo mobilization in the cell membrane during fusion does not depend on the value of their mobile fractions. Further support for this notion is supplied by the distributions of the D values of the viral glycoproteins, which remain normal at the lower fusion temperatures despite the transfer of the R_f values to skewed distributions (Figure 3).

Previous reports (Sekiguchi & Asano, 1978; Lalazar & Loyter, 1979) indicated that human erythrocyte ghosts prepared by hypotonic lysis and resealing fuse with each other under the influence of Sendai virus to a much lower degree than intact erythrocytes. Since the rate of cell–cell fusion was inhibited at reduced fusion temperatures to a higher degree than the rate of virally mediated hemolysis (Figure 1), it was possible that the reduction in the maximal (final) level of cell–cell fusion at lower fusion temperatures was due to an increase in the rate of ghost formation (hemolysis) relative to the rate of cell–cell fusion. To examine this possibility, fusion

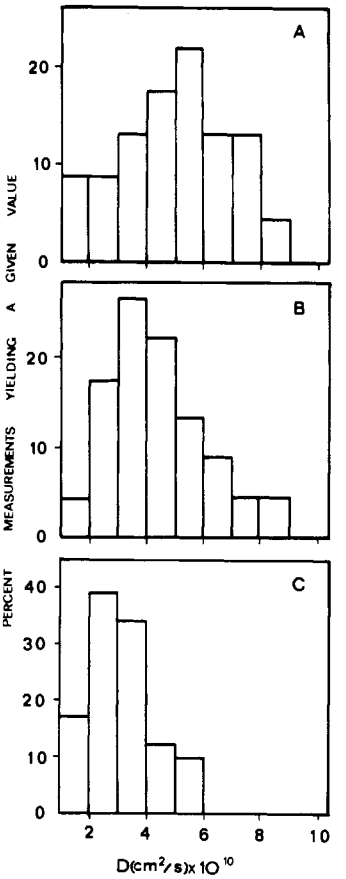

FIGURE 3: Distribution of D values of the viral F proteins over the population of human erythrocytes ghosts after fusion at 37 (A), 31 (B), and 25 °C (C). Experiments were performed as in Figure 2. Fifty to sixty cells were measured in each case. Similar results were obtained for the viral HN proteins.

was allowed to proceed to the final maximal level at 25 °C (500 min), and the temperature was then shifted to 37 °C for an additional 60-min period. This treatment resulted in a large increase in cell–cell fusion back to the level obtained by fusion at 37 °C (Table III), demonstrating that the hemolysis induced by the incubation with the virions at 25 °C does not significantly interfere with the ability of the cells to undergo cell–cell fusion. In this context, Peretz et al. (1974) reported that ghosts prepared by slow and gradual hemolysis were efficiently fused with each other by Sendai virus. The above experiments further emphasize the correlation between the lateral mobilization of the viral glycoproteins in the cell membrane and the ability of the virions to induce cell–cell fusion, since the shift to 37 °C after the incubation at 25 °C restored the average R_f values (Table III) and the distribution of the R_f

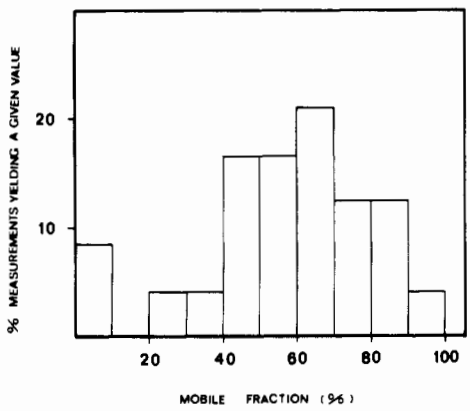

FIGURE 4: Distribution of the mobile fractions of F proteins over the population of human erythrocyte ghosts after fusion at 25 °C followed by further incubation at 37 °C. Fusion was allowed to proceed to completion at 25 °C (500 min), and the cells were further incubated at 37 °C for 60 min. FPR measurements were performed at 37 °C after labeling with anti-F TMR-Fab′ fragments (see Materials and Methods). Sixty cells were measured. Similar results were obtained for the HN proteins.

values of the viral glycoproteins (Figure 4) to the values and pattern observed following fusion at 37 °C (Table III; compare Figures 4 and 2).

The concomitant reduction in the lateral mobilization of the viral glycoproteins and in the final level of cell–cell fusion upon reduction of the fusion temperature supports the hypothesis that lateral motion of the viral envelope proteins in the target cell membrane is essential for the induction of cell–cell fusion. If this were true, the concentration of *laterally mobile* viral glycoproteins in the target cell membrane should correlate with the level of cell–cell fusion. This parameter can be measured by the product of two quantities—the number of fused virions per cell and R_f (the fraction of laterally mobile viral glycoproteins). Indeed, when the amount of viral glycoproteins which are laterally mobile in the cell membrane following fusion at 25 °C was increased by fusing the cells with a higher concentration of virions (resulting in a higher number of fused virions per cell), the final level of cell–cell fusion increased in direct correlation (Table III). In fact, the percentage of fused cells (30%) was in excellent agreement with the product of fused virions per cell times R_f (21), considering that fusion at 31 °C yielded a value of 18 for this parameter vs a final cell–cell fusion level of 26% (Table III). The D values of the viral glycoproteins at 25 °C were not altered by the use of 1200 HAU/mL virus [$(3.3 \pm 0.3) \times 10^{-10}$ cm^2/s for either the F or HN proteins].

Discussion

The results depicted in Figure 1 and in Table I demonstrate that changes in the fusion temperature have different effects on the final levels (after the plateau of the various fusion parameters is reached) of virus–cell and of cell–cell fusion. The maximal, final level of cell–cell fusion is markedly reduced along with the fusion temperature, while the final levels of virus–cell fusion and of the accompanying hemolysis are either not affected or affected only slightly (see first section under Results). A lack of dependence of the final extent of virus–cell fusion on the fusion temperature (studied in the range of 32–39 °C) was also reported by Lyles and Landsberger (1979) for the fusion of Sendai virions with human erythrocyte ghosts, as measured by ESR. The selective decrease in the level of cell–cell fusion without a parallel reduction in the final level of virus–cell fusion at lower fusion temperatures suggests that virus–cell and cell–cell fusion are separable processes and the first can occur without proceeding to the latter. This conclusion is supported by former studies which demonstrated selective inhibition of Sendai virus mediated cell–cell fusion by cytochalasins or high saccharide concentrations (Asano & Okada, 1977; Maeda et al., 1977; Miyake et al., 1978) and by the finding that RSVE prepared under specific conditions were defective in their cell–cell fusion activity but not in their ability to fuse with the cells (Henis & Gutman, 1987). These results clearly argue against the suggestion that simultaneous fusion of a viral envelope with the plasma membrane of two adjacent cells (Apostolov & Almeida, 1972; Knutton, 1977; Maeda et al., 1977) is a major mechanism for Sendai virus mediated cell–cell fusion. If this were the case, virus–cell and cell–cell fusion should have been inhibited simultaneously. The latter conclusion is strongly supported by experiments with other enveloped viruses, where the expression of cDNA encoding specific viral glycoproteins in transfected cells endowed them with cell–cell fusion activity (Kondor-Koch et al., 1983; Florkiewicz & Rose, 1984).

There are several possible explanations for the reduced final levels of cell–cell fusion at lower fusion temperatures: (a) An obvious reason could have been a reduction in the amount of fused viral spike proteins inserted per cell by virus–cell fusion as the fusion temperature is lowered. However, this is clearly not the case, since the number of fused virions/cell is similar for fusion at 37 and 31 °C and only slightly reduced following fusion at 25 °C (Table I). As discussed in the first section under Results, this slight reduction cannot be the cause for the dramatic drop in the level of cell–cell fusion at 25 °C: fusion at 37 °C with a lower number of virions (yielding a similar number of fused virions/cell as after fusion at 25 °C under the standard incubation conditions) did not inhibit significantly the final level of cell–cell fusion. Moreover, a further incubation at 37 °C after the completion of fusion at 25 °C did not increase the number of fused virions per cell but raised the percentage of fused cells close to the level oberved following fusion at 37 °C (Table III). (b) Since human erythrocyte ghosts prepared by hypotonic lysis and resealing are defective in their ability to undergo cell–cell fusion by Sendai virus (Sekiguchi & Asano, 1978; Lalazar & Loyter, 1979), the increase in the ratio of the hemolysis rate to the cell–cell fusion rate as the fusion temperature is reduced could result in a lower degree of cell–cell fusion. This possibility is excluded by the demonstration that the ghosts formed by fusion at 25 °C could be fused with each other to the normal level by a further incubation at 37 °C (Table III). (c) If enzymatic activites of the viral envelope proteins were involved in the fusion process, they could be inhibited at lower fusion temperatures. However, no indications for such activities are available. Moreover, temperature-dependent inhibition of fusion-promoting enzymatic activities would be expected to affect virus–cell fusion as well as cell–cell fusion; in addition, a slower rate of catalytic enzymatic activity is expected to slow down the rate of the catalyzed reaction (in this case, cell–cell fusion) but not to reduce the final level of the reaction product (fused cells). (d) The reduction in the fraction and level of viral glycoproteins that become laterally mobile in the cell membrane at fusion temperatures below 37 °C in spite of the similar fractions and rather close levels of fused virions/cell (Tables I and II; Figure 2) demonstrates that lateral mobilization of the viral glycoproteins in the target cell membrane is not an immediate and obligatory result of viral envelope–cell fusion. The finding that reduced levels of viral glycoprotein mobilization are accompanied by a parallel reduction in the final extent of cell–cell fusion suggests that

lateral mobilization of the viral glycoproteins in the cell membrane after the stage of viral envelope–cell fusion plays a crucial role in the induction of cell–cell fusion.

In view of the considerations discussed above, the last possibility appears to be the most likely. The notion that fusion is not necessarily followed by free lateral motion of the fused proteins in the target membrane is also in line with reports on restricted lateral mobilization of fused membrane proteins in other experimental systems, such as poly(ethylene glycol)-mediated erythrocyte–fibroblast fusion or lipid vesicle–mouse L cell fusion (Wojcieszyn et al., 1983; Bauman et al., 1980).

Lower fusion temperatures result not only in lower R_f values of the viral glycoproteins; these proteins also display lower D values at lower temperatures, most likely due to the reduction in the membrane lipid fluidity with temperature (Table II and Figure 3; discussed under Results). However, the lower diffusion rate (a twofold reduction in D at 25 vs 37 °C) does not determine the *final* level of cell–cell fusion; this was demonstrated by fusing the cells at 25 °C with a higher concentration of virions (1200 instead of 400 HAU/mL) (Table III). Under these conditions, the D values of F and HN remained constant [$(3.2–3.4) \times 10^{-10}$ cm^2/s], while the amount of laterally mobile viral glycoproteins per cell increased (Table III). The final level of cell–cell fusion showed a parallel elevation, in spite of the unaltered D values. These findings are in accord with the expectation that a slower diffusion rate may reduce the rate, but not the final extent, of a diffusion-dependent process.

In view of the marked correlation between the concentration of laterally mobile viral glycoproteins in the target cell membrane and the final attainable extent of cell–cell fusion (Table III), we would like to propose that lateral motion of the viral glycoproteins in the cell membrane after viral envelope–cell fusion has occurred is required for the induction of cell–cell fusion. This hypothesis is in accord with our former studies with RSVE (Henis & Gutman, 1987), where the failure of the reconstituted viral envelopes to induce cell–cell fusion was correlated with the low fraction of their viral glycoproteins that became laterally mobile in the cell membrane following fusion. The lateral motion of the fusion-promoting viral glycoproteins in the cell membrane may be needed to perturb the membranes of adjacent cells in order to enable cell–cell fusion. It may also be required to enable them to achieve high concentrations in cell–cell contact regions in order for cell–cell fusion to take place.

At this stage, it is not possible to pinpoint the cause for the reduced mobilization of the viral glycoproteins in the cell membrane at fusion temperatures below 37 °C. It is clear that this phenomenon is not due to increased fractions of adhered, unfused virions, since the fraction of fused virions is not altered (Table I) and removal of the remaining unfused virions by DTT treatment does not significantly alter R_f of either F or HN (see Results). Not all of the fused viral glycoproteins become laterally mobile in the cell membrane even after fusion at 37 °C, and the immobile population (about 40% after fusion at 37 °C) may be due to interactions with cellular entities (e.g., the cytoskeleton), as was proposed for a variety of endogenous cell membrane proteins (Koppel et al., 1981; Henis & Elson, 1981; Tank et al., 1981; Edidin, 1981). Alternatively, the inability of some of the viral glycoproteins organized in spikes to detach from the original sites of virus–cell fusion (possibly due to interactions with other viral components, such as the matrix protein) could also contribute to the immobile fraction. Alterations in either type of interactions as a function of temperature could explain the temperature dependence of the lateral mobilization of the fused viral glycoproteins in the cell membrane. In this context, temperature-dependent changes in the surface structure of cell-associated virions (which could reflect altered interactions within the viral envelope) were observed by electorn microscopy (Knutton, 1978), and rotational motion of Sendai virus glycoproteins on the virion itself was found to be temperature dependent and to correlate with the ability of the virions to induce hemolysis (Lee et al., 1983).

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Transmembrane Calcium Movements Mediated by Ionomycin and Phosphatidate in Liposomes with Fura 2 Entrapped†

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ABSTRACT: A novel liposomal method permits studies of Ca movements across the bilayers of multilamellar vesicles (MLV) which had entrapped the Ca-dependent, fluorescent indicator dye Fura 2. Ionomycin-mediated Ca translocation across MLV of phosphatidylcholine (PC)/dicetyl phosphate (DCP), 9:1, obeyed simple first-order kinetics since log–log plots of initial rates versus ionomycin or Ca concentration yielded slopes of approximately 1. Since Ca is translocated in a Ca-dependent fashion in the course of stimulus–response coupling of cells which form diacylglycerol (DAG) and phosphatidate (PA) from polyphosphoinositides, we compared effects of PA with those of DAG. PA and DAG were preincorporated in PC/DCP vesicles, in which trace amounts of ionomycin provided transmembrane potential (due to Ca^{2+}/H^+ exchange). Significant increases in Ca movements were observed in the presence of egg lecithin PA, dioleoyl-PA, and dipalmitoyl-PA when compared with DCP- or DAG-containing MLV. DAGs such as 1-oleoyl-2-acetoylglycerol or 1,2-dioleoylglycerol in liposomes decreased rates of Ca translocation. Ca influx into PA-containing MLV was dependent on the mole percent of the PA in bilayers; the complex kinetics of Ca influx were compatible with the formation of nonbilayer states. Incorporation of cholesterol into the liposomes inhibited initial rates of Ca uptake by MLV presumably by condensing the bilayers. Ca influx increased with increasing pH of the external medium from 6.9 to 7.9 in liposomes with an internal pH of 7.4. The results not only indicate that transmembrane pH gradients and the extent of ionization of the ionophore affect rates of Ca translocation across lipid bilayers but also demonstrate that in model systems PA but not DAG promotes Ca translocation, the rate of which is altered by lipid composition of the bilayer and the pH of the suspension.

The levels of free cytoplasmic Ca ($[Ca]_i$)[1] play a critical role in mediating cell responses to extracellular signals (Berridge & Irvine, 1984; Kikkawa & Nishizuka, 1986; Spat et al., 1986). These responses are probably initiated by ligand-induced hydrolysis of inositol phospholipids (Hokin & Hokin, 1953; Nishizuka, 1984a; Berridge, 1984) followed by a rise in the $[Ca]_i$ (Michell, 1975). In absence of phospholipid remodeling, $[Ca]_i$ may be modulated by ionophores (Serhan et al., 1983; Ransom & Cambier, 1986). The ionophore-

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[1] Abbreviations: $[Ca]_i$, Ca concentration in cytoplasm or internal medium of liposomes; PA, phosphatidic acid or phosphatidate; DAG, diacylglycerol; IP_3, inositol 1,4,5-triphosphate; H_{II}, inverted hexagonal phase; MLV, multilamellar vesicle(s); PC, egg L-α-phosphatidylcholine; DOPA, dioleoyl-L-α-phosphatidic acid; DPPA, dipalmitoyl-L-α-phosphatidic acid; OAG, 1-oleoyl-2-acetoyl-*sn*-3-glycerol; DOG, 1,2-dioleoyl-*sn*-3-glycerol; DCP, dicetyl phosphate; TX-100, Triton X-100; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; $[Ca]_o$, Ca concentration in the external medium.

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