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Effects of Temperature on Viral Glycoprotein Mobility and a Possible Role of Internal "Viroskeleton" Proteins in Sendai Virus Fusion

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Abstract. The effect of temperature on fusion of Sendai virus with target membranes and mobility of the viral glycoproteins was studied with fluorescence methods. When intact virus was used, the fusion threshold temperature (20–22°C) was not altered regardless of the different types of target membranes. Viral glycoprotein mobility in the intact virus increased with temperature, particularly sharply at the fusion threshold temperature. This effect was suppressed by the presence of erythrocyte ghosts and/or dextran sulfate in the virus suspension. In these cases also, no change in the fusion threshold temperature was observed. On the other hand, reconstituted viral envelopes (virosomes) bearing viral glycoproteins but lacking matrix proteins were capable of fusing with erythrocyte ghosts even at temperatures lower than the fusion threshold temperature and no fusion threshold temperature was observed over the range of 10–40°C. The mobility of viral glycoproteins on virosomes was much greater and virtually temperature-independent. The intact virus treated with an actin-affector, jasplakinolide, reduced the extent of fusion with erythrocyte ghosts and the mobility of viral glycoproteins, while the treatment of virosomes with the same drug did not affect the extent of fusion of virosomes with erythrocyte ghosts and the mobility of the glycoproteins. These results suggest that viral matrix proteins including actins affect viral glycoprotein mobility and may be responsible for the temperature threshold phenomenon observed in Sendai virus fusion.

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Key words: Sendai virus — Virosomes — Temperature-dependent fusion — Mobility of viral glycoproteins

Introduction

The envelope of Sendai virus (SV) contains two glycoproteins, hemagglutinin-neuraminidase proteins (HN) and fusion (F) protein, which play important roles in the entry of the virus into target cells. The HN protein serves for the virion to bind to the receptor molecule on the target cell membranes, which brings both virion and target cell into close proximity, and the F protein mediates fusion of the viral envelope with the target cell plasma membrane (Hoekstra, 1990; White, 1992). The binding of Sendai virions to erythrocyte membranes occurs at temperatures over the range of 0°C to 40°C, and is relatively little temperature-dependent (Hoekstra et al., 1985; Wagner et al., 1998). In contrast to binding, the process of fusion is strongly temperature-dependent (Maeda et al, 1975; Haywood & Boyer, 1982; Hoekstra et al., 1985,1989). There is no appreciable fusion below the threshold temperature of about 20°C. Above this temperature (fusion threshold temperature), fusion rates increase sharply.

It has been reported that the mobility of the glycoproteins increases considerably at and above the fusion threshold temperature (Lee, Cherry & Bachi, 1983; Aroeti, Jovin & Henis, 1990, Hoekstra et al., 1989). A high degree of mobility of and conformational change in these glycoproteins is considered to be required for fusion to occur (Hoekstra, 1990; Lee et al., 1983, Henis et al., 1989). Electron microscopic studies of both Sendai virus and influenza virus-target cell systems indicate that there is aggregation and/or

reorganization of viral glycoproteins before fusion proceeds (Asano & Sekiguchi, 1978; Kim et al., 1979; Burger, Knoll & Verkleij, 1988; Kanaseki et al, 1997). Fusion-pore formation during virus fusion with target membranes has been suggested from patch-clamp experiments that measure the impedance of the interacting membranes (Spruce, Iwata & Almers, 1991; Zimmerberg, Curran & Cohen, 1991), and which would involve rearrangement of lipids and proteins in both virus and target membranes.

If such a fusion-pore formation is necessary for fusion to occur, it should require an increase in movement of viral glycoproteins during the pore formation. Since Sendai virus fusion strongly depends on temperature and especially shows a great increase in the fusion extent at the threshold temperature, the protein movement would be greatest around this temperature. If the rearrangement of the pore-forming proteins is due to their own characteristics with respect to temperature, a restriction of movement of viral glycoproteins by binding with target membranes or externally applied polymers would therefore alter the temperature characteristics of virus fusion. Dextran sulfate, which binds preferentially to the virus surface, suppresses the fusion of Sendai virus with erythrocyte ghosts (Ohki et al., 1992; Wagner et al. 1998). So far, these quantities (threshold temperature characteristics and mobility in viral glycoproteins) have not been measured systematically with use of various virus-target membrane systems and with various fusion affectors.

In addition, it has been known with biological cells (Bennett & Gilligan, 1993) that the cytoskeleton proteins underlying the plasma membranes affect the movement of plasma-membrane proteins. Sendai virus contains matrix (M) proteins (Peeples, 1991), which resemble cell cytoskeleton proteins underlying viral envelopes and actins (Lamb, Mahy & Choppin, 1976). It has been reported that the matrix proteins have some interaction with HN and F proteins (Sanderson, McQueen & Nayak, 1993; Sanderson, Wu & Nayak, 1994). Therefore, studies using reconstituted viral envelops bearing envelope glycoproteins HN and F, but not containing viral matrix proteins, on fusion and protein mobility with respect to temperature, may provide some information regarding involvement of viral matrix proteins in the threshold phenomenon observed in fusion of Sendai virus with target membranes.

In this paper, therefore, we have studied the effect of temperature on virus fusion and mobility of viral glycoproteins, using intact Sendai virus and its reconstituted viral envelopes (virosomes) with and without treatment with actin-affectors, (e.g., cytochalasins and jasplakinolide) and various target membranes (erythrocyte ghosts and liposomes bearing viral receptors) in the presence and absence of dextran sulfate.

Materials and Methods

Chemicals

All fluorescent probes were purchased from Molecular Probes, Eugene, OR. Dextran sulfate of molecular weight 40,000 was obtained from ICN Biomedicals, Costa Mesa, CA. Phospholipids (egg-phosphatidylcholine (PC), phosphatidylethanolamine derived from egg-PC (PE) and lysophosphatidylcholine (l-sterol,-2 hydroxyglycero-3 phosphatidylcholine (SLPC)) were obtained from Avanti Polar Lipids, Alabaster, AL. Sephadex G-75 was from Pharmacia Fine Chemicals. Octylglucoside (n-Octyl β-D-Glucopyranoside), Glycophorin A (from human blood, type MM), trypsin (from soybean) and trypsin inhibitor (from soybean) were all purchased from Sigma Chemical, St. Louis, MO. The actin-affecting drugs jasplakinolide and cytochalasin D were purchased from Calbiochem, San Diego, CA and Sigma, respectively. All other chemicals used were of reagent grade.

Preparation of Sendai Virus

The Cantell strain of Sendai virus was grown in the allantoic cavity of embryonated chicken eggs, harvested at 72 hrs after inoculation, and purified by a method similar to those previously described (Al-Ahdal, Abidi & Flanagan, 1986). Briefly, the infected allantoic fluid was clarified by low-speed centrifugation, and virus in the supernatant was pelleted through a 20% sucrose buffer layer (20% sucrose in 0.15 M NaCl/10 mM Tris, pH 7.4 (NTB)) by centrifugation at 91,000 $\times$ g in a Beckman rotor (SW28) for 1 h at 4°C. The pellet was then resuspended in the 20% sucrose buffer and centrifuged at 150,000 $\times$ g in a Beckman rotor (SW41) for 2 h through a discontinuous sucrose density gradient. The virus at the interface between the 40% and 45% sucrose layers was collected. The collected virus suspensions were dialyzed against in NTB and stored at −70°C. The preparations had titers of 10,000–20,000 hemagglutinating units and protein concentrations of 0.5–1.0 mg/ml. Virus stocks for experiments were prepared by sedimenting the virus from NTB and resuspending them in NTB or if necessary, another buffer solution, NPB (150 mM NaCl/10 mM phosphate, pH 7.4), to a specified concentration as needed. The virus stocks were kept at −70°C until use.

Preparation of Erythrocyte Ghosts

Human erythrocyte ghosts were prepared from freshly drawn human blood according to the published method (Steck & Kant, 1974; Leonards & Ohki, 1983) and resuspended in NTB at 4°C. The prepared erythrocyte ghosts were used for the experiments within a week. The protein concentrations of virus and erythrocyte ghost preparations were determined by the method of Bradford (1976), using bovine plasma γ-globulin as the protein standard.

Reconstituted Viral Envelopes (Virosomes)

Reconstituted viral envelopes bearing both HN and F proteins were prepared by a similar procedure as published (Bagai et al, 1993); Briefly, octylglucoside (OG) was added to 1 ml of virus (3 mg/ml) suspension in NTB containing 1 mM Ca^{2+} and 1 mM Mg^{2+} to give a final concentration of 1% (wt/wt) OG. The mixture was mixed well and held at room temperature for 30 min and further kept for 2 h at 4°C and then centrifuged at 63,600 $\times$ g for 1 h at 4°C. The pellet contained nucleocapsid and other internal viral proteins. The supernatant, which contained the viral envelope proteins and lipids, was placed into dialysis tubing (mol. wt. cut off:

12,000–14,000) and dialyzed over 24 h with 4 exchanges of NTB with constant stirring at 4°C. During dialysis, the viral envelope vesicles (virosomes = HN-F virosomes) were formed. The size of the HN-F virosomes was measured with a submicron particle analyzer (Coulter, Model N4) and found to be on average 250 nm in diameter. The proteins in the intact virus and virosomes were analyzed by SDS-PAGE with Coomassie blue (BioRad Mini-Protein II with Laemmli buffer system (Laemmli, 1970)) and also by use of silver staining (Wray et al., 1981). The position corresponding to the M protein band on the gel was determined by comparing with the protein bands of the intact Sendai virus and that of the M protein isolated according to the published method (Hewitt & Nermut, 1977). No M-protein band was visually observed on the gel for virosomes. The hemagglutinin titer of such virosome preparations had about one hemagglutinin unit (about 2 times in concentration) lower than the intact virus. To make virosomes that lacked the F protein (HN virosomes), first, the intact Sendai virus was treated with trypsin. For this, the intact virus (3 mg) was incubated with 4% (w/w) of trypsin in 1.0 ml NTB at 37°C for 30 min, and then the reaction was terminated by adding a 2-fold excess of trypsin inhibitor into the virus suspension at room temperature for 20 min. The trypsin-treated virus was then suspended in 10 ml of NTB and washed three times by centrifugation at $18,000 \times g$. The pellet was resuspended in 1 ml of NTB containing 1 mM Ca^{2+}, 1 mM Mg^{2+} and 1% OG. Hereafter, we used the same procedure as for preparation of HN-F virosomes from intact virus. The SDS-PAGE gels of the trypsin-treated virosomes (HN virosomes) and the HN-F virosomes were scanned by an Imaging Densitometer (BioRad, Model GS-700) and analyzed by use of the program "Molecular Analyst" (BioRad). The analysis showed a loss of about 80% of F protein for the HN virosomes compared to HN-F virosomes using the ratio of F to HN in each sample. The HN-F and HN virosomes were stored at −20°C until the experiments.

Treatment of Sendai Virus and Virosomes with Actin Affectors

The treatment of Sendai virus or virosomes with actin-affectors was done as follows: an aliquot of 1.0 mM jasplakinolide in DMSO was added to 0.2 ml NTB containng 20 μg of fluorescent-labelled virus (either R_{18} or fluorescamine) or 10 μg virosomes, and mixed well by vortexing briefly and then the mixture was left at room temperature for 30 min. Then, the incubated mixture was added to 1.8 ml of NTB prepared at a specified temperature in a cuvette in the spectrofluorimeter for fusion or polarization experiments. The concentrations of jasplakinolide indicated in the experimental results were those at the incubation stage. Therefore, the final concentration of the drug was one tenth of that at the incubation stage. This drug is considered to exert an effect similar to phalloidin on actin filaments by stabilizing the filament organization, and it is permeable to cell membranes. Another actin-affector, cytochalasin D, was also applied to Sendai virus in a similar manner as jasplakinolide. However, this drug is considered to destabilize the actin filament when it binds to actin.

Liposomes Bearing Glycophorin

The lipids (2.5 mg of PC or PC/PE (2:1) with or without 10 mol% LSPC) were mixed in chloroform/methanol (2:1), and then the solvent was evaporated completely in vacuum. Then the lipids were suspended in 1 ml NTB containing 1% (w/w) OG. After the lipid suspension became clear, 2.5 (w/w) % glycophorin (GP) with respect to the total lipids was added to the lipid suspension and mixed well. This lipid-GP suspension was kept at room temperature for 1 h. Then the lipid-GP suspension was dialyzed over 24 h with 4 changes of NTB (500 times volume of the dialysate) with constant stirring at 4°C. During dialysis, the liposomes bearing GP were formed. The size of the GP-liposomes was measured with a submicron particle analyzer (Coulter, Model N4) to be on average 180 nm in diameter. As far as the size of the GP-liposomes was concerned, there was no difference between those with and without lysophospholipids (LSPC) within its standard error (±20 nm). GP is considered a receptor for Sendai virus (Wybenga et al., 1996). Liposomes without GP were prepared in the same manner but skipping the process of GP addition in the above procedures described for the liposomes bearing GP. The size of these liposomes was on average 150 ± 15 nm in diameter. Lipid concentration was determined by phosphate analysis (Allen, 1940).

Fluorescence Probe Labelling

Octadecylrhodamine B (R_{18}) Labelling of Virus (Hoekstra et al, 1984)

A 10 μl aliquot of an ethanolic solution of R_{18} (1 mg/ml) was rapidly injected into 1 ml of Sendai virus (1 mg virus protein/ml in NTB) while vortexing. After incubation of the suspension for 1 h at room temperature, the unincorporated R_{18} was removed by gel-filtration through a Sephadex G-75 column with NTB as an elution buffer.

Fluorescamine (FLCN) Labelling of Viral Envelope Proteins (Wagner et al, 1998)

5 μl of an acetonitrile solution of fluorescamine (5 mg/ml) was added to 0.5 ml of Sendai virus (1 mg virus protein/ml NTB) while vortexing and then the suspension was incubated for 1 h at room temperature. These procedures were done in the dark. Then, the unbound flurophore was removed by passing the virus suspension through a Sephadex (G-75) column as above.

DPH (1,6-Dipheny1-1,3,5-hexatriene) Labelling of Viral Lipids

Sendai virus (1 mg protein/ml of NTB) was incubated with 2 μg of DPH in DMSO (1mg/ml) at 37°C for 1 h. Then, the virus suspension was passed through a Sephadex (G-75) column to remove the unincorporated fluorophores.

Fluorescence probe (R_{18} and FLCN) labelling of virosomes was also performed in a manner similar to that described for the intact virus, respectively, except for the protein concentration of 0.2–0.4 mg/ml. The amount of fluorescence probe in virosome incubation medium was reduced proportionally to its suspension volume.

Fusion Assay

The fusion of Sendai virus with target membranes was measured by the R_{18} dequenching assay (Hoekstra et al., 1984). The assay is based on the relief of self-quenching of the fluorescence probe as it is diluted into the target membrane as a result of fusion. In the assay, aliquots of the R_{18}-labelled virus (20 μg) or virosomes (20 μg) and of target cells (60 μg erythrocyte ghosts or 100 μg liposomes) were mixed in 2 ml of NTB with and without dextran sulfate at a given temperature (10°C–40°C). The increase in fluorescence signal resulting from dequenching was monitored with respect to time, using a spectrofluorimeter (Perkin-Elmer, LS-5)

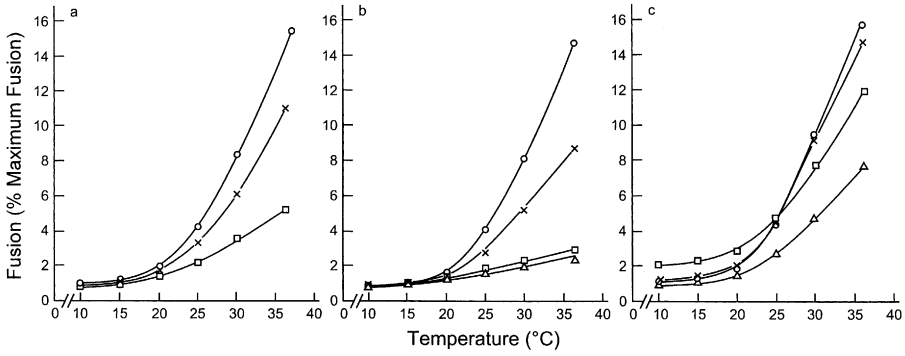

Fig. 1. The effect of temperature on Sendai virus (SV) fusion with erythrocyte ghosts and liposomes as target membranes. The fusion activity is represented by percent of maximum fusion during the first 10 minutes of SV-target membrane interaction. (*a*) Fusion of SV (10 μg/ml) with erythrocyte ghosts (30 μg/ml): SV and erythrocyte ghosts alone (○), in the presence of 0.2 mg/ml (X) and 0.5 mg/ml (□) of dextran sulfate. (*b*) Fusion of SV with liposomes (50 μg/ml): SV with 2.5% GP-liposomes (PC/PE = 2:1) (X), with 2.5% GP-liposomes (PC/PE = 2:1) bearing 10%LPC (□), with PC liposomes (Δ), and with erythrocyte ghosts as control (○). (*c*) Fusion of drug-treated SV(10 μg/ml) with erythrocyte ghosts: SV treated with 2 μM cytochalasin D (□), 2 μM jasplakinolide (Δ), 0.5% DMSO (X), and untreated SV (10 μg/ml) as control (○).

with $\lambda_{excitation}$ = 550 nm and $\lambda_{emission}$ = 585 nm. The fluorescence signal of the self-quenched state of the virus was first recorded as I_0, and then the time-dependent fluorescence signal (I) at 585 nm was recorded for about 20 min as fusion proceeded. At the end of each experiment, 0.2% of Triton X-100 was added to the reaction mixture, and the maximum value (I_{tri}) of fluorescence was obtained. This value is considered as 100% fusion of the virus with the erythrocyte ghosts. The extent of fusion (F) is proportional to % fluorescence dequenching and is defined as

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

It has been found that when the initial rate of dequenching is measured (e.g., within 2 min), the dequenching of R_{18} is due mostly to the fluorophore transferred to the target membrane by membrane fusion (Ohki, Flanagan & Hoekstra, 1998).

Fluorescent Polarization Measurements

Fluorescent polarization, P, of fluorescamine (FLCN) bound to viral protein was obtained by measuring the parallel and perpendicular components of the fluorescence upon excitation with vertically polarized light at an appropriate wavelength (380 nm for fluorescamine) with a steady-state fluorometer (Perkin-Elmer LS-5):

$$P = (I_{\parallel} - GI_{\perp})/(I_{\parallel} + GI_{\perp}) \quad (2)$$

where $I_{\parallel}$ and $I_{\perp}$ are the parallel and perpendicular components of the emission with respect to the excitation light, respectively, and G is a factor to correct the anisotropic geometry of the instrument (Lakowicz, 1983). The samples containing FLCN were excited by linearly polarized light of 380 nm and the respective parallel and perpendicular components of fluorescence with respect to exciting light were measured at 475 nm at various temperatures in the range of 10°C to 40°C. An aliquot of labelled virus (20 μg protein) alone or mixed with aliquots of erythrocyte ghosts (60 μg protein) were suspended in 2 ml of NTB with or without dextran sulfate (0.2 mg/ml) at each specified temperature (10, 15, 20, 25, 30 and 37°C) for 2–3 min for equilibration. Then, each component of fluorescence was measured. The time needed to record one of the fluorescence-polarization components was at least 1–2 min to assure a stable fluorescence signal. At each temperature, a new sample was mixed and then the polarization measurement was made as described above. For experiments using virosomes, fluorescamine-labelled virosomes (10 μg) alone or the labelled virosomes (10 μg) and erythrocyte ghosts (30 μg) were suspended in 2 ml of NTB with and without dextran sulfate (0.2 mg/ml) at each specified temperature and the polarization measurement was performed in a manner similar to those for the intact virus mentioned above. The polarization measurement on drug-treated virus was also performed in similar manner to that for drug-untreated virus.

The experimental points presented in all figures in the result section are the average values of at least three measurements for a preparation of the samples and the experimental errors (SE) were all within 10%.

Results

Effect of Temperature on Fusion of Sendai Virus with Target Membranes

The fusion of Sendai virus with various target membranes (erythrocyte ghosts and lipid vesicles bearing viral receptor) was measured at temperatures ranging from 10–40°C.

In the first series of this type of experiments, the extent of fusion (% maximum fusion for the first 10 min) of SV with erythrocyte ghosts was measured at different temperatures. The results are shown in Fig. 1*a*. Below 20°C, the extent of fusion activity detected was not significant or very low. Fusion activity increased significantly between 20 and 25°C and this is called "fusion threshold temperature" (*see* Fig. 1*a*, ○). In the presence of dextran sulfate (0.2 mg/ml) in the same virus-erythrocyte ghost suspension, the initial rate of fusion was suppressed by approximately 30% (Fig. 1*a*, x). A higher concentration of dextran sulfate (0.5 mg/ml) suppressed fusion more than 2-fold (Fig. 1*a*, □) compared to that with dextran sulfate of 0.2 mg/ml. However, the presence of dextran sulfate did not alter the fusion threshold temperature.

In order to examine the nature of target membranes involved in the temperature-threshold phenomenon of virus fusion, for the second series of the experiments, liposomes that lacked cytoskeleton proteins were used as target membranes instead of erythrocyte ghosts. When the liposomes (PC/PE(2:1)) bearing glycophorin (GP-liposomes) were used, a similar temperature dependence of fusion was obtained as for the virus-erythrocyte system. Glycophorin has been considered a receptor for Sendai virus (Wybenga et al, 1996). Although the concentration of glycophorin on the liposomes was approximately the same as that of erythrocyte ghosts, the extent of fusion was less than that of virus-erythrocyte ghosts. However, the extent of fusion was increased by raising the GP-liposome concentration (*data not shown*). The important finding with these experiments was that the threshold temperature was unchanged with this system (Fig. 1*b*, x). This suggests a possibility that the threshold-temperature phenomenon is not due to the cytoskeleton of target membranes since GP-liposomes do not contain cytoskeleton proteins. For the case of the GP-lipid vesicles containing 10 mol % lysophospholipid (SLPC), virus fusion was almost completely suppressed, as indicated in Fig.1*b* (□), to an extent similar as for phosphatidylcholine (PC) vesicles without glycophorin (indicated by Δ in Fig. 1*b*).

In the third series of the experiments, the effect of actin-affecting drugs (cytochalasin D and jasplakinolide) on the temperature-dependent fusion activity of Sendai virus with erythrocyte ghosts was investigated. It is known that cytochalasins disrupt actin filament organization, while jasplakinolide stabilizes the actin filament organization similarly to phalloidin. At 37°C, the extent of fusion of the virus treated with 2 μM jasplakinolide was reduced to about 50% of that for the intact virus with erythrocyte ghosts, while that for the virus treated with 2 μM cytochalasin D was reduced by 16%. The effect of temperature on the fusion inhibition by these drugs at 2 μM is shown in Fig. 1*c*. Jasplakinolide suppressed viral fusion more effectively than cytochalasin D in the higher temperature range above the threshold temperature. However, at temperatures lower than the threshold temperature, the cytochalasin D-treated virus showed a slightly greater extent of fusion than the untreated virus (*see* Fig. 1*c*).

Fusion experiments of Sendai virus with erythrocyte ghosts as a function of concentration of actin-affecting drugs were performed. The degree of the reduction effusion was dependent on the concentration of the drugs, which is shown in Fig. 2. Similar experiments using GP-liposomes as the target membranes instead of erythrocyte ghosts were also performed under the same experimental conditions. The results (the relative inhibitory effect of the drugs) were similar to those for virus-erythrocyte ghosts, as shown in Fig. 2. These results suggest that the actin-effecting drugs affect the "viroskeleton" components of virus, but not those of the target membranes. The effect of drug solvent, DMSO, on fusion of Sendai virus with erythrocyte ghosts was also investigated, as a control. The results show that in the presence of 0.5% DMSO, which was the maximum concentration used in our experiment, the temperature-dependent fusion activity of the virus was similar to that in the absence of the drug, indicating that the presence of DMSO used in these experiments had no effect on the virus-mediated fusion activity within experimental errors (*see* Fig. 1*a*).

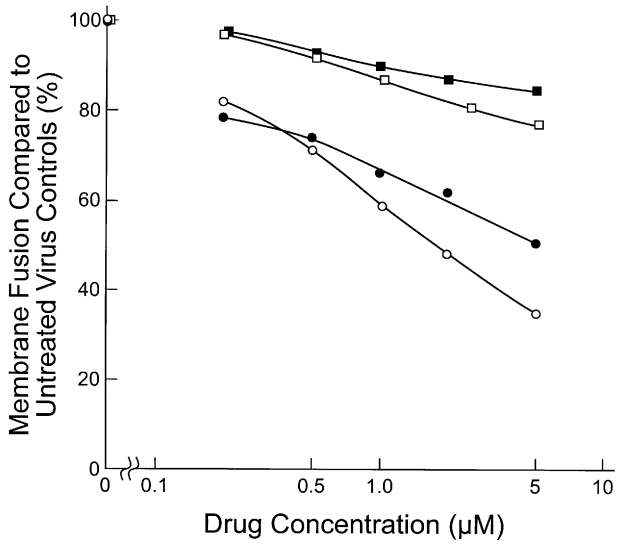

Fig. 2. Relative fusion of Sendai virus treated with actin affectors (cytocholashin D and jaspalakinolide) with erythrocyte ghosts (30 μg/ml) (*empty symbols*) or 2.5% GP-liposomes (50 μg/ml) (*filled symbols*) in NTB at 37°C are shown as a function of drug concentrations. cytochalasin D (□ and ■) or jasplakinolide R18-SW (10 μg/ml) treated with (○ and ●).

Effect of Temperature on the Mobility of Viral Glycoproteins, HN and F, in Intact Sendai Virus

The effect of temperature on the steady-state polarization of fluorescamine (FLCN) bound to the viral glycoproteins on the envelope of Sendai virus was investigated in the following four cases; i) virus alone, ii) the virus interacting with target cells; iii) in the presence of dextran sulfate in virus-erythrocyte ghost suspension and iv) the virus treated with actin-affecting drugs, cytochalasin D and jasplakinolide. The results are shown in Fig. 3.

In the case of intact Sendai virus alone, the polarization of the fluorophore decreased with increase of temperature, which suggests an increase in the random motion of the outer segments of the viral glycoproteins with temperature, since the fluorophore was applied to virus particles in suspension. A major decrease in polarization was observed between 15°C

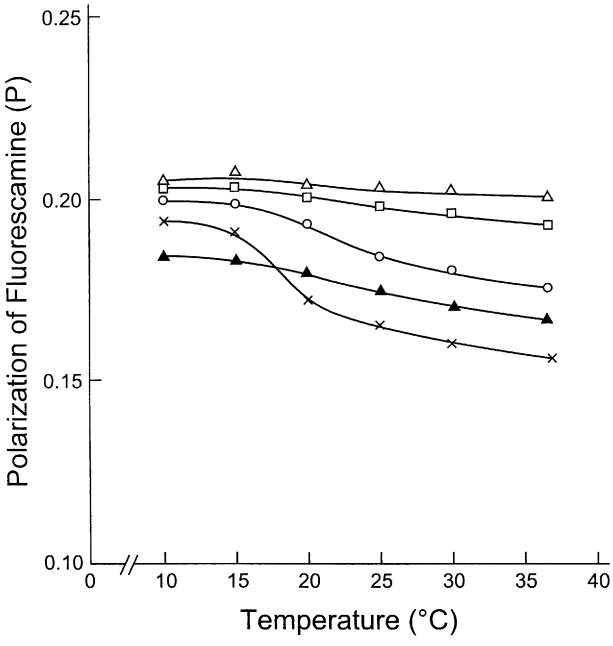

Fig. 3. The effect of temperature on mobility of Sendai virus (SV) glycoproteins, HN and F. The SV was labelled with fluoresamine (FLCN) and the polarization of the fluorescamine was measured as described in Materials and Methods. FLCN-SV (10 μg/ml) alone (X); FLCN-SV and erythrocyte ghosts (30 μg/ml) in the absence (○) and presence (□) of 0.2 mg/ml dextran sulfate; FLCN-SV treated with 2 μM cytochalasin D (▲) or 2 μM jasplakinolide (Δ).

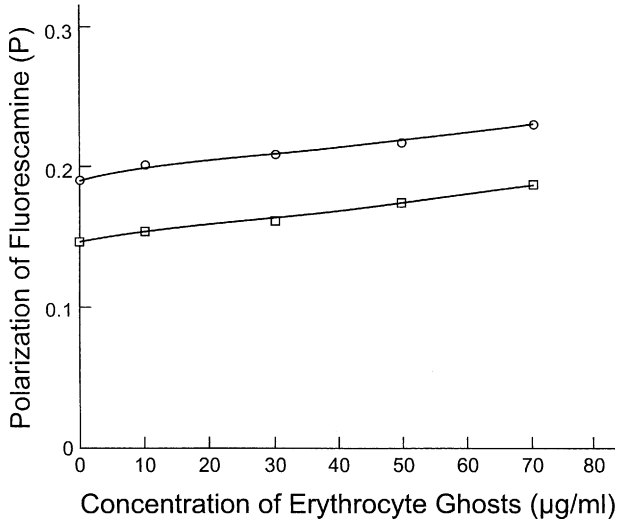

Fig. 4. Fluorescence polarization of FLCN attached to Sendai virus (10 μg/ml) with respect to various amounts of erythrocyte ghosts at 10°C (○) or 30°C (□).

and 25°C, which is in the range of the fusion-threshold temperature. This implies that protein motion increased greatly in this temperature range. A rather small but steady decrease in polarization was observed above 25°C. The decrease in polarization was retarded in the case of virus interacting with erythrocyte ghosts and the magnitude of the polarization was further retarded in the presence of dextran sulfate in the virus-erythrocyte-interacting system, indicating further restriction in mobility of viral envelope glycoproteins in comparison with the case of virus alone. Sendai virus treated with 2 μM jasplakinolide showed similar restriction of the protein motion as that in the presence of both erythrocyte ghosts and dextran sulfate. On the other hand, for the virus treated with 2 μM cytochalasin D, the FLCN polarization was lower than that for the intact virus alone in the temperature range of 10–20°C, while in the temperature range above the fusion threshold, it was slightly greater than those of the intact virus alone (Fig. 3).

The results presented in Fig. 4 show that there was an increase in the restriction of mobility of Sendai virus glycoproteins with increasing concentration of erythrocyte ghosts, which contain receptors for Sendai virus. Polarization (P) measurements for the virus-erythrocyte ghost system were performed at 10°C and 30°C. There was an increase in polarization at both temperatures as the amount of erythrocyte ghosts increased, indicating an increasing restriction of molecular mobility as a result of interaction of more viral proteins with their receptors (glycophorin) on erythrocyte membranes. The magnitude of difference in P between 10°C and 30°C is consistent with differences shown in Fig. 3.

Effect of Temperature on the Mobility of Lipids in Sendai Virus Envelope

To examine the effect of temperature on the mobility of lipids in the viral envelope, the virus was labelled with DPH as described in Materials and Methods. The polarization of DPH at various temperatures is shown in Fig. 5. The polarization of fluorophore decreased monotonically in the temperature range from 10°C to 37°C. The presence of dextran sulfate did not affect the polarization of DPH, suggesting that dextran sulfate has no significant effect on lipid mobility in the viral envelope. These results are in contrast to those obtained with fluorescamine-labelled virus in the fusion "threshold temperature" region, where the polarization of the fluorophore attached to the envelope glycoproteins decreased greatly (Fig. 3). DPH mobility in membranes is regarded as a measure of lipid mobility (Shinitzky & Barenholz, 1974) and, therefore, the non-linear reduction in the polarization obtained with FLCN reflects those due to mobility of protein segments.

Effect of Temperature on Virosome Fusion Activity

In this case, the fusion of various virosomes (HN-F-virosomes and HN-virosomes) with erythrocyte ghosts was performed in comparison to that of the

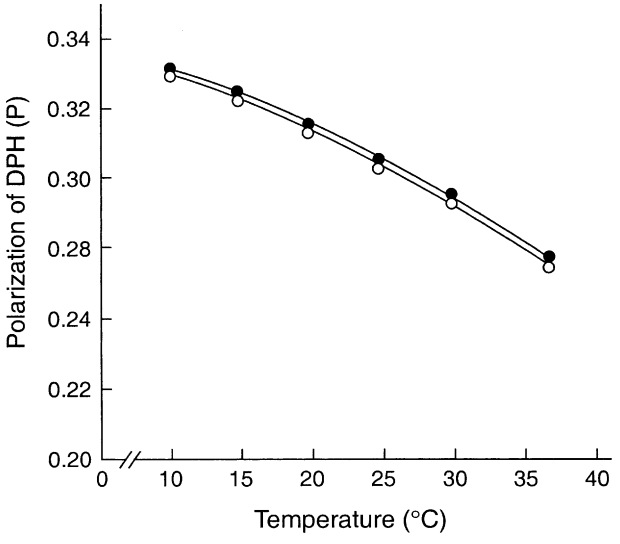

Fig. 5. The effect of temperature on the mobility of Sendai virus (SV) lipids in the viral envelope. The polarization of DPH incorporated into Sendai virus envelope lipids was measured as a function of temperature in the absence (○) and presence (●) of 0.2 mg/ml dextran sulfate.

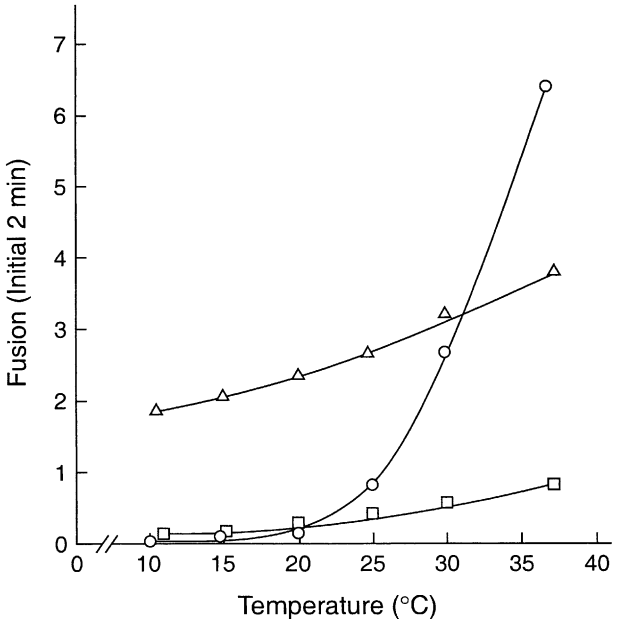

Fig. 6. Fusion of R_{18} -labelled Sendai virosomes (10 μg/ml) with erythrocyte ghosts (30 μg/ml) in 2 ml of NTB as a function of temperature. Fusion (% of maximum fusion) measured during the initial 2 min is plotted with respect to temperature. Symbol Δ refers to HN-F virosomes; symbol □, to HN virosomes (trypsin-treated virosomes) and symbol ○, to untreated, intact Sendai virus.

intact Sendai virus with erythrocyte ghosts. The results of fusion of intact virus and virosomes (HN-F- and HN-virosomes) with erythrocyte ghosts measured over the initial 2 min are shown in Fig. 6. Symbol ○ corresponds to control, the intact Sendai virus. Symbols Δ and □ correspond to use of HN-F virosomes and HN-virosomes, respectively. In order to determine fusion events clearly, the data obtained at the initial 2 min was plotted in the Fig. 6, which is mainly due to the true membrane fusion according to our earlier work (Ohki, et al., 1998). As seen in Fig. 6, the distinct fusion of the HN-F-virosomes with erythrocyte ghosts was observed at all temperatures (10–37°C). It is significant to note that the fusion occurred even at 10°C (Δ), while the intact virus did not fuse with erythrocyte ghosts at this temperature (○), and that no fusion threshold temperature was observed for the HN-F-virosome case (Δ) (Fig. 6). On the other hand, the HN-virosomes did not show any appreciable fusion with erythrocyte ghosts (□). This indicates that the presence of the F protein in the viral envelope was necessary for virus fusion to occur in this fusion system and also that the observed fusion of the HN-F-virosome with target cells was not due to the effect of detergent, which was used in the preparation of the extent of fusion of virosomes with erythrocyte ghosts. The extent of fusion of HN-F-virosomes treated with either 2 μM cytochalasin D or 2 μM jasplakinolide with erythrocyte ghosts was not different within the experimental errors (*data not shown*) from those obtained using the untreated virosomes. These results suggest that the drugs do not significantly suppress viral membrane fusion by acting directly on viral HN and F proteins, but may act primarily through viral internal proteins such as actin, which is a part of the matrix proteins within the virus particles.

Effect of Temperature on Mobility of Viral Envelope Glycoproteins on Virosome Membranes

Polarization of fluorophore (FLCN) attached to the viral glycoproteins of the virosomes was measured in the range of 10–37°C. The results are shown in Fig. 7. The magnitude of polarization measured was much smaller at all temperatures than that of the intact virus at 37°C, indicating considerable mobility of glycoproteins, and there was little change over the temperature range (10–37°C). This is different from the large decrease in polarization observed for the fluorophore attached to the viral glycoproteins of the intact virus at around the fusion threshold temperature (*compare* Fig. 3 with Fig. 7). The presence of erythrocyte ghosts as well as dextran sulfate in the virosome suspension increased the magnitude of polarization of the fluorophore, the trend of which was similar to that observed for the intact virus systems. However, the application of the actin-affecting agent, jasplakinolide, up to 5 μM, to the virosome suspension resulted in virtually no effect on the mobility of viral glycoproteins as compared with that in untreated virosomes. Together with the results shown in Fig. 3, this experimental result suggests that

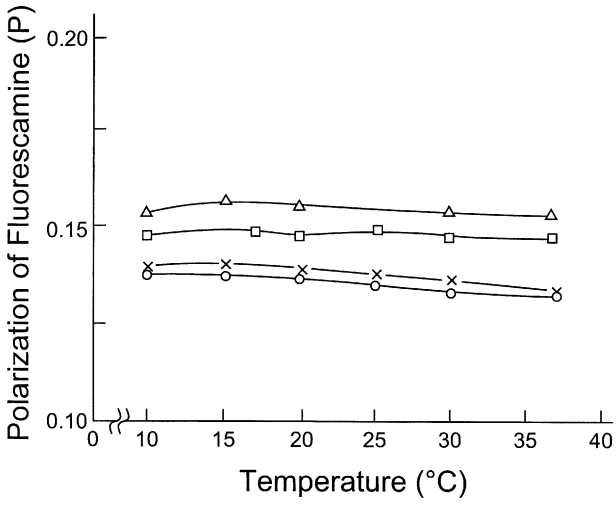

Fig. 7. The effect of temperature on mobility of glycoproteins on virosomes. Fluorescence polarization of fluorescamine (FLCN) attached to HN-F virosomes (5 μg/ml) are shown as a function of temperature. Symbol ○ refers to virosomes alone; symbol □, to virosomes with erythrocyte ghosts (15 μg/ml); symbol Δ, to virosomes in the presence of 0.2 mg/ml dextran sulfate; and symbol, X, to virosomes treated with 2 μM jasplakinolide.

jasplakinolide affects the mobility of envelope glycoproteins only in the presence of viral skeletal proteins, including the M-proteins in viral particles.

Discussion

In the present studies, the following findings were made: 1) When the intact Sendai virus was used for fusion with target membranes bearing the viral receptors, the fusion threshold temperature was observed and it was not altered regardless of use of different types of target membranes. 2) The binding of Sendai virion to viral receptors of target membranes, as well as to dextran sulfate, restricted the motion of the viral glycoproteins, but the fusion threshold temperature was not altered. 3) The reconstituted viral envelopes (virosomes) showed fusion activity with erythrocyte ghosts even at temperatures lower than 20°C and no threshold temperature of fusion was observed in the temperature range of 10–37°C. 4) The motion of viral glycoproteins on the virosomes was virtually temperature-independent over the range of 10–37°C, while those of the intact virus increased with increase in temperature, particularly at the fusion threshold temperature. 5) An actin affector, jasplakinolide, inhibited the fusion of Sendai virus with erythrocyte ghosts as well as GP-liposomes in a similar manner and reduced the mobility of viral glycoproteins over the range of 10–37°C, but did not affect the fusion of virosomes with erythrocyte ghosts. 6) For jasplakinolide-treated Sendai virus, the mobility (segmental motion) of viral glycoproteins was reduced over the range of 10–37°C, whereas virtually no change in mobility of viral proteins was observed for virosomes treated with the same agent.

The initial rate of fusion between Sendai virus and erythrocyte ghosts was virtually zero below 20°C, particularly when it was measured within 2 min (the fluorescence dequenching signal measured within the initial 2 min is mainly due to the true membrane fusion, as mentioned in Materials and Methods), and increased sharply at around 20°C (fusion threshold temperature) and increased further with increasing temperature (*see* Fig. 6, ○). In this work, we have shown that this temperature behavior was observed regardless of the target membranes, as long as the intact virus was used. It has been suggested (Lee et al., 1983; Aroeti et al., 1990; Hoekstra, 1990) that the temperature-induced virus fusion is due to the mobility of viral glycoproteins. However, when the virus interacted with target membranes bearing its receptors (e.g., erythrocyte ghosts), the mobility of viral glycoproteins was suppressed (Fig. 3), while virus fusion occurred (Fig. 1). The degree of suppression of mobility was dependent on the concentration of target membranes (Fig. 4); the more target membranes, the more reduction in viral glycoprotein mobility. When dextran sulfate was present in free virus suspension, the mobility of glycoproteins was reduced (*data not shown*) and when dextran sulfate was present in virus-ghost suspension, the reduction of mobility was further enhanced (*see* Fig. 3), while virus fusion was suppressed (Fig. 1 *a*). Therefore, the mere mobility (segmental motion) of viral glycoproteins does not correlate well with the rate of virus fusion. In both cases, however, the fusion threshold temperature was not altered. Also, the temperature behavior of virus fusion with lipid vesicles bearing glycophorin, which is considered one of the Sendai virus receptors (Wybenga et al. 1996), was similar to that when erythrocyte ghosts were the target membranes (*see* Fig. 1 *b*, x). Since the target membranes, GP-liposomes, which do not contain cytoskeleton proteins, exhibited the same temperature threshold as erythrocyte ghost membranes (Fig. 1 *a* and *b*) and since also the target liposomes (ganglioside (G_{D1a} - liposomes) bearing receptor molecules different from glycophorin have been shown to demonstrate a similar temperature threshold (Haywood & Boyer, 1982), the temperature threshold fusion behavior most likely is not due to the nature of the target membranes.

It should be noted, however, that when liposomes consisting of acidic lipids (e.g., cardiolipin or phosphatidylserine) were used as target membranes for Sendai virus, it was found that Sendai virus fused with the liposomes to some degree at temperatures lower than the threshold temperature, 20°C, (Hoekstra et al 1989, Zschornig, Arnold & Ohki, 1993) and the temperature threshold behavior was not so sharp

as for virus-cell fusion (Hoekstra et al. 1989). In this regard, Amselem et al. (1986) showed that the HN-virosomes fused with acidic lipid (phosphatidylserine) membranes, whereas in our present experiments, the HN virosomes did not fuse with erythrocyte ghosts or with the GP-liposomes. It is possible that the fusion of Sendai virus with acidic lipid membranes may have a slightly different mechanism from that for the fusion of Sendai virus with target membrane bearing virus receptors.

Externally applied dextran sulfate inhibited the mobility of viral glycoproteins for intact virus, which is reasonable because they probably bind to the envelope glycoproteins, according to previous work (Ohki et al., 1992 and Wagner et al., 1998). Furthermore, an actin-binding drug, jasplakinolide, which is considered to stabilize the actin filament network, inhibited the mobility of the viral glycoproteins of the intact virus (Fig. 3) and also reduced the extent of virus fusion (Fig. 1 *c*). Both agents, dextran sulfate and jasplakinolide, inhibited the fusion of Sendai virus with erythrocyte ghosts. However, the site of action of these agents on viral glycoproteins may be different: dextran sulfate interacts with glycoproteins externally and probably interferes with the close approach of the virion to the target membrane. On the other hand, jasplakinolide seems not to interact with the viral glycoproteins directly, judging from the experimental results with virosomes, where jasplakinolide did not affect the fusion of virosomes with erythrocyte ghosts and did not suppress the glycoprotein mobility (*see* Fig. 7). This infers that the jasplakinolide acts on the actin filament network to which the M-proteins are associated and stabilizes it and, thus, in turn, the viral glycoproteins will be stabilized. On the other hand, at temperatures lower than the fusion threshold temperature, cytochalasin D-treated viruses showed greater mobility of their glycoproteins than untreated viruses alone. This suggests that cytochalasin D disrupts the actin network, which in turn affects the M protein network, which influences the glycoprotein mobility. Regarding the effect of cytochalasins on virus fusion, there have been two studies; one of them showed that cytochalasin B at 4 μM inhibited Sendai virus-induced cell-cell fusion of Lettrée ascites cells (Pasternak & Micklem, 1973) and the other showed that Sendai virus-induced cell-cell fusion of Ehrlich ascites tumor cells was inhibited completely by cytochalasin D at 5 μg/ml (Miyake, Kim & Okada, 1978).

Thus, the experimental results described above indicate in general that the mobility of viral glycoproteins parallels the extent of viral fusion, but there are a few unparallel experimental results. These may be due to the fact that the protein mobility may be affected by at least two factors; due to local binding between virus and target membranes, which lead to fusions, and due to overall or non-local binding, e.g., binding with dextran sulfate. This may result in some unparallel results between the measured protein mobility and virus fusion. It is possible to discern the two contributions (overall binding and local binding) to the protein mobility by performing two different sets of experiments with different mixing of particles, e.g., mixing virus and target membrane first and after incubation, applying dextran sulfate, or mixing virus and dextran sulfate first and after incubation, adding target membranes, since such different mixing and incubation resulted in different extents of virus fusion (Ohki et al, 1992). Another general conclusion is that the temperature threshold characteristics of fusion of Sendai virus with target membranes seems to be involved with internal "viroskeleton" proteins and not mere mobility of envelope glycoproteins.

Previous studies have investigated the effect of temperature on the mobility of the viral glycoproteins of intact virus but have not studied the reconstituted virus envelopes, virosomes, and also have not examined the effect of dextran sulfate and actin-affectors (e.g., jasplakinolide or cytochalasins). Lee et al. (1983) found that the steady-state fluorescence anisotropy of another fluorophore (eosin-5-isothiocyanate) attached to viral glycoproteins decreased with increasing temperature, which was similar to our finding, but they did not examine the behavior at temperatures between 10 and 20°C. We detected a sharp increase in protein mobility around 20°C for the intact free virus. Lee et al. (1983) also found that there was a large break in rotational mobility at around 30–35°C, which they attributed to hemolysis of erythrocytes. Aroeti et al. (1990) showed significant changes in glycoprotein rotational mobility at 22°C in bound and fused Sendai virions with phosphorescence emission anisotropy measurements. The latter authors attributed this change to possible aggregation of the HN and F glycoproteins at that temperature. Although there was general agreement among the previous studies that the mobility of viral glycoproteins is affected by temperature, there were considerable differences in the details. These differences may be due to the use of different methods and the use of bound versus free virions. Hoekstra et al. (1989) labelled viral glycoproteins with eosin-5-maleimide on virions and found that at 22°C there was a relief of self-quenching, which they interpreted as protein self-clustering occurring at lower temperatures, which then got relieved as the temperature increas above 22°C.

The degree of polarization of the fluorophore attached to viral glycoproteins measures the mobility of viral glycoproteins, particularly for the portion of the proteins extending to the ectodomain, since the fluorophore was applied only externally. It does not measure the mobility of the lipids in the viral envelope, since control experiments for lipid mobility (Fig. 5) indicated that there was no sharp decrease in

fluorophore polarization around the fusion threshold temperature.

In contrast to the intact virus, we have found that the reconstituted viral vesicles (virosomes), which lacked the M proteins, fused with erythrocyte ghosts even at low temperatures (Fig. 6) at which the intact virus did not fuse; also, for the virosomes and erythrocyte-ghost interaction system, there was no fusion threshold temperature in the temperature range studied. The treatment of virosomes with the actin-affectiong drug, jasplakinolide, did not affect the fusion of virosomes with erythrocyte ghosts, nor the mobility of viral glycoproteins up to high concentrations (5 μM), at which the fusion of intact virus with erythrocyte ghosts or GP-liposomes and viral glycoprotein mobility were both suppressed considerably. These results suggest that the fusion-threshold phenomenon and the sharp increase in mobility of viral glycoproteins at the fusion threshold temperature are related to the temperature characteristics of internal viroskeleton proteins, such as M-proteins and actins.

It has been shown that Sendai viral M-proteins bind not only the viral nucleocapsid (Shimizu & Ishida, 1975; Narayanan et al., 2000) but also the virus envelope glycoproteins, HN and F (Sanderson et al., 1993,1994). Such binding was shown with other viruses (Garcia-Sastre, Cabezas & Villan 1989; Lyles, McKenzie & Parce, 1992). There have been a number of studies suggesting that the M-proteins play an important role for virus-budding from the host cells (Yu et al, 1992; Mebatsion, Weiland & Conzelman, 1999; Takimoto et al, 2001; Ali & Nayak, 2001). It was also reported that actins were found in various viruses (Lamb et al., 1976; Tyrrell & Norrby, 1978; Ott et al, 1996). There have been a few reports that actin associates with the viral M proteins (Giuffre et al., 1982). It is well recognized that the internal cytoskeleton proteins often control the movement of proteins on the cell plasma-membranes (Bennett & Gilligan, 1993; Repasky & Gregorio, 1992). The temperature-dependent mobility of viral glycoproteins, especially those in the fusion threshold region, may be related to the temperature characteristics of the M-protein together with other contractile proteins (e.g., actins) underlying the viral envelope membrane, which control the mobility as well as organization of surface envelope glycoproteins.

References

Al-Ahdal, M.N., Abidi, T.F., Flanagan, T.D. 1986. The interaction of Sendai virus glycoprotein-bearing recombinant vesicles with cell surfaces. *Biochim. Biophys. Acta* **854:**157–168

Ali, A., Nayak, D.P. 2000. Assembly of Sendai virus: M protein interacts with F and HN proteins and with the cytoplasmic tail and transmembrane domain of F protein. *Virol.* **276:**289–303

Allen, R.J.L. 1940. The estimation of phosphorus. *Biochem. J.* **34:**858–865

Amselem, S., Barenholz, Y., Loyter, A., Nir, S., Lichtenberg, D. 1986. Fusion of Sendai virus with negatively charged liposomes as studied by pyrene-labelled phospholipid liposomes. *Biochim. Biophys. Acta.* **860:**301–313

Aroeti, B., Jovin, T.M., Henis, Y.I. 1990. Rotational mobility of Sendai virus glycoprotein in membranes of fused human erythrocytes and in the envelopes of cell-bound virions. *Biochemistry* **29:**9119–9125

Asano, A., Sekiguchi, K. 1978. Redistribution of intramembrane particles of human erythrocytes induced by HVJ (Sendai virus): A prerequisite for the virus-induced cell fusion. *J. Supermolec. Struct.* **9:**441–452

Bagai, S., Puri, A., Blumenthal, R., Sarkar, D.P. 1993. Hemagglutinin-neuraminidase enhances F protein-mediated membrane fusion of reconstituted Sendai virus envelopes with cells. *J. Virol.* **67:**3312–3318

Bennett, V., Gilligan, D.M. 1993. The spectrin-based membrane skeleton and micro-scale organization of the plasma membrane. *Annu. Rev. Cell Biol.* **9:**27–66

Bradford, M.M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. *Biochem.* **78:**248–254

Burger, K.N., Knoll, G., Verkleij, A.J. 1988. Influenza virus-model membrane interactions: A morphological approach using modern cryotechniques. *Biochim. Biophys. Acta* **939:**89–101

Garcia-Sastre, A., Cabezas, J.A., Villar, E. 1989. Protein of Newcastle disease virus envelope: Interaction between the outer hemagglutinidase glycoprotein and the inner non-glycosylated matrix protein. *Biochim. Biophys. Acta* **999:**171–175

Giuffre, R.M., Tovell, D.R., Kay, C.M., Tyrrell, D.L.J. 1982. Evidence for an interaction between the membrane protein of a paramyxovirus and actin. *J. Virol.* **42:**963–968

Haywood, A.M., Boyer, B.P. 1982. Sendai virus membrane fusion: time course and effect of temperature, pH, calcium, and receptor concentration. *Biochemistry* **21:**6041–6046

Henis, Y.I., Herman-Barhom, Y., Aroeti, B., Gutman, O. 1989. Lateral mobility of both envelope proteins (F and HN) of Sendai virus in the cell membrane is essential for cell-cell fusion. *J. Biol. Chem.* **264:**17119–17125

Hewitt, J.A., Nermut, M.V. 1977. A morphological study of the M-protein of Sendai virus. *J. Gen. Virol.* **34:**127–136

Hoekstra, D. 1990. Membrane fusion of enveloped virus: Especially a matter of proteins. *J. Bioenerg. Biomembr.* **22:**121–155

Hoekstra, D., De Boer, T., Klappe, K., Wilschut, J. 1984. A fluorescence method for measuring the kinetic analysis of viral binding, dissociation and fusion, J. *Virol.* **23:**5675–5681

Hoekstra, D., Klappe, K., de Boer, T., Wilschut, J. 1985. Characterization of the fusogenic properties of Sendai virus: Kinetics of fusion with erythrocyte membranes. *Biochemistry* **24:**4739–4745

Hoekstra, D., Klappe, K., Hoff, H., Nir, S. 1989. Mechanism of fusion of Sendai virus: Role of hydrophobic interaction and mobility constrains of viral membrane proteins. *J. Biol. Chem.* **264:**17119–17125

Kanaseki, T., Kawasaki, K., Murata, M., Ikeuchi, Y., Ohnishi, S. 1997. Structural features of membrane fusion between influenza virus and liposomes as revealed by quick-freezing electron microscopy. *J. Cell Biol.* **137:**1041–1056

Kim, J., Hama, K., Miyake, Y., Okada, Y. 1979. Transformation of intramembrane particles of HVJ (Sendai virus) envelopes from an invisible to visible form on aging of virions. *Virology* **24:**523–535

Lakowicz, J.R. 1983. Principles of Fluorescence Spectroscopy. Plenum NY

Lamb, R.A., Mahy, B.W.J., Choppin, P.W. 1976. The synthesis of Sendai virus polypeptides in infected cells. *Virology* **69:**116–131

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227:**680–685

Lee, P.M., Cherry, R.J., Bachi, T. 1983. Correlation of rotational mobility and flexibility of Sendai virus spike glycoproteins with fusion activity. *Virology* **128:**65–76

Leonards, K., Ohki, S. 1983. Isolation and characterization of large (0.5–1.0 μm) cytoskeleton-free vesicles from human and rabbit erythrocytes. *Biochim. Biophys. Acta* **728:**383–393

Lyles, D.S., McKenzie, M., Parce, J.W. 1992. Subunit interactions of vesicular stomatitis virus envelope glycoprotein stabilized by binding to viral matrix protein. *J. Virology* **66:**349–358

Maeda, T., Asano, A., Ohki, K., Okada, Y., Ohnishi, S. 1975. A spin-label study on fusion of red blood cells induced by hemagglutinating virus of Japan. *Biochemistry*. **14:**3736–3741

Mebatsion, T., Weiland, F., Conzelmann, K.K. 1999. Matrix protein of rabies virus is responsible for the assembly and budding of bullet-shaped particles and interacts with the transmembrane spike glycoprotein G. *J. Virol*. **73:**242–250

Miyake, T., Kim, J., Okada, Y. 1978. Effect of cytochalasin D on fusion of cells by HVJ (Sendai virus). *Exp. Cell Res*. **116:**167–178

Narayanan, K., Maeda, A., Maeda, J., Makino, S. 2000. Characterization of the coronovirus M protein and nucleocapside interaction in infected cells. *J. Virol*. **74:**8127–8134

Ohki, S., Flanagan, T.D., Hoekstra, D. 1998. Probe transfer with and without membrane fusion in a fluorescence fusion assay. *Biochemistry* **37:**7496–7503

Ohki, S., Arnold, K., Srinivasakumar, N., Flanagan, T.D. 1992. Effect of anionic polymers on fusion of Sendai virus with human erythrocyte ghosts. *Antiviral Research* **18:**163–177

Ott, D.E., Coren, L.V., Kane, B.P., Busch, D.G., Johnson, D.G., Sowder II, R.C., Chertova, E.N., Arthur, L.O., Henderson, L.E. 1996. Cytoskeletal proteins inside human immunodeficiency virus type I virion. *J. Virol*. **70:**7734–7743

Pasternak, C.A., Micklem, K.J. 1973. Permeability changes during cell fusion. *J. Membrane Biol*. **14:**293–303

Peeples, M.E. 1991. Paramyxovirus M proteins: Pulling it all together and taking it on the road. *In:* Kingsbury, D.W., (editor) Paramyxoviruses. pp 427–456, Plenum Press, New York

Repasky, E.A., Gregorio, C.C. 1992. Plasma membrane skeletons. *In:* Yeagle, P., (ed) The Structure of Biological Membranes. pp 449–505, CRC Press, Boca Raton, FL

Sanderson, C.M., McQueen, N.L., Nayak, D.P. 1993. Sendai virus assembly: M protein binds to viral glycoproteins in transit through the secretory pathway. *J. Virol*. **67:**651–663

Sanderson, C.M., Wu, H.-H., Nayak, D.P. 1994. Sendai virus M protein binds independently to either the F or the HN glycoprotein in vivo. *J. Virol*. **68:**69–76

Shimizu, K., Ishida, N. 1975. The smallest protein of Sendai virus: its candidate function of binding nucleocapsid to envelope. *Virology* **67:**427–436

Shinitzky, M., Barenholz, Y. 1974. Dynamics of the hydrocarbon layer in liposomes of lecithin and sphingomyelin containing diacetylphosphate, J. *Biol. Chem*. **249:**2652–2657

Spruce, A.E., Iwata, A., Almers, W. 1991. Patch clamp studies of single cell-fusion events mediated by a viral fusion protein, Proc. *Natl. Acad. Sci. USA*. **88:**3623–3627

Steck, T.L., Kant, J.A. 1974. Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes. *Methods Enzymol*. **31:**172–180

Takimoto, T., Gopal Murti, K., Bousse, T., Scroggs, R.A., Porter, A. 2001. Role of matrix and fusion proteins in budding of Sendai virus. *J. Virol*. **75:**11384–11391

Tyrrel, D.L., Norrby, E. 1978. Structural polypeptides of measles virus. *J. Gen. Virol*. **39:**219–229

Wagner, M., Flanagan, T.D., Ohki, S. 1998. Effects of anionic and nonionic polymers on fusion and binding of Sendai virus to human erythrocyte ghosts. *Antiviral Res*. **39:**113–127

White, J.M. 1992. Membrane fusion. *Science* **258:**917–924

Wray, W., Boulikas, T., Wray, V.P., Hancock, R. 1981. Silver staining of proteins in polyacrylamide gels. *Analytical Biochem*. **118:**197–203

Wybenga, L.E., Epand, R.F., Nir, S., Chu, J.W.K., Sharon, F.J., Flanagan, T.D., Epand, R. 1996. Glycophorin as a receptor for Sendai virus. *Biochemistry* **35:**9513–9518

Yu, X., Yuan, X., Matsuda, Z., Lee, T.H., Essex, M. 1992. The matrix protein of human immunodeficiency virus type 1 is required for incorporation of viral envelope protein into mature virions. *J. Virol*. **66:**4966–4971

Zimmerberg, J., Curran, M., Cohen, F.S. 1991. A lipid/protein complex hypothesis for exocytotic fusion pore formation, Ann. *N.Y. Acad. Sci*. **635:**307–317

Zschornig, O., Arnold, K., Ohki, S. 1993. Effect of glycosaminoglycans and PEG on fusion of Sendai virus with phosphatidylserine vesicles. *Biochim. Biophys. Acta*. **1148:**1–6