

# Interaction of Sendai virions with resealed human erythrocyte ghosts

## Lateral mobility of the viral glycoproteins in the cell membrane following fusion

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Two independent methods demonstrated that resealed human erythrocyte ghosts undergo Sendai virus-mediated cell-cell fusion to a much lower degree (about 4%) than intact erythrocytes, in spite of similar levels of viral envelope-cell fusion in the two preparations. Fluorescence photobleaching recovery (FPR) showed similar lateral mobilities of the viral glycoproteins following fusion with either ghosts or whole erythrocytes. It is suggested that although viral glycoprotein mobilization in the cell membrane is essential for cell-cell fusion, the target cell properties are also important; in the absence of the required cellular parameters, the mobilization may not be a sufficient condition.

Fusion; Sendai virus; Photobleaching; Diffusion; Erythrocyte

### 1. INTRODUCTION

Employing fluorescence photobleaching recovery (FPR), we have recently demonstrated that the ability of Sendai virus to induce cell-cell fusion of human erythrocytes correlates with the lateral mobilization of the viral envelope glycoproteins (F, fusion protein; HN, hemagglutinin-neuraminidase protein) in the cell membrane following viral envelope-cell fusion [1,2]. However, it was not clear whether this lateral mobilization is a sufficient condition for the induction of cell-cell fusion, or whether cellular factors may also be required. This question can be explored by studies on the lateral mobility of the viral glycoproteins in a system where fusion stops after the viral envelope-cell fusion stage due to altered cellular properties. Hypotonically lysed resealed human erythrocyte ghosts provide such a system,

since the resealed ghosts were reported to fuse with Sendai virions but not with each other [3-5].

Here, we report studies on the lateral mobility of Sendai virus glycoproteins in the membranes of resealed ghosts following fusion. Under conditions where the resealed ghosts fused with each other to a negligible degree as compared with intact erythrocytes, the viral glycoproteins underwent lateral mobilization to the same extent in the two preparations. It is therefore proposed that although lateral mobilization of the viral glycoproteins in the cell membrane is essential for cell-cell fusion, it is not always a sufficient condition, and cellular factors could also be involved in the latter process.

### 2. EXPERIMENTAL

#### 2.1. Reagents

Tetramethylrhodamine (TMR) 5-isothiocyanate was purchased from Research Organics (Cleveland, OH). *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (N-NBD-PE) was from Avanti (Birmingham, AL), and octadecylrhoda-

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mine B chloride ( $R_{18}$ ) from Molecular Probes (Junction City, OR). Trypsin and dithiothreitol (DTT) were from Sigma.

## 2.2. Virus

Sendai virus (Z-strain) was grown in embryonated chicken eggs and harvested 48 h after injection [1,5]. The virus was resuspended in 160 mM NaCl, 20 mM Tricine, pH 7.4 (solution A) and stored at  $-70^{\circ}\text{C}$ . Protein was determined by a modified Lowry procedure [6]. Hemagglutinating units (HAU) were measured as in [5]. Treatment of virions with trypsin or with phenylmethylsulfonyl fluoride (PMSF) to eliminate their fusogenic activities was as detailed [7,8].

## 2.3. Interaction of Sendai virions with human erythrocytes

Fresh human erythrocytes (group O, Rh-positive) or resealed ghosts prepared by hypotonic lysis and resealing [9] were incubated with virions at  $4^{\circ}\text{C}$  (2%, v/v, cells with 400 HAU/ml Sendai virions, 15 min, in solution A). After washing twice with solution A, fusion was achieved by a further incubation (30 min) at  $37^{\circ}\text{C}$ .

## 2.4. Fluorescence photobleaching recovery

Lateral diffusion coefficients ( $D$ ) and mobile fractions ( $R_f$ ) were measured by FPR [10,11] at  $22^{\circ}\text{C}$ , using an apparatus described in [12]. FPR was performed on ghosts attached to polylysine-coated coverslips and wet-mounted in solution A, exactly as in [1,2].

## 2.5. Virus-cell fusion

Viral envelope-cell fusion was measured by incorporating  $R_{18}$  into the viral envelope and determining the fusion-dependent

$R_{18}$  fluorescence dequenching [13,14]. In experiments with erythrocytes, ghosts were prepared prior to the fluorescence measurements by the viral hemolytic activity or by hypotonic lysis, and the hemoglobin was washed away with solution A. A similar wash was applied also after fusion with resealed ghosts, to remove virions that may detach during the incubation. The percentage of fused virions was determined as described [14].

An alternative method involved the removal of adsorbed (but not fused) virions by DTT treatment [15]. After incubation with the virions, cells were incubated with 50 mM DTT, 2 mM EDTA in solution A (15 min at  $4^{\circ}\text{C}$ , followed by 30 min at  $22^{\circ}\text{C}$ ), vortex-mixed and washed to remove released virions. The cells were then labelled with TMR-Fab' fragments (described in [1]) directed against the F and HN proteins ( $100\ \mu\text{g}/\text{ml}$ , 30 min,  $22^{\circ}\text{C}$ ). Comparison of the fluorescence levels before and after DTT treatment (using the FPR instrumentation under non-bleaching conditions) yielded the fraction of fused viral glycoproteins [2].

## 3. RESULTS AND DISCUSSION

### 3.1. Fusion of erythrocyte ghosts by Sendai virus

Native Sendai virions fused with ghosts as efficiently as with intact erythrocytes, as demonstrated both by  $R_{18}$  fluorescence dequenching and by DTT treatment to determine the fraction of fused virions (table 1). Lipid exchange did not contribute to the fluorescence dequenching experiments, as indicated by the lack of dequenching in the case of

Table 1  
Sendai virus-mediated fusion of resealed ghosts and of intact human erythrocytes

Cells	Treatment	Fraction of fused virions		% agglutinated cells after DTT treatment	% increase in fluorescent cells
		DTT treatment	$R_{18}$ fluorescence dequenching		
Erythrocytes	$4^{\circ}\text{C}$	$0.07 \pm 0.06$	$0.08 \pm 0.05$	$2 \pm 2$	$8 \pm 3$
	$4^{\circ}\text{C} + 37^{\circ}\text{C}$	$0.86 \pm 0.15$	$0.84 \pm 0.04$	$78 \pm 5$	$56 \pm 8$
	trypsin, $4^{\circ}\text{C} + 37^{\circ}\text{C}$	$0.08 \pm 0.06$	$0.09 \pm 0.06$	$5 \pm 4$	$7 \pm 2$
	PMSF, $4^{\circ}\text{C} + 37^{\circ}\text{C}$	$0.08 \pm 0.05$	$0.09 \pm 0.05$	$6 \pm 4$	$8 \pm 3$
Ghosts	$4^{\circ}\text{C}$	$0.08 \pm 0.05$	$0.08 \pm 0.04$	$1 \pm 2$	$3 \pm 2$
	$4^{\circ}\text{C} + 37^{\circ}\text{C}$	$0.79 \pm 0.14$	$0.80 \pm 0.04$	$3 \pm 2$	$12 \pm 3$
	trypsin, $4^{\circ}\text{C} + 37^{\circ}\text{C}$	$0.08 \pm 0.04$	$0.07 \pm 0.05$	$2 \pm 1$	$7 \pm 3$
	PMSF, $4^{\circ}\text{C} + 37^{\circ}\text{C}$	$0.09 \pm 0.05$	$0.08 \pm 0.04$	$3 \pm 2$	$7 \pm 3$

Cells were incubated with virions as described in section 2 at  $4^{\circ}\text{C}$  (rows 1,5) or at  $4^{\circ}\text{C}$  followed by  $37^{\circ}\text{C}$  (all other rows). Virus-cell fusion (the fraction of fused virions) was determined by  $R_{18}$  fluorescence dequenching or by DTT treatment, as described in section 2.5. Cell-cell fusion was evaluated by the percentage of agglutinated cells that could not be detached from each other by DTT treatment, or by the increase in the percentage of fluorescent cells upon fusion of N-NBD-PE labelled cells with unlabelled cells (see text). 25–35 cells were scored in experiments of virus-cell fusion, and 200–300 cells in cell-cell fusion experiments. Each experiment was repeated 3 times. Results are means  $\pm$  SE

virions pre-treated by trypsin or PMSF to eliminate their fusogenic activity. The second method does not include any lipid exchange contributions since the TMR-Fab' fragments employed to label the fused viral glycoproteins do not bind to lipids. The level of TMR-Fab' fluorescence in the latter experiments was similar on ghosts and on whole erythrocytes, suggesting that the amounts (and not only the fractions) of the fused viral glycoproteins were also essentially similar.

On the other hand, major differences were observed between the two cellular systems in their ability to undergo Sendai virus-mediated cell-cell fusion. Since phase-contrast microscopy cannot detect local cell-cell fusion, which was proposed to occur between resealed erythrocyte ghosts [16], we developed other methods which can detect such fusion. One method involved the use of DTT treatment to separate agglutinated (unfused) erythrocytes from each other. The treatment was performed exactly as described in section 2.5, except that the percentage of aggregated ghosts which could not be separated by DTT was determined (rather than the amount of remaining virions). The results (table 1) clearly indicate that ghost-ghost fusion occurred to a negligible extent as compared to erythrocyte-erythrocyte fusion. The validity of the method is supported by the fact that the cell-cell fusion level detected by phase contrast in the case of intact erythrocytes coincided with the percentage of agglutinated cells after the DTT treatment, and hardly any cell-cell fusion was detected using non-fusogenic virions (trypsin- or PMSF-treated).

The second method was based on measuring the increase in the number of cells labelled with the fluorescent lipid N-NBD-PE following fusion. N-NBD-PE-containing cells (labelled as in [17]) were mixed at a 1:4 ratio, and incubated with the virions. Samples were observed by phase contrast and fluorescence microscopy, and the increase in the percentage of fluorescent cells (which should follow even local bridge formation between cells) was determined. The results (table 1) demonstrate a much lower increase in the percentage of labelled cells under fusion-promoting conditions in the case of resealed ghosts as compared with whole erythrocytes. The increase in labelled cells was negligible at 4°C or using non-fusogenic virions

Table 2

Lateral mobility of Sendai virus envelope glycoproteins following fusion with human erythrocytes or with resealed erythrocyte ghosts

Cells	Envelope protein labelled	Mobile fraction (%)	$D$ (cm <sup>2</sup> /s) ( $\times 10^{10}$ )
Erythrocytes	F + HN	55 $\pm$ 3	3.2 $\pm$ 0.2
	F	60 $\pm$ 4	3.2 $\pm$ 0.1
	HN	55 $\pm$ 5	3.3 $\pm$ 0.2
Ghosts	F + HN	58 $\pm$ 5	2.9 $\pm$ 0.2
	F	56 $\pm$ 4	3.1 $\pm$ 0.2
	HN	55 $\pm$ 5	2.9 $\pm$ 0.2

Whole erythrocytes or resealed erythrocyte ghosts were fused with Sendai virions (400 HAU/ml) as described in section 2.3. The FPR experiments were performed as described in section 2. The envelope proteins were labelled with 100  $\mu$ g/ml of anti-F, anti-HN, or anti-RSVE (directed against F + HN) TMR-Fab' fragments (described in [1]). The values in the table are means  $\pm$  SE of 50–60 measurements in each case. Very low mobile fractions (2–5%) were obtained using both erythrocytes and resealed ghosts under conditions that do not promote fusion (incubation with virions at 4°C only, or the use of trypsinized or PMSF-treated virions)

for both cell systems (table 1), demonstrating that exchange of N-NBD-PE between cells is not significant. Thus, the results obtained by this semi-quantitative method are in good agreement with those obtained by the DTT treatment protocol.

The demonstration of no appreciable ghost-ghost fusion by both methods contrasts with a former report on Sendai virus-mediated local fusion between ghosts [16], concluded from some transfer of fluorescence-labelled albumin trapped within one ghost to another. A possible explanation for this discrepancy is virally mediated leakage of trapped proteins without the formation of actual cell-cell fusion, as Sendai virions are known to induce transient permeabilization of various cell types [18].

### 3.2. Lateral motion of viral glycoproteins

The lateral mobility of F and HN proteins in the cell membrane following fusion of erythrocytes or ghosts with Sendai virions was investigated. As expected, no lateral mobility was observed in either case in the absence of fusion, since under these conditions the viral glycoproteins are limited within the viral membrane and cannot diffuse to

micron distances over the cell surface ([1,2]; table 2). Following fusion-promoting conditions, F and HN became laterally mobile to the same extent (as reflected in both the  $D$  and  $R_f$  values) on both erythrocytes and resealed ghosts (table 2). It is therefore apparent that the lack of ghost-ghost fusion is not due to restricted lateral motion of the viral glycoproteins in the ghost membrane. This differs from the fusion of intact erythrocytes by reconstituted Sendai virus envelopes, where the inability of the envelopes to induce cell-cell fusion coincided with a large reduction in the fraction of laterally mobile viral glycoproteins in the cell membrane as compared with native virions [2]. We therefore proposed [2] that lateral motion of the viral envelope proteins in the cell membrane is required for the induction of cell-cell fusion. It appears that the properties of the target cells are also important for the ability of the cells to be fused by the virions, and the lateral mobilization of the viral glycoproteins is not always a sufficient condition for mediating cell-cell fusion, at least in the system of resealed ghosts. Since sequestering of certain macromolecules within ghosts partially restored their capacity to fuse with each other [3,4,16], it is possible that osmotic swelling conditions, which are lacking in hypotonically lysed resealed ghosts, represent an additional requirement for cell-cell fusion.

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