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Fusogenic properties of reconstituted hybrid vesicles containing Sendai and influenza envelope glycoproteins: fluorescence dequenching and fluorescence microscopy studies

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Co-reconstitution of influenza and Sendai virus phospholipids and glycoproteins resulted in the formation of membrane vesicles containing the envelope glycoproteins from both viruses within the same membrane. Reconstituted influenza-Sendai hybrids (RISH) were able to lyse human erythrocytes and fuse with their membranes or with living cultured cells at pH 5.0 as well as at pH 7.4, thus exhibiting the fusogenic properties of both viruses. This was also inferred from experiments showing that the fusogenic activity of RISH was inhibited by anti-influenza as well as by anti-Sendai virus antibodies. Fusion of FISH and of reconstituted influenza (RIVE) or reconstituted Sendai virus envelopes (RSVE) with recipient membranes was determined by the use of fluorescently labeled envelopes and fluorescence dequenching methods. Observations with the fluorescence microscope were used to study localization of fused reconstituted envelopes within living cells. Incubation of RISH and RSVE with living cells at pH 7.4 resulted in the appearance of fluorescence rings around the cell plasma membranes and of intracellular distinct fluorescent spots indicating fusion with cell plasma membranes and with membranes of endocytic vesicles, respectively. The fluorescence microscopy observations clearly showed that RIVE failed to fuse, at pH 7.4, with cultured cell plasma membranes, but fused with membranes of endocytic vesicles.

Introduction

Penetration of enveloped viruses into animal cells is mediated by a virus-membrane fusion step. Fusion is promoted by the viral envelope glycoproteins whose detailed function in the process is still obscure [1]. Reconstituted membrane vesicles containing isolated viral glycoproteins are a convenient tool to study the molecular mechanism of virus-membrane fusion as well as an excellent model system for investigating structure-function relationships of transmembrane proteins [2].

Recently [3], we have shown that reconstituted influenza virus envelopes (RIVE), namely, vesicles containing the viral hemagglutinin and neuraminidase glycoproteins, are as fusogenic as intact virus particles. RIVE fused with isolated biological membranes or with liposomes only at low pH values which are required for activation of the hemagglutinin glycoprotein [1,4]. Due to this requirement, envelopes of influenza virions have been claimed to fuse, in living cells, with membranes of endocytic vesicles following receptor-mediated endocytosis [1,5]. A pH-dependent conformational change was demonstrated in the viral hemagglutinin glycoprotein and was suggested to be required for the proper expression of its fusogenic activity [6].

Reconstituted envelopes of Sendai virus particles (RSVE) have also been shown to fuse – similar to intact virus particles – with cell plasma membranes at a wide range of pH values [2]. Fusion of RSVE with recipient membranes requires the participation of the two viral envelope glycoproteins namely the hemagglutinin/neuraminidase (HN) and the fusion (F) polypeptides [7,8]. The presence of the two envelope glycoproteins within the same membrane allows their mutual interaction, which was suggested to be required for proper expression of the viral fusogenic activity [8]. Indeed,

Abbreviations: SV, Sendai virus; RSVE, reconstituted Sendai virus envelopes; HN, Sendai hemagglutinin/neuraminidase polypeptide; F, Sendai fusion polypeptide; IV, influenza virus; RIVE, reconstituted influenza virus envelopes; RISH, reconstituted influenza Sendai hybrid; DQ, fluorescence dequenching; DTT, dithiothreitol; PBS, phosphate-buffered saline.

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circular dichroism (CD) studies clearly revealed that the conformation of the viral glycoproteins in reconstituted envelopes containing both glycoproteins is different from its conformation in vesicles bearing only individual glycoproteins, namely HN and F vesicles [8].

Thus, it appears that in order to be active in the fusion process, the envelope glycoproteins of both Sendai and influenza virions should possess a specific structure. The conformational change which allows such structure is promoted in the case of influenza virions (IV) by the low pH environment and in Sendai virus (SV) by the specific interaction between the HN and F polypeptides [1,6,8]. Specific interaction between other transmembrane proteins, such as hormone or polypeptide receptors, have also been suggested to be required for their proper function [9].

An experimental approach to gain a better understanding on the role that the interaction between viral glycoproteins plays in allowing expression of their fusogenic activity is to insert new membrane polypeptides into the viral envelopes [1,10]. Estimation of the hemolytical and fusogenic activities of such 'hybrid' vesicles may show whether the presence of additional transmembrane proteins decreases or stimulates its biological activity.

In the present work, we have reconstituted vesicles containing the influenza and Sendai viruses' glycoproteins. Our results clearly show that both the influenza and Sendai glycoproteins preserved their fusogenic activities within such 'hybrid' vesicles.

Materials and Methods

Chemicals. Triton X-100 (scintillation grade) was obtained from Koch and Light Laboratory Ltd (U.K.). SM-2 Bio-Beads (20–50 mesh) were from Bio-Rad, and octadecylrhodamine B chloride (R_{18}) was obtained from Molecular Probes (Junction City, OR, U.S.A.).

Virus. Influenza (A_0PR_8 strain) and Sendai (Z strain) were isolated from the alantoic fluid of fertilized chicken eggs [11,12]. The viral hemagglutinating units were determined as previously described [11,12].

Cells. Human blood, type O, Rh^+, recently outdated was washed three times in phosphate-buffered saline (PBS) (pH 7.4) and the final pellet obtained was suspended in PBS to give 2.5% (v/v).

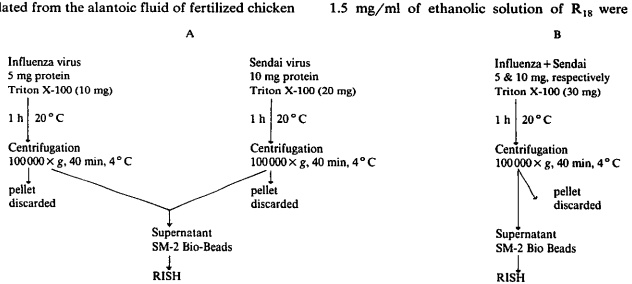
Human erythrocyte ghosts were obtained following hemolysis of the human erythrocytes with 40 vol. of 5 mM phosphate buffer (pH 8.0) [13]. After three washings with the same buffer, the final pellet of white human erythrocyte ghosts was suspended in PBS (pH 7.4) to give 4 mg of protein/ml.

Hela cells were grown in Eagle's minimum essential medium + 10% fetal calf serum, as described previously [14]. Prior to use, the cells were washed twice with Eagle's minimum essential medium without serum.

Preparation of reconstituted viral vesicles. RIVE and RSVE were obtained essentially as described previously [3,15]. Briefly, 10 mg of intact pelleted Sendai or influenza virions were solubilized with 20 mg of Triton X-100 in a final volume of 500 µl PBS (pH 7.4). The detergent was removed from the clear supernatant obtained after centrifugation by direct addition of SM-2 Bio-Beads [3,15].

Reconstituted influenza Sendai hybrids (RISH) were prepared either by mixing a detergent solution containing glycoproteins and phospholipids (the supernatant obtained after solubilization and centrifugation) of influenza and Sendai virus particles (Scheme IA) or by solubilizing a pellet containing both viruses (Scheme IB). These two methods gave essentially the same results. Our experiments showed that RISH obtained from a mixture containing influenza and Sendai virus particles at a ratio (w/w) of 1 : 2, respectively, exhibited the highest fusogenic activity.

Preparation of fluorescently labeled viral vesicles. RIVE, RSVE and RISH were labeled with R_{18} essentially as described previously [3,16]. Briefly, 2–3 µl of 1.5 mg/ml of ethanolic solution of R_{18} were rapidly



Scheme I. Methods used for reconstitution of influenza-Sendai hybrid envelopes: schematic summary.

injected into 250 μl of PBS (pH 7.4) containing 400 μg of viral protein. After 15 min of incubation at room temperature in the dark, the viral preparations were washed with 60 vol. of PBS (Eppendorf centrifuge, 15 min). Under such conditions, the R_{18} was inserted into the viral membranes at self-quenching surface density (about 3 mol% of total viral phospholipids), and its decrease was shown to be proportional to the fluorescence dequenching [3].

Fluorescence measurements. Fluorescent RIVE, RSVE and RISH (5 μg protein each) were incubated with human erythrocyte ghosts (200 μg protein) or living cultured cells ($4 \cdot 10^6$ cells) in a final volume of 200 μl PBS (pH 7.4). Following 15–30 min of incubation at 4°C, the pH of the medium was adjusted to the desired pH value by the addition of 40 μl of sodium acetate (0.15 M (pH 5.0)), and the suspension obtained was than incubated at 37°C. At the end of the incubation period, a volume of 1.5 ml PBS (pH 7.4) was added to the reaction mixture, and the degree of fluorescence (excitation at 560 nm, emission at 590 nm) of each sample was measured before and after solubilization with 0.1% Triton X-100. The extent of fluorescence obtained in the presence of the detergent was considered to represent 100% dequenching, i.e., infinite dilution of the probe [3]. All fluorescence measurements were carried out with a Perkin-Elmer MFP-4 spectrofluorimeter. Virus preparations were also incubated under the same experimental conditions in the absence of recipient membranes. The extent of fluorescence dequenching (DQ) was calculated as described previously [3].

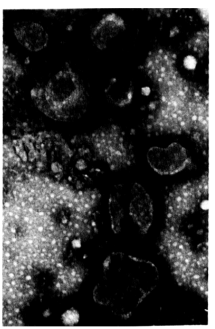


Fig. 1. Electron microscopic observation of RISH. RISH were obtained, negatively stained and prepared for electron microscopic observations as described in Materials and Methods and above [3]. Magnification×84000.

Determination of the degree of hemolysis. RIVE, RSVE and RISH (3 μg protein each) were incubated with 2.5% (v/v) human erythrocytes in a final volume of 800 μl at pH 7.4. Following 15 min of incubation at 4°C, the pH of the medium was adjusted to the desired value by the addition of 120 μl of sodium acetate (0.15 M (pH 5.0)) and the erythrocytes were further incubated for 20 min at 37°C. At the end of the incubation period, the degree of hemolysis was estimated at 540 nm, as previously described [3,16].

Anti-virus antibodies. Rabbit anti-Sendai or anti-influenza virus antibodies were obtained following injection of reconstituted viral envelopes as described previously [17]. The IgG fraction of the rabbit anti-serum were obtained after precipitation in ammonium sulfate.

Radiolabeling. Sendai virus was labeled with $Na^{125}I$ and iodogen as described by Markwell and Fox [18].

Protein was determined by the method of Lowry et al. [19] with bovine serum albumin as standard. Phospholipid concentration was estimated by the method of Stewart [20] with phosphatidylcholine as a standard.

Results

Hybrid reconstituted vesicles bearing Sendai and influenza virus glycoproteins: electron microscopy and immunoprecipitation studies

Removal of Triton X-100 from a solution containing a mixture of Sendai and influenza virus phospholipids and glycoproteins resulted in the formation of resealed membrane vesicles whose morphological characteristics are very similar to those of RSVE or RIVE (Fig. 1 and see also Refs. 3 and 15). From negatively stained preparations and electron microscopy studies, it appeared that the RISH obtained was relatively homogeneous and its size was similar to that of intact virus preparation, ranging in diameter from 600 to 2000 Å. In addition, similar to our previous preparations of RSVE and RIVE [3,15], the RISH obtained in the present work appeared to be composed of a single phospholipid bilayer in which viral glycoproteins are inserted (Fig. 1, Refs. 3 and 15). As can be seen, each vesicle obtained has numerous spikes (viral glycoproteins) whose size and appearance are very similar to those of intact Sendai or influenza virus (Fig. 1). However, as opposed to intact virus particles, in some of the RISH – as was inferred from thorough analysis of negatively stained preparations – the viral glycoproteins appear to be inserted on both sides of the RISH membrane. This indicates a partial symmetry in the localization of glycoproteins in reconstituted viral envelopes [3,15,16].

Immunoprecipitation studies confirmed that the vesicles obtained contained Sendai and influenza envelope glycoproteins within the same membrane (Table I). This is evident from the results showing that anti-influenza antibodies precipitated ^{125}I-Sendai virus glyco-

TABLE I

Immunoprecipitation of ^{125}I-RISH by anti-influenza virus antibodies

^{125}I-RISH (spec. act., 20000 cpm/μg) were prepared using ^{125}I-Sendai virus and non-labeled influenza virus particles. ^{125}I-RSVE (spec. act. 24000 cpm/μg) and RIVE were prepared as described before [3,15]. Viral envelopes (4 μg protein of ^{125}I-RSVE, ^{125}I-RISH or a mixture of RIVE and ^{125}I-RSVE) were incubated for 60 min at 37°C with (+Ab) or without (−Ab) 100 μg of anti-influenza antibodies in a final volume of 60 μl. At the end of the incubation period, the reaction mixture was suspended to 500 μl of PBS (pH 7.4), centrifuged (15000 × g, 2 min), and the radioactivity in the supernatant and pellet was determined in a γ-counter.

Viral envelopes	Precipitated (% of total)	
	−Ab	+Ab
^{125}I-RSVE	11	16
RIVE + ^{125}I-RSVE	12	18
^{125}I-RISH	14	88

proteins (88% of total) only in vesicles obtained by co-reconstitution with influenza glycoproteins (Table I). Under the same conditions, the anti-influenza antibodies practically failed to precipitate ^{125}I-Sendai viral glycoproteins from a suspension containing only ^{125}I-RSVE or a mixture of ^{125}I-RSVE and RIVE (Table I).

Functional interaction of hybrid vesicles (RISH) with intact human erythrocytes and human erythrocyte ghosts

It was well established that fusion of viruses belonging to the ortho and paramyxovirus groups, such as influenza and Sendai, respectively, with recipient cells, is accompanied by a process of virus-induced lysis [1]. As can be seen (Table II), RIVE was able, as expected to induce hemolysis only at pH 5.0, while RSVE induced hemolysis at pH 5.0 as well as at pH 7.4. A pH-dependency curve showed that the lytic activity of RIVE was expressed mainly between pH 5.0 and 5.5, while that of RSVE as well as RISH was expressed at a wide range of pH values, namely between pH 5.0 and 7.5 (Fig. 2A). At low pH values, the profile of the RISH lytic activity was found to be similar to that exhibited by RIVE rather than by RSVE (Fig. 2A).

Chemical reduction of the Sendai virus glycoproteins by dithiothreitol (DTT) or its partial proteolysis with trypsin have been shown to cause complete inactivation of the viral fusogenic and hemolytic activities [21,22]. These treatments do not have any affect on the influenza virus biological activities [23]. Indeed, a high degree of hemolysis was induced by RIVE which had been treated with DTT or with trypsin, as opposed to very little, if any, hemolysis observed with treated RSVE. The hemolytic activity of RIVE was, however, affected by anti-influenza antibodies (Table II). The results in Table II also show that RISH, namely, vesicles containing Sendai and influenza viral glycoproteins, induced hemolysis at pH 7.4 as well as at pH 5.0. This by itself does not indicate the presence of active glycoproteins from both viruses since RSVE alone were able to induce lysis at both pH values (see also Fig. 2A). However, treatment of RISH with DTT or trypsin completely abolished the lytic activity induced by this preparation at pH 7.4 but not that observed at pH 5.0 (Table II). Essentially, the same results were obtained when a suspension containing a mixture of RSVE and RIVE was treated with either DTT or trypsin (Table II). It may therefore be inferred that the hemolytic activity which was expressed at pH 5.0 by treated RISH or by a treated mixture of RSVE and RIVE is probably due to the presence of an active influenza hemagglutinin glycoprotein.

The view that in RISH the influenza and Sendai glycoproteins are inserted in the same membrane is supported by the results showing that anti-Sendai virus

TABLE II

Induction of hemolysis by reconstituted influenza-Sendai 'hybrid' envelopes

For inactivation of its biological activities, RIVE, RSVE and RISH (100 μg protein each) were incubated for 30 min at 37°C either with DTT (10 mm) or with trypsin (25 μg), and then washed once with PBS as previously described [21,22]. Immunoprecipitation was performed by incubating viral envelopes (3 μg) with 50 μl of anti-influenza antibodies (75 μg) or anti-Sendai antibodies (60 μg) for 30 min at 37°C. Following the addition of human erythrocytes, the degree of hemolysis was determined as described in Materials and Methods.

System	pH	Hemolysis (%)				
	Treatment:	none	trypsin	DTT	anti-SV Ab	anti-IV Ab
RISH	5.0	95	82	85	0	0
	7.4	80	0	0	0	0
RSVE	5.0	70	0	0	0	70
	7.4	98	0	0	0	95
RIVE	5.0	98	96	96	98	0
	7.4	0	0	0	0	0
RIVE + RSVE	5.0	97	80	70	80	50
	7.4	95	0	0	0	95

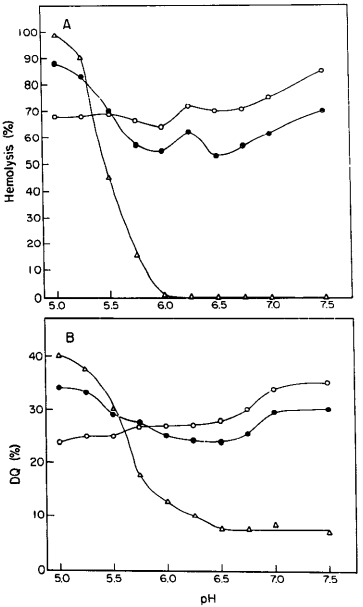


Fig. 2. Interaction of RSVE, RIVE and RISH with human erythrocytes and human erythrocyte ghosts: pH curve. RSVE (○), RIVE (△) and RISH (●) were incubated with intact human erythrocytes for determination of hemolysis (A) or with human erythrocyte ghosts for estimation of the degree of fluorescence (DQ) (B) as described in Materials and Methods. The pH of the incubation medium was adjusted to the indicated pH values by the addition of 40 μl (A) or 120 μl (B) of 0.15 M sodium acetate as described in Materials and Methods.

antibodies blocked completely the lytic activity of this preparation. As can be seen, RISH failed to induce hemolysis, either at pH 7.4 or at pH 5.0 in the presence of anti-Sendai virus antibodies (Table II). The same antibodies blocked only the lytic activity which was expressed at pH 7.4 when added to a mixture of RSVE and RIVE (Table II).

Essentially the same results were obtained following the addition of anti-influenza antibodies (Table II). Such antibodies inhibited the lytic activity of the RISH preparation but not of a suspension containing a mixture of RSVE and RIVE. The magnitude of the lytic activity expressed by the mixture of RSVE and RIVE in the presence of anti-influenza antibodies was very close to that expressed by the RSVE preparation alone (Table II).

The results in Fig. 2B show that the various reconstituted envelope preparations namely, RSVE, RIVE and RISH, were able to fuse with human erythrocyte membranes. This is inferred from the increase in the degree of fluorescence (fluorescence dequenching, DQ) observed following incubation of fluorescently labelled envelopes with human erythrocyte ghosts (Fig. 2B). The pH profile of the viral envelopes fusogenic activity is very similar to the pH profile of its lytic activity (compare results in Fig. 2A to those in Fig. 2B).

The results in Table III confirm previous results [21–23] showing that chemical reduction with DTT or proteolysis with trypsin abolished the RSVE but not the RIVE fusogenic activity. Only treatment with glutaraldehyde blocked completely RIVE as well as RSVE fusogenic activities (Table III). Treatment of RISH with glutaraldehyde also totally inactivated its fusogenic activity, while reduction with DTT or trypsinization inhibited only the fusion activity observed at pH 7.4 but not that expressed at pH 5.0 (Table III). The fusogenic activity expressed by the DTT or trypsin-treated RISH is due probably to the presence of an active influenza hemagglutinin glycoprotein.

The presence of functional-active glycoproteins from both viruses within RISH is also evident from the results summarized in Table IV. As can be seen, the fusogenic activity of RISH, which was expressed at pH 5.0 or pH 7.4, was significantly and similarly affected by the addition of either anti-Sendai or anti-influenza antibodies (Table IV). Different results were obtained when the same antibodies were added to a mixture of RSVE and RIVE. Addition of anti-Sendai virus anti-

TABLE III

Fusion of RISH with human erythrocyte ghosts: effect of inhibitors

Fluorescently labelled viral envelopes (100 μg) were incubated for 30 min at 37 °C either with glutaraldehyde (0.2%, v/v), DTT (10 mM) or with trypsin (25 μg), and washed once with PBS (pH 7.4) as previously described [3,21,22]. All other experimental conditions were as described in Materials and Methods.

System	pH	Fluorescence dequenching (%)			
	Treatment:	none	trypsin	DTT	glutaraldehyde
RISH	5.0	30	26	22	4
	7.4	26	7	6	2
RIVE	5.0	34	30	31	5
	7.4	8	7	7	1
RSVE	5.0	20	12	10	7
	7.4	32	8	6	2

TABLE IV

Fusion of viral envelopes with human erythrocyte ghosts: effect of antiviral antibodies

Fluorescently labelled viral envelopes (5 μg) were incubated with anti-Sendai antibodies (anti-SV Ab) (100 μg) or anti-influenza antibodies (anti-IV Ab) (120 μg) for 30 min at 30 °C. Incubation with human erythrocyte ghosts and determination of the degree of DQ were as described in Materials and Methods.

System	pH	Fluorescence dequenching (%)		
	Incubated with:	none	anti-SV Ab	anti-IV Ab
RISH	5.0	32	8	7
	7.4	27	5	5
RIVE	5.0	35	33	8
	7.4	7	6	4
RSVE	5.0	18	4	16
	7.4	30	5	30
RIVE + RSVE	5.0	30	23	17
	7.4	26	6	25

bodies to such a mixture inhibited only the fusogenic activity of RSVE, as can be inferred from the high degree of DQ obtained at pH 5.0 in the presence of such antibodies. On the other hand, anti-influenza antibodies inhibited only the activity of the influenza virus glycoproteins, as is evident from the high degree of DQ seen at pH 7.4 following its addition to a mixture of RSVE and RIVE (Table IV).

Fusion of RISH with living cultured cells

The results in Table V show that a relatively high degree of DQ was obtained following the incubation of RISH as well as of RIVE and of RSVE with Hela cultured cells. An increase in the degree of fluorescence was observed at low pH values (pH 5.0) as well as at pH 7.4 (Table V). Treatment of RSVE with DTT or with trypsin inhibited its fusogenic activity, as can be inferred from the low degree of DQ obtained following incubation of treated virions with the Hela cells (Table V). The same treatments did not affect the fusogenic activity of RIVE (not shown). Chemical reduction with DTT or proteolysis with trypsin significantly affected 'the pH 7.4 fusogenic activity' of RISH but not that which was expressed at pH 5.0 (Table V). The lysosomotrophic agent NH_4Cl and the respiratory inhibitor NaN_3 drastically reduced the susceptibility of Hela cells to fuse with RIVE at pH 7.4, but not at pH 5.0, as can be inferred from the degree of DQ seen under these conditions (Table V). Lysosomotropic agents are known to increase the low pH of intracellular organelles, while NaN_3 inhibits endocytosis [1,16]. Thus, it may be concluded that the DQ observed with RIVE at pH 7.4 could be due to fusion with intracellular organelles whose pH was shown to be acidic [24]. On the other hand, the ability of RISH or RSVE to fuse with Hela cells was only slightly affected by these reagents (Table V). It is noteworthy that DTT- or trypsin-treated RISH were able to fuse, at pH 7.4, with untreated but not with $NH_4Cl + NaN_3$ treated Hela cells (Table V). These results certainly indicate that RISH are able to fuse at pH 7.4 with plasma membranes as well as with membranes of intracellular organelles following receptor-mediated endocytosis.

Fluorescence microscopy observations were used in order to confirm these observations and to study the localization of fused RISH as well as of RIVE and RSVE. Dequenching of the virus-associated self-quenched R_{18} – due to virus-membrane fusion – should allow observation of fusion phenomena at the level of the fluorescence microscope. The results in Fig. 3 clearly show that incubation of fluorescently labelled RSVE with Hela cells at either pH 5.0 or pH 7.4 resulted in the appearance of highly fluorescence plasma membranes. The view that the fluorescence observed indeed results from virus-membrane fusion can be inferred from our observations showing that very little, if any, fluorescence was seen in cells incubated with glutaraldehyde-treated Sendai virus particles (Fig. 3c). It has been shown that glutaraldehyde-treated virus particles are unfusogenic but allow lipid–lipid exchange processes to occur [25].

The results in Fig. 3 also show that in cells incubated with RSVE at pH 7.4, in addition to the fluorescence rings seen around the plasma membranes, also distinct fluorescent spots are visible. These spots appear to be either associated with the cell surface or localized within the cells. The observation (Fig. 3c) that very little fluo-

TABLE V

Fusion of RISH with HeLa cells

Fluorescently labelled viral envelopes were treated with DTT or trypsin as described in Table III and in Materials and Methods. The various viral envelope preparations (5 μg) were incubated with Hela cells at pH 7.4 or pH 5.0 as described before [3] and in Materials and Methods. Before addition of the viral envelopes, the Hela cells were incubated in the absence or presence of a mixture of NH_4Cl and NaN_3 (50 mM each) [3].

	Fluorescence dequenching (%)			
Hela cells incubated with:	none		$NH_4Cl + NaN_3$	
pH of incubation:	5.0	7.4	5.0	7.4
Viral envelopes				
RISH	35	30	30	25
DTT-treated RISH	25	20	23	5
Trypsinized RISH	30	18	27	4
RSVE	28	37	n.d.	29
DTT-treated RSVE	10	7	n.d.	5
Trypsinized RSVE	14	6	n.d.	3
RIVE	39	30	35	10

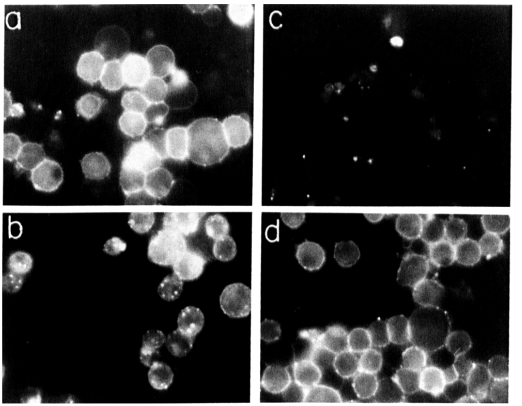


Fig. 3. Fusion of self-quenched R_{18}-labelled RSVE with Hela cells: fluorescence microscopy studies. All experimental conditions of preparation of R_{18}-labeled RSVE and incubation with Hela cells were as described in Materials and Methods and in legends to Tables III–V. At the end of the 30 min incubation at 37 °C with RSVE, the cells were washed with PBS and then observed by a fluorescence microscope (Lietz). RSVE were incubated with Hela cells either at pH 5.0 (a) or at pH 7.4 (b–d). (c) Incubation with glutaraldehyde-treated RSVE. (d) Incubation in the presence of NH_4Cl and NaN_3 (50 mM each) as described in Table V. Magnification × 360.

rescence has appeared at pH 7.4 following incubation with glutaraldehyde-treated virus may indicate that also the fluorescence spots result from a virus-membrane fusion process. (Compare the highly fluorescence cells seen in Fig. 3b to the very few unspecific fluorescence spots seen in Fig. 3c.) Essentially the same results, namely, very low and weak fluorescence-stained recipient cells, were obtained when RSVE were rendered unfusogenic also by treatment with trypsin or DTT (not shown).

The pattern of the fluorescence in Fig. 3d further strengthens our view that the fluorescent spots seen are due to a fusion process and result from staining of membranes of intracellular organelles. Our results show that the fluorescent spots seen in Fig. 3b are almost absent in the cells seen in Fig. 3d. In these cells (Fig. 3d), which have been incubated with the virus in the presence of NH_4Cl and NaN_3, the fluorescence appears to be associated mainly with the cell plasma membrane. The combination of NH_4Cl and NaN_3 was shown to inhibit fusion with membranes of intracellular organelles, but not with cell plasma membranes [1,3,16,24].

Incubation of RIVE or RISH with Hela cells at pH 5.0 also resulted in fluorescent staining of the cell plasma membranes (Fig. 4a, e). Very little, if any fluorescence was associated with the cells following incubation with glutaraldehyde-treated RIVE, again showing that the fluorescence seen is due to a process of virus-membrane fusion (Fig. 4b). Interestingly, the fluorescence observed in Hela cells incubated with RIVE at pH 7.4 appeared almost conclusively as distinct spots and not as rings around the plasma membrane (Fig. 4c). Most of the fluorescent spots are absent from cells incubated with RIVE at pH 7.4, but in the presence of NH_4Cl and NaN_3 (Fig. 4d), clearly indicating that these spots result from fusion of RIVE with membranes of intracellular organelles. The residual fluorescence seen in these cells – mainly as weak spots (Fig. 4d) – may be due to a very low degree of virus-membrane fusion still seen in the presence of these reagents (see Table V).

Incubation of RSVE with Hela cells at pH 7.4 and in the presence of NH_4Cl and NaN_3 (Fig. 3d), as opposed to incubation of RIVE (Fig. 4d), resulted in fluorescence staining of the cell plasma membranes. It was well-established that Sendai virions as well as RSVE, but not RIVE, possess fusogenic activity at pH 7.4 and therefore are able to fuse with cell plasma membranes even in the presence of NH_4Cl and NaN_3.

Incubation of fluorescently labelled RISH with Hela cells at pH 7.4 resulted in the appearance of fluorescent rings around the plasma membrane as well as intracellu-

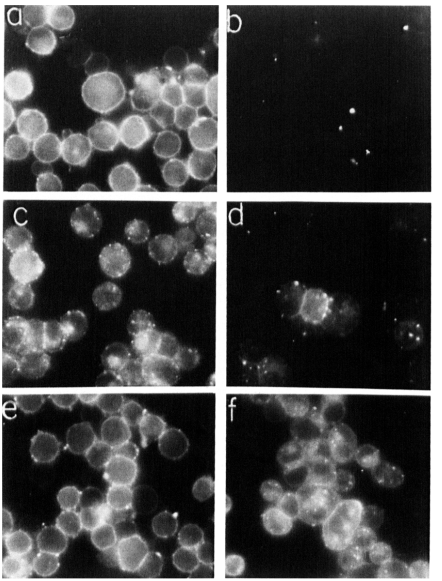


Fig. 4. Fusion of fluorescently labeled RIVE and RISH with Hela cells: observation by fluorescence microscopy. Fluorescently labelled RIVE (a–d) or RISH (e–f) were incubated with Hela cells either at pH 5.0 (a, b, e) or at pH 7.4 (c, d, f) as described in Materials and Methods and in the legend to Fig. 3. (b) Incubation with glutaraldehyde-treated RIVE. (d) Incubation in the presence of NH_4Cl and NaN_3 (50 mM each) as described in Table V. Magnification×360.

lar fluorescent spots (Fig. 4f). On the other hand, when RISH were incubated with Hela cells in the presence of NH_4Cl and NaN_3, fluorescence was observed only around the cell plasma membrane (not shown), similar to what has been observed with RSVE under these conditions (Fig. 3d).

Discussion

The results of the present work show that removal of Triton X-100 from a detergent solution containing a mixture of Sendai and influenza envelope glycoproteins and phospholipids resulted in the formation of reconstituted 'hybrid' vesicles. The vesicles obtained resemble in their size and especially by the appearance of packed populations of external spikes the structure of intact virus particles.

These hybrid vesicles possessed the glycoproteins of both viruses within the same membrane. This conclusion is based, first of all, on our experiments showing that anti-influenza antibodies were able to precipitate Sendai virus glycoproteins only from a suspension containing hybrid vesicles. These antibodies failed to immunoprecipitate the Sendai virus glycoproteins from a mixture containing RSVE and RIVE, indicating that under the experimental conditions used, the anti-influenza antibodies did not cross-react with the Sendai virus glycoproteins.

Formation of hybrid vesicles could also be inferred from experiments showing that both anti-Sendai or anti-influenza antibodies totally inhibited the lytic and fusogenic activities of only hybrid vesicles, but not of RSVE or RIVE. These results can be explained by assuming the presence of influenza hemagglutinin and

neuraminidase as well as Sendai HN and F glycoproteins within the same membrane.

Our results also show that the biological activities of the envelope glycoproteins of both viruses were preserved in the hybrid vesicles. The presence of biologically active influenza glycoproteins was inferred from results showing that DTT- or trypsin-treated hybrid vesicles still lysed and fused at pH 5.0 with human erythrocytes. The presence of active Sendai virus glycoproteins is evident from the ability of untreated hybrid vesicles to lyse and fuse with human erythrocytes at pH 7.4. It was well established that neither intact influenza virions nor RIVE are able to functionally interact, namely to lyse and to fuse at pH 7.4 with biological membranes. It appears therefore that insertion of glycoproteins from two different viruses into the same membrane does not effect their biological activity. This certainly indicates that each of the envelope glycoproteins preserve its three-dimensional structure, which is required for the appropriate expressing of its activity. Also the fluorescence dequenching and fluorescence microscopic studies clearly showed that the presence of Sendai viral glycoproteins did not affect the activity of influenza glycoproteins and vice versa.

Fusion of RISH as well as of RIVE and RSVE was determined by following the increase in DQ of R_{18}-labelled viral envelopes with non-labelled recipient membranes [3,16,26]. This method allows quantitative estimation of the extent of virus-membrane fusion but does not give any information regarding the localization of the recipient membranes with which the virus fused. Such information can, evidently, be obtained from electron microscopic studies, from which it was suggested that fusion of Sendai virions occurs with cell plasma membranes and fusion of influenza viruses with membranes of endocytic vesicles [1]. The recognition of viral envelope components and of virus-membrane fusion processes by electron microscopic techniques requires the use of specific staining methods [27].

In the present work, we have made use of previous observations showing that fusion of self-quenched R_{18}-labelled virus particles with recipient membranes results in fluorescence dequenching [26] for the development of a quick assay to follow virus-membrane fusion processes by fluorescence microscopic observations. The present fluorescence microscopy studies confirm our view claiming that RISH possess active glycoproteins of Sendai as well as of influenza virus particles. Our microscopic studies may also suggest that Sendai virus particles fuse not only with cells' plasma membranes, but also with intracellular membranes. This conclusion is based on the results showing the appearance of distinct intracellular fluorescence spots following incubation of R_{18}-labelled Sendai virions with cultured Hela cells.

Based on these observations, it may be suggested that Sendai virus particles are fusogenic at the intraendosomal low pH environment. This is not surprising, since it was well established that Sendai virus particles, as well as RSVE, are fusogenic at a wide range of pH values, including pH 5.0. Sendai virus glycoproteins, as opposed to the influenza hemagglutinin glycoprotein, are also fusogenic at neutral pH values, namely at pH 7.4 [1,2,28]. Indeed, fusion of fluorescently labelled RSVE, as well as of intact virions (not shown), with Hela cultured cells whose endocytosis was inhibited resulted in the appearance of clearly distinguished fluorescence rings, indicating exclusive fusion with the cells' plasma membranes. Essentially, the same results – fluorescence staining of the cell plasma membrane – was observed following incubation of RSVE with human erythrocyte ghosts (not shown).

The possibility that the fluorescence spots seen in Hela cells following incubation with RSVE at pH 7.4 are due to translocation of the fluorescence probe from the cell plasma membranes to intracellular membranes and not to fusion with endocytic vesicles cannot be excluded. Following their insertion into cell plasma membranes, fluorescence lipids have been shown to be translocated rapidly into other membranes, such as those of the Golgi apparatus [29]. Thus, it is certainly possible that following virus-plasma membrane fusion at pH 7.4 and fluorescence staining of the plasma membrane, the inserted R_{18} is translocated, similar to other lipids – to intracellular membranes, resulting in the appearance of fluorescence spots. A study in which NaN_3 and NH_4Cl will be used separately is required to resolve this question.

The fluorescent spots observed following incubation of RIVE with Hela cells at pH 7.4 must on the other hand, reflect our results from fusion with membranes of intracellular organelles, most probably with endocytic vesicles whose pH was shown to be acidic [24]. This conclusion is based on the following results: (1) in contrast to the results obtained with RSVE, no staining of the cell plasma membrane has been observed with RIVE at pH 7.4; (2) the presence of NH_4Cl and NaN_3 drastically reduced the degree of the fluorescence staining. Also, in this case, the possibility that following fusion with membranes of endocytic vesicles, the fluorescence probe is translocated into other intracellular membranes cannot be excluded. Fusion of intact influenza virus particles, in a low pH environment, with membranes of endocytic vesicles was suggested and observed before using other experimental methods [1].

In addition, the use of unfusogenic – inactivated virus particles and cells whose endocytic activity was inhibited – enabled us to distinguish between fluorescence dequenching due to virus-membrane fusion processes or to lipid–lipid exchange or other unspecific phenomena.

Potentially, the RISH whose properties were studied in the present work can be used as an efficient vehicle

for fusion-mediated microinjection of macromolecules into living cells. RSVE have been used previously as a biological carrier for the introduction of polypeptides or specific functional genes into cultured cells [2]. However, the presence of influenza virus glycoproteins in RISH may greatly increase its efficiency mainly due to its ability to fuse with endocytic vesicles. In addition, influenza virus glycoproteins are resistant to proteolytic digestion. This may be of great advantage, especially for fusion-mediated delivery of macromolecules in vivo following intravenous injection of fusogenic vesicles. Currently, experiments in our laboratory are performed in order to compare the efficiency of RISH as a biological carrier to that of RIVE or RSVE.

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