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Hemagglutinin-Neuraminidase Enhances F Protein-Mediated Membrane Fusion of Reconstituted Sendai Virus Envelopes with Cells

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Reconstituted Sendai virus envelopes containing both the fusion (F) protein and the hemagglutinin-neuraminidase (HN) (F,HN-virosomes) or only the F protein (F-virosomes) were prepared by solubilization of the intact virus with Triton X-100 followed by its removal by using SM2 Bio-Beads. Viral envelopes containing HN whose disulfide bonds were irreversibly reduced (HN_{red}) were also prepared by treating the envelopes with dithiothreitol followed by dialysis (F,HN_{red}-virosomes). Both F-virosomes and F,HN_{red}-virosomes induced hemolysis of erythrocytes in the presence of wheat germ agglutinin, but the rates and extents were markedly lower than those for hemolysis induced by F,HN-virosomes. Using an assay based on the relief of self-quenching of a lipid probe incorporated in the Sendai virus envelopes, we demonstrate the fusion of both F,HN-virosomes and F-virosomes with cultured HepG2 cells containing the asialoglycoprotein receptor, which binds to a terminal galactose moiety of F. By desialylating the HepG2 cells, the entry mediated by HN-terminal sialic acid receptor interactions was bypassed. We show that both F-virosomes and F,HN-virosomes fuse with desialylated HepG2 cells, although the rate was two- to threefold higher if HN was included in the viral envelope. We also observed enhancement of fusion rates when both F and HN envelope proteins were attached to their specific receptors.

Paramyxoviruses contain two glycoproteins (HN [hemagglutinin-neuraminidase] and F [fusion factor]) in the outer leaflet of the lipid bilayer (28). The F protein is absolutely required for the entry of viruses into the host cells and for cell-cell fusion (36). The HN binds to terminal sialic acid (TSA)-containing receptors (TSA-R) and is a neuraminidase (28). Studies of syncytium formation following expression of envelope proteins on the cell surface indicate that both HN and F are required for paramyxovirus-induced cell fusion (8, 16, 24, 25, 33, 40), although the F protein alone has been reported as sufficient to cause cell-cell fusion (1, 14, 30). Further evidence for the role of HN in paramyxovirus-induced cell fusion was obtained by showing that Sendai virus-induced cell fusion was blocked by a monoclonal anti-HN protein antibody that does not inhibit hemagglutination activity (23).

The role of HN in viral envelope-cell fusion is not so clear. A Sendai virus mutant deficient in HN protein has been shown to infect HepG2 cells (22), a line that retains the asialoglycoprotein receptor (ASGP-R) in continuous culture (37). The F protein contains a terminal galactose moiety (42) and thus is a likely ligand for recognition by the ASGP-R, a mammalian lectin that specifically recognizes glycoproteins terminal in galactose or *N*-acetylgalactosamine (3). In addition, reconstitution of viral envelopes with F alone was sufficient to cause envelope-cell fusion in the presence of lectins or antibodies which attach the virosomes to the cells (15, 38). In studies using similar reconstitution and targeting techniques, it has been claimed that HN also plays a role in the fusion of viral envelopes with cells (10, 20, 28) or with liposomes (7).

To shed further light on this issue, we used an assay based on lipid mixing (13, 19, 29, 34) to examine the kinetics of fusion of reconstituted Sendai virus envelopes. We prepared viral envelopes containing the individual glycoproteins from Sendai virus and a fluorescent lipid and show that F and HN cooperate in mediating the fusion of viral envelopes with cells.

MATERIALS AND METHODS

Reagents. *N*-4-Nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (NBD-PE) was purchased from Avanti (Birmingham, Ala.). SM2 Bio-Beads were obtained from Bio-Rad (Richmond, Calif.). Triton X-100 (TX-100) was obtained from Aldrich (Milwaukee, Wis.). Orosomucoid and asialo-orosomucoid were a generous gift from Gilbert Ashwell, National Institutes of Health. Neuraminidase, wheat germ agglutinin (WGA), dithiothreitol (DTT), and trypsin were procured from Sigma Chemical Co., St. Louis, Mo. Carrier-free $Na^{125}I$ was obtained from BARC, Trombay, India. All other reagents used were of analytical grade.

Virus. Sendai virus (Z strain), initially obtained from A. Loyter, Hebrew University, Jerusalem, was propagated in the allantoic sac of 10- to 11-day-old embryonated chicken eggs (34). The diluted viral stocks in phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 150 mM NaCl [pH 7.4]) containing 2 to 4 hemagglutinating units were used to infect the eggs. After infection, the eggs were incubated at 37°C for 48 h and then left at 4°C for 24 h. The virus was harvested and purified in accordance with the method described by Peretz et al. (32). The allantoic fluid was centrifuged for 10 min at 1,500 rpm in a tabletop centrifuge, and the clear supernatant was collected. Virus was pelleted by centrifuging the clear supernatant for 60 min at 100,000 × *g* at 4°C in

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a L8-70 M ultracentrifuge, using a 70 Ti rotor. The virus pellet was suspended in a small volume of PBS and finally dispersed with a Teflon-coated tissue homogenizer (Wheaton Industries, Millville, N.J.). The viral yield from each egg was 0.5 to 1 mg of protein, as estimated by the method of Markwell et al. (21). The purified virus was aliquoted in batches of 5 mg of protein (2 mg of viral protein per ml of PBS) and stored at −70°C until further use.

Cells. HepG2 cells (a human hepatoblastoma cell line), obtained from the American Type Culture Collection, Rockville, Md., were grown at 37°C in a 10% CO_2 atmosphere in minimal essential medium (GIBCO) containing 25 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin sulfate per ml in 75-cm^2 plastic bottles (Costar, Cambridge, Mass.). Single-cell suspensions of HepG2 cells were made by lifting them from monolayers with 5 mM EDTA in Dulbecco's PBS (DPBS; pH 7.4). Fresh erythrocytes (RBCs) were obtained from healthy Swiss albino mice stock (University of Delhi, South Campus). Desialylation of cultured HepG2 cells was performed by incubation with neuraminidase (type V; final concentration, 0.5 U/ml; Sigma) and soybean trypsin inhibitor (type 1-S; 1 mg/ml; Sigma) in a 4-ml suspension of 10^7 cells per ml in Dulbecco's modified Eagle's medium (DMEM) without serum for 1 h at room temperature with constant shaking. Then 10 ml of DMEM with 10% fetal calf serum was added, and the cells were washed three times with DMEM without serum at 4°C. The cells were finally resuspended either in DPBS with 2 mM $CaCl_2$ and 2 mM $MgCl_2$ or 5 mM EDTA-containing DPBS.

Preparation of fusogenic F,HN-, F-, and F,HN_{red}-virosomes. Reconstituted Sendai virus envelopes containing the F protein (F-virosomes) were prepared as described by Tomasi and Loyter (39), with modifications. A suspension of 20 mg of Sendai virus in PBS was centrifuged, and the pellet obtained was resuspended in 4 ml of buffer A (150 mM NaCl, 20 mM Tris [pH 8.4]) containing 3 mM DTT. The suspension was incubated at 37°C for 2 h and then dialyzed at 4 to 10°C for 16 h against three changes of 2 liters of buffer B (150 mM NaCl, 10 mM Tris [pH 7.4], 2 mM Ca^{2+}, 2 mM Mg^{2+}). The viral particles were centrifuged, and the pellet obtained was resuspended in 2 ml of buffer B containing 40 mg of TX-100. After incubation at 20°C for 1 h, the suspension obtained was centrifuged (100,000 × *g* for 1 h at 4°C) to remove the detergent-insoluble substances, which presumably contain reduced HN and nucleocapsid. From the clear supernatant, the detergent was removed by stepwise addition of SM2 Bio-Beads. Briefly, 320 mg of methanol-washed SM2 Bio-Beads was added to 2 ml of supernatant and incubated at 4°C with gentle rocking. After 2 h, an additional 320 mg of SM2 Bio-Beads was added, and incubation continued at 20°C for 2 h. Incubation was further continued for 2 h at 20°C with an additional 640 mg of SM2 Bio-Beads. The turbid suspension was separated from the Bio-Beads with a 26-gauge needle and centrifuged as described above. The pellet containing F-virosomes was resuspended (0.5 to 1 mg of protein) in 1 ml of buffer B and stored at 4°C. Viral envelopes containing both F and HN (F,HN-virosomes) were made as described above but without DTT treatment (11). F,HN_{red}-virosomes (HN_{red} denotes HN whose disulfide bonds were irreversibly reduced) were prepared by treating the F,HN-virosomes with 3 mM DTT at 37°C for 2 h in buffer A followed by the removal of DTT by dialysis against buffer B. After centrifugation, the F,HN_{red}-virosomes were suspended (1.6 to 2 mg of protein) in 1 ml of buffer B and stored at 4°C.

NBD-PE was incorporated into the virosomes as described earlier (29), with minor modifications. Briefly, the NBD-PE dissolved in chloroform was dried in a glass vial under nitrogen to form a thin film. The supernatant from the detergent extract, containing only the viral proteins and lipids, was added to the NBD-PE film and incubated at 20°C for 30 min with gentle shaking. The detergent was removed by SM2 Bio-Beads as described above. The amount of NBD-PE used was estimated to be 10 mol% of the total viral lipid. The virosomes were passed through 26-gauge needle 20 times and filtered through 0.22-μm-pore-size Millipore type Millex GV filters to remove large aggregates before use.

Intact Sendai virus was radiolabeled with ^{125}I as described by Wolf et al. (41). The hemagglutinating units and hemolytic activity of the ^{125}I-labeled virus were checked as reported earlier (32). Radiolabeled F- and F,HN-virosomes were prepared from ^{125}I-labeled intact virus.

Composition of the virosomes. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was used to check the purity of F,HN-, F-, and F,HN_{red}-virosomes. The analysis was done in a separating gel containing 10% acrylamide and 0.27% bisacrylamide in the presence of 1.0% SDS. The method of sample preparation and the discontinuous buffer system used were as reported earlier (18), with a constant current of 30 mA for 6 h. The gel was stained with 0.25% Coomassie blue in 35% methanol–10% acetic acid. The stained gel was subjected to autoradiography for further characterization of the virosomal preparations.

The ratio of F and HN in F,HN-virosomes and F,HN_{red}-virosomes was measured as follows. F,HN-virosomes were reduced with 3 mM DTT to form F,HN_{red}-virosomes as described above. The F,HN_{red}-virosomes were then solubilized with TX-100 and centrifuged as described above to separate the detergent-insoluble HN_{red} from the detergent-solubilized F protein. The amount of protein in the pellet and supernatant was measured according to Markwell et al. (21). The ratio of F and HN in F,HN-virosomes and F,HN_{red}-virosomes was found to be 1:1 (wt/wt) in all preparations. The amount of lipid in the virosomes was measured by phosphorus analysis (4).

Hemolysis assay. RBCs were drawn from healthy Swiss albino mice in the presence of heparin (50 U/ml) and washed three times with PBS. Washed RBCs were finally resuspended at a concentration of 0.5% (vol/vol) in PBS. After 0.5 ml of 0.5% RBCs was mixed with 0.5 ml of virosomes, WGA was added to a final concentration of 6 μg/ml. The mixture was incubated on ice for 40 min with occasional shaking and then at 37°C for 2 h with periodic shaking at an interval of 15 min. The reaction was terminated by chilling the mixture on ice and spun at 1,000 × *g* for 10 min. The amount of hemoglobin released in the presence of 0.3% TX-100 was taken as 100% lysis. Virosomes treated at 50°C for 20 min or with trypsin (29, 39) served as control for spontaneous and WGA-induced leakage during incubation.

Spectrofluorometric measurements. The fusion of NBD-PE-labeled virosomes with HepG2 cells was carried out as reported earlier (29). The virosomes (25 μg of protein) were mixed with 10^7 HepG2 cells in 1 ml of DPBS (GIBCO) containing 2 mM Ca^{2+} and 2 mM Mg^{2+} and incubated at 4°C for 40 min. The virosome-cell complexes were washed three times at 300 × *g* in the same buffer to remove unbound virosomes. To determine the specificity of interaction of F-virosomes with HepG2 cells, the binding and fusion assay was carried out in the presence of orosomucoid (20 μg/ml), asialo-orosomucoid (20 μg/ml), or 5 mM EDTA in DPBS.

Fluorescence changes as a result of fusion of NBD-PE-

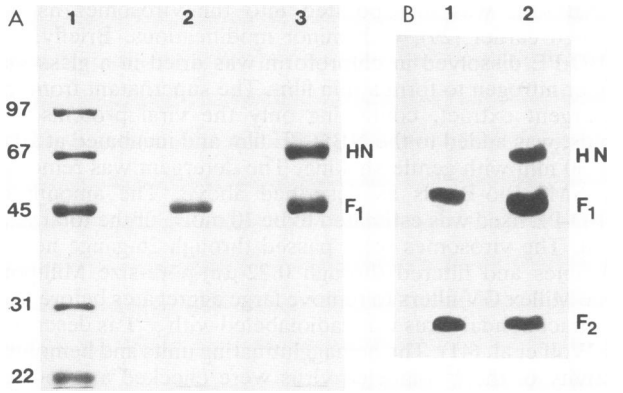

FIG. 1. Electrophoretic analysis of virosomes. (A) Coomassie-stained gel following SDS-PAGE of F-virosomes (lane 2) and F,HN-virosomes (lane 3). Molecular weight markers (indicated in kilodaltons) are shown in lane 1. (B) Radioautographs of gels following SDS-PAGE of ^{125}I-labeled F-virosomes (lane 1) and F,HN-virosomes (lane 2). In both panels, 25 and 50 μg of total protein were loaded for F-virosomes and F,HN-virosomes, respectively.

labeled virosome-cell complexes were measured continuously with a spectrofluorometer (model 8000; SLM-Aminco, Urbana, Ill.). Fifty microliters of the virosome-cell suspension (from a final suspension of 300 μl in DPBS after washing) was placed into a cuvette containing 2 ml of DPBS prewarmed to 37°C. Fluorescence measurement was started with 2-s time resolution at 473/535-nm (excitation/emission) wavelength. A 515-nm cutoff high-pass filter was placed in the emission optical path to reduce scattering. The fluorescence dequenching (% FDQ) data were normalized and calculated according to the following equation: % FDQ = 100 × (F-F_0/F_t-F_0), where F and F_t are the fluorescence (arbitrary) units at a given time point and in the presence of 0.1% TX-100, respectively (35). In the kinetic experiments presented in Fig. 3 and 4, F_0 is fluorescence at time zero. In the single-time-point experiments presented in Table 2, F_0 is the observed fluorescence after incubation at 4°C for a given amount of time.

RESULTS

Characterization of virosomes. The protein composition of the virosomes was examined by SDS-PAGE in the presence of β-mercaptoethanol. Coomassie blue staining of the gel revealed prominent bands corresponding to F and HN proteins (Fig. 1A). F,HN_{red}-virosomes exhibited the same SDS-PAGE pattern as did F,HN-virosomes (data not shown). The F protein is synthesized as a precursor, F0, which is subsequently cleaved by a host cell protease into its biologically active form, composed of F1 and F2 subunits linked by disulfide bonds (36), which are resolved on SDS-PAGE. The F-virosomes were found to be free of any detectable contamination by other proteins. Staining of F2 was very weak, in agreement with previous reports (15, 27). To detect the presence of any trace amount of HN protein in the F-virosomes, ^{125}I-labeled virosomes were subjected to SDS-PAGE under the same conditions followed by autoradiography. Figure 1B shows that the F- and F,HN-virosome preparations were free of contamination with extraneous proteins. Moreover, no HN was detected in the F-virosome preparation, which is consistent with the fact that the F-virosomes did not have any hemagglutinating activity (data not shown).

TABLE 1. Protein and phospholipid composition of Sendai virus and virosomes

Prepn	Protein/PL ratio[a]		Molar F/PL ratio[b]
	By wt	Molar	
F-virosome	0.49 ± 0.04	1/158	1/158
F,HN-virosome	0.76 ± 0.07	1/106	1/196
Intact virus	2.83 ± 0.20		

[a] The protein/phospholipid (PL) weight ratio was determined as described in Materials and Methods. The averages are from four separate virosome preparation. The molar ratios were calculated by using molecular sizes of 60,000, 67,000, and 780 Da for F, HN, and phospholipid, respectively (28).
[b] The calculated ratio in the mixed virosomes is based on the measurement F/HN (wt/wt) = 1 as described in Materials and Methods.

The protein and phospholipid compositions of the virosomes and virus are summarized in Table 1. The data indicate that the F virosomes are enriched in F protein, although the total protein/phospholipid ratio was higher in the mixed virosomes.

The virosomes were found to retain calcein, fluorescein isothiocyanate-dextran of M_r 4,400, and lysozyme over a period of 48 to 72 h (data not shown) both at 4 and 37°C. Electron microscopy of negatively stained preparation of virosomes revealed their spherical shape with sizes varying between 100 and 300 nm in diameter (data not shown). These results are indications of the stability and structural integrity of the virosome preparations.

Interaction of virosomes with RBCs. The F protein of the paramyxovirus envelope is required for virus-cell fusion but exhibits other activities such as hemolysis (15). Figure 2 shows that the F protein is sufficient to cause hemolysis if WGA is added to provide attachment. However, the presence of intact HN in the F,HN-virosomes markedly enhanced the lytic activity, both in rate and in extent (Fig. 2). Note that the amount of F protein required for hemolysis induced by F-virosomes or F,HN_{red}-virosomes was about 25 times higher than the amount required for hemolysis induced by F,HN-virosomes. WGA did not affect the hemolytic activity of F,HN-virosomes (Fig. 2). We also examined hemolysis of mouse RBCs induced by virosomes containing F and HN_{red}. The effect of DTT on F is reversible, but HN_{red} loses its binding function (20). Figure 2 shows that the presence of HN_{red} in the viral membrane did not have a marked effect on the lytic activity, even though the total protein/phospholipid ratio was higher in the mixed virosomes. HN-virosomes, prepared by selective inactivation of the F protein with galactose oxidase, trypsin, or phenylmethylsulfonyl fluoride, did not have any hemolytic activity, although they were able to hemagglutinate RBCs (data not shown).

Fusion of virosomes with HepG2 cells. It has been shown that a mutant Sendai virus deficient in HN enters cultured liver cells through the interaction of the terminal galactose of F with the ASGP-R (22). In the absence of Ca^{2+} or in the presence of competitive inhibitors, the ligands do not bind to the ASGP-R (3). The ASGP-R contains TSA moieties (31) to which HN could bind. However, those binding sites could be removed by treating HepG2 cells with neuraminidase, yielding TSA^- cells. We could therefore manipulate the system to allow fusion of F,HN-virosomes via the ASGP-R pathway (TSA^- cells plus Ca^{2+}-Mg^{2+}), the TSA-R pathway (TSA^+ cells plus EDTA), or both pathways (TSA^+ cells plus

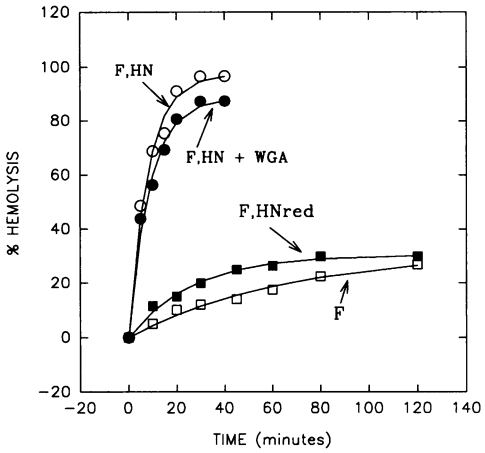

FIG. 2. Kinetics of hemolysis induced by virosomes. Virosomes were added to mouse RBCs (0.5% [vol/vol]) in a total volume of 1 ml in PBS (pH 7.4), and hemolysis was measured as a function of time at 37°C as described in Materials and Methods. The points are averages of three independent experiments. Open circles, F,HN-virosomes (2 μg of F protein); closed circles, F,HN-virosomes (2 μg of F protein) in the presence of 6 μg of WGA; closed squares, F,HN_{red}-virosomes (50 μg of F protein) in the presence of WGA (6 μg/ml); open squares, F-virosomes (50 μg of protein) in the presence of WGA (6 μg/ml). The lines through the points are best fits to single exponential curves with rate constants of 0.123/s. 0.113/s, 0.037/s, and 0.015/s for F,HN-virosomes, F,HN-virosomes plus WGA, F,HN_{red}-virosomes, and F-virosomes, respectively. The F,HN_{red}-virosomes and F-virosomes had no hemolytic activity in the absence of WGA (not shown).

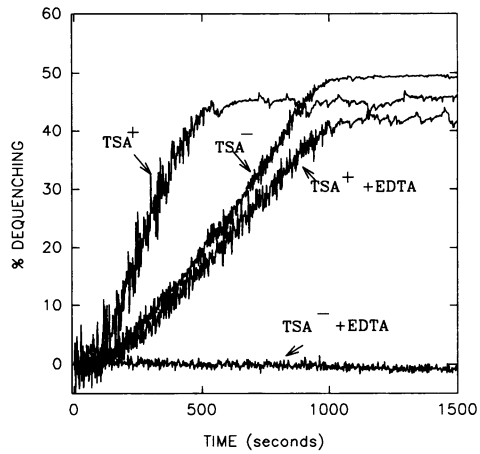

FIG. 3. Kinetics of fusion of F,HN-virosomes with HepG2 cells via different receptor pathways. The virosomes (25 μg of F protein) were incubated with 10^7 untreated (TSA^+) or desialylated (TSA^-) HepG2 cells at 4°C for 40 min in PBS (pH 7.4) containing either 2 mM Ca^{2+} and 2 mM Mg^{2+} or 5 mM EDTA. Desialylation of cultured HepG2 cells was performed by incubation with neuraminidase as described in Materials and Methods. The virosome-cell complexes were washed three times at 300 × *g* in the same buffer to remove unbound virosomes. Fifty microliters of the virosome-cell suspension was placed into a cuvette containing 2 ml of the appropriate buffer prewarmed to 37°C, and fluorescence measurements were made within 2 s as described in Materials and Methods. Percent dequenching was calculated as described in Materials and Methods. The untreated HepG2 cells in the presence of Ca^{2+} and Mg^{2+} (marked TSA^+) admitted fusion via both the ASGP-R and TSA-R pathways. Desialylated HepG2 cells in the presence of Ca^{2+} and Mg^{2+} (marked TSA^-) allowed fusion via the ASGP-R pathway only, and untreated HepG2 cells in the presence of 5 mM EDTA (marked TSA^+ + EDTA) permitted fusion via the TSA-R pathway only. The control (marked TSA^- + EDTA) was performed with desialylated HepG2 cells in the presence of 5 mM EDTA. Delay times calculated from the intercept of a line drawn through the steepest portion of the curve (linear regression) with the time axis were 63, 181, and 170 s for both pathways, the ASGP-R pathway, and the TSA-R pathway, respectively.

Ca^{2+}-Mg^{2+}). Figure 3 shows that the kinetics of fusion of the F,HN-virosomes via both pathways were more rapid than via either the ASGP-R or TSA-R pathway. The kinetics were similar in the presence of sodium azide, indicating that the fusion was at the plasma membrane (data not shown). Figure 3 also shows that TSA^- cells in the presence of EDTA allow no fusion, since neither F nor HN was able to bind to the respective receptors. Similar results were obtained in the presence of the inhibitory ligand asialo-orosomucoid (3) (data not shown). Other controls which showed no increase in fluorescence dequenching were heat-treated and trypsinized virosomes (data not shown).

By using TSA^- HepG2 cells, it is possible to examine in a quantitative way the role of HN in fusion of virosomes in the absence of HN binding to its specific TSA-R. Both F-virosomes and F,HN-virosomes bind TSA^- HepG2 cells by interaction of the terminal galactose moiety on F with the ASGP-R. Figure 4 shows that both types of virosome fuse with the TSA^- HepG2 cells. However, the presence of HN in the virosomes resulted in a two- to threefold-larger fusion rate. The curve's yield delay times (intercept of a line drawn through the steepest portion of the curve with the time axis) were 181 and 466 s for the F,HN- and F-virosomes, respectively. F- and F,HN_{red}-virosomes fused with HepG2 cells with the same rate (data not shown). This finding indicates that fusion mediated by HN,F-virosomes is not due to the higher protein density in the mixed virosomes (Table 1).

To provide some indication of the reproducibility and variation between experiments, we performed additional experiments (Table 2). Table 2 shows the percent dequenching after 10 min, which gives a measure of the rate, and 30 min, which gives the extent of fusion. The extents of fusion were about the same for all fusion-active preparations. There was no fusion in the absence of binding. However, the data in Table 2 confirm our finding that rates of fusion were enhanced if F and HN bind to both receptors, compared with rates of fusion mediated by F–ASGP-R or HN–TSA-R interactions. Moreover, the rate of fusion was higher via the ASGP-R pathway when both F and HN were present in the virosome than when F alone was present. NBD-PE-labeled HN-virosomes, prepared by selective inactivation of the F protein with galactose oxidase, trypsin, or phenylmethylsulfonyl fluoride, did not have any fusion activity with HepG2 cells, although they were able to bind to those cells via the TSA-R (data not shown).

DISCUSSION

We have used a continuous assay to demonstrate that Sendai virus F protein is sufficient for viral envelope protein-mediated membrane fusion. The binding role of HN can be replaced either by lectins or by a receptor for the F protein on the target membrane. The continuous fusion assays offer a highly sensitive direct method to obtain quantitative information on the fusion process during its very early stages (6, 13, 19, 35).

SDS-PAGE analysis and autoradiographic visualization of

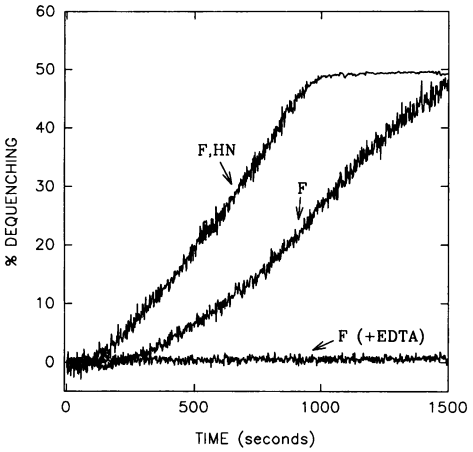

FIG. 4. Kinetics of fusion of F,HN-virosomes and F-virosomes with desialylated HepG2 cells via the ASGP-R pathway. For details about the desialylation of HepG2, incubation with virosomes, and fluorescence measurements, see the legend to Fig. 3. F,HN and F, experiments with F,HN-virosomes and F-virosomes in the presence of 2 mM Ca^{2+} and 2 mM Mg^{2+}; F (+EDTA), the control with F-virosomes in presence of 5 mM EDTA. The virosomes contained 25 μg of F protein. Delay times calculated from the intercept of a line drawn through the steepest portion of the curve (linear regression) with the time axis were 181 and 466 s for the HN,F- and F-virosomes, respectively.

the radiolabeled virosomes demonstrate the complete absence of HN protein in the F-virosomal preparation (Fig. 1). The F-virosomes were enriched in F protein, although the total protein/phospholipid ratio was higher in the mixed virosomes (Table 1). Since the hemolytic activity of Sendai virus is closely related to its membrane fusion activity (28), we first examined the hemolytic activity of the various virosome preparations. Figure 2 shows that the hemolytic activity of F,HN-virosomes is significantly higher than that of F-virosomes and F,HN_{red}-virosomes in the presence of WGA, indicating that fusion mediated by F protein in the presence of attachment factors is much less efficient than fusion mediated by F and HN together (9, 15, 27, 38). WGA did not affect the fusion when both F and HN were incorporated into the virosome (Fig. 2). The higher protein/lipid ratio might be responsible for the higher hemolytic activity of the F,HN-virosomes (Fig. 2). However, the observation that the activity of F,HN_{red}-virosomes was not significantly higher than that of F-virosomes indicates that the total protein/phospholipid ratio is not the determining factor. The F,HN_{red}-virosomes did not hemagglutinate RBCs (data not shown), indicating that HN had lost its binding property, but did hemolyze RBCs (Fig. 2) and fused with HeLa cells in the presence of WGA (data not shown) at about the same rate as did F-virosomes, indicating that the inactive HN did not affect the function of the fusion protein.

HepG2 cells provide a system with which to study fusion resulting from two independent modes of virosome binding. Fusion of the F-virosomes with HepG2 cells is achieved by an alternative route that bypasses the native interaction of HN with its TSA-R (Fig. 3 and 4). The inhibitory effects of EDTA and asialo-orosomucoid indicate that the asialoglycoprotein receptor of HepG2 cells is involved in binding of the virosomes. The terminal galactose moiety of the F protein interacts with the galactose receptor of liver cells, as shown earlier (22). We used desialylated HepG2 cells to compare the activities of F- and F,HN-virosomes in the absence of a receptor for HN. It has been reported that desialylation of HepG2 cells inhibits binding of ligands to the ASGP-R (37). However, those experiments were done with confluent cells whose ASGP-R could interact with terminal galactose moieties on adjacent cells. When cells are in suspension, there are apparently sufficient ASGP-R molecules available on the surface to bind to terminal galactose on F.

We show that both F-virosomes and F,HN-virosomes fuse with desialylated HepG2 cells, although the rate was two- to threefold higher if HN was included in the viral envelope (Fig. 3 and Table 2). The data shown in Fig. 4 and Table 2 provide another indication of such cooperation. The ASGP-R has TSA moieties (31) and therefore may also provide a binding site for HN. At the same time, it binds to the terminal galactose moiety on F via the receptor binding site. In the case of such dual attachment, the fusion rates were threefold higher than when F or HN was separately attached either to the ASGP-R or the TSA-R.

Studies of paramyxovirus-induced syncytium formation indicate that both HN and F are required for paramyxovirus-induced cell fusion (8, 16, 24, 25, 33, 40), although in the case of simian virus 5 (14, 26, 30) and measles virus (1), the F protein alone has been reported as sufficient to cause cell-cell fusion. Attachment factors such as lectins or antibodies appear to be ineffective in facilitating F-mediated cell fusion (16, 25), but they do facilitate fusion of reconstituted viral envelopes mediated by F (15, 38). The inability to induce cell fusion following coexpression of F and HN genes from

TABLE 2. Fusion of F,HN- and F-virosomes with HepG2 cells[a]

Virosomes[b]	Buffer[c]	Cells	Attachment	% FDQ (mean ± SD)[d]	
				10 min	30 min
F,HN	Ca^{2+}-Mg^{2+}	TSA^+	Both	58.3 ± 2.5	55.0 ± 5.1
F,HN	Ca^{2+}-Mg^{2+}	TSA^-	ASGP-R	36.1 ± 2.0	60.3 ± 3.1
F,HN	EDTA	TSA^+	TSA-R	29.8 ± 2.6	64.2 ± 3.0
F,HN	EDTA	TSA^-	None	0	0
F	Ca^{2+}-Mg^{2+}	TSA^+	ASGP-R	15.2 ± 1.2	45.8 ± .5
F	Ca^{2+}-Mg^{2+}	TSA^-	ASGP-R	17.0 ± 1.6	44.0 ± 3.2
F	EDTA	TSA^+	None	0	0
F	EDTA	TSA^-	None	0	0

[a] For details about the measurement of fusion based on fluorescence dequenching, see the legends to Fig. 3 and 4.
[b] The virosomes contained 25 μg of F protein.
[c] The experiments were carried out in PBS (pH 7.4) containing either 2 mM Ca^{2+} and 2 mM Mg^{2+} or 5 mM EDTA.
[d] Calculated as described in Materials and Methods. are from three different virosome preparations. For each virosome preparation, the determinations at the different time points were done in triplicate.

different human parainfluenza virus types (types 2 and 3) indicates a high degree of specificity between the homologous pair in mediating cell fusion (16). Why, then, is the requirement for HN more stringent for cell fusion than for virosome fusion?

A possible answer could be found by considering studies of paramyxovirus-induced cell fusion by Henis and coworkers (2, 12), who found a strict correlation between lateral mobility of viral envelope proteins and their ability to induce cell fusion. Lectins have been shown to immobilize viral envelope proteins on cell surfaces. Whereas such lateral mobility is not crucial for fusion of intact virus, immobilization of viral envelope proteins might be detrimental for cell fusion. Moreover, it was shown that both the Sendai virus F and HN proteins accumulate in cell-cell contact regions in the course of fusion (2). This finding indicates that migration of the viral glycoproteins to cell contact regions and accumulation at the contact sites are essential parts of the fusion mechanism and form the basis of the requirement for their lateral motion in the fusion event. Presumably, a homologous pair of F and HN proteins is required for the recruitment of the F protein to the appropriate contact region for cell fusion (16). In the virosome, on the other hand, the F protein is recruited in a limited area of the vesicle by the reconstituted technique itself, and therefore HN is not absolutely required. However, the presence of HN in the reconstituted particle did enhance the rate of fusion (Fig. 3 and 4), presumably by recruiting F to its proper position. The method of reconstitution, the size of the virosome, and the nature of the encapsulated molecules are very important factors in determining whether transfer of F-virosome contents into cells can be mediated by other forms of attachment to the target cell than HN (9, 15, 27, 38).

The notion that there may be different requirements for viral fusion and viral envelope protein-mediated cell fusion is consistent with the finding that receptor requirements for human parainfluenza virus type 3 to infect cells are different from those for fusion between cells (26). The level of TSA-R on the susceptible cells could be modulated by the viral neuraminidase or an externally added neuraminidase. By infecting cells with the virus at different multiplicities of infection, Moscona and Peluso (26) showed that cells with a low level of TSA-R are susceptible to viral fusion, whereas cell fusion is induced when cells have a high level of TSA-R. This finding is consistent with the notion that massive recruitment of HN–TSA-R complexes is required for cell fusion, whereas only a limited amount of such complexes is required for viral fusion.

Observations on the modification of intact Sendai viruses to generate specificity toward target cells for binding and fusion are indeed encouraging. In recent years, reports on the possibility of using virosomes and fusogenic liposomes as an efficient delivery vehicle in both in vitro and in vivo conditions have appeared (5, 17). The observations presented here and the conclusion that F in the presence of a targeting ligand is sufficient for virosome-cell fusion have provided substantive support for this suggestion. F-virosomes have also been found to efficiently deliver entrapped aqueous markers to HepG2 cells by means of membrane fusion (3a). Studies to provide a critical evaluation of the efficiency of F-virosomes and fusogenic liposomes with defined specificities to deliver enzymes, polypeptide toxins, and genetic materials into selected cell types both in vitro and in vivo are in progress.

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