Biochimica et Biophysica Acta 854 (1986) 157–168
Elsevier

BBA 72867

The interaction of Sendai virus glycoprotein-bearing recombinant vesicles with cell surfaces

Mohammed N. Al-Ahdal *, Tanveer F. Abidi ** and Thomas D. Flanagan ***

Department of Microbiology, Schools of Medicine and Dentistry, State University of New York at Buffalo, Buffalo, NY 14214 (U.S.A.)

(Received June 10th, 1985)

Key words: Viral glycoprotein; Membrane reconstitution; Membrane-virus interaction; (Sendai virus)

Sendai virus glycoproteins HN and F were purified by immunoaffinity chromatography from virions disrupted by β-D-octylglucoside. The purified glycoproteins were reconstituted in recombinant vesicles with phosphatidylcholine or phosphatidylethanolamine and phosphatidylserine. P815 or EL-4 cells treated with glycoprotein HN/F-phosphatidylcholine recombinant vesicles acquired the glycoproteins and retained them in the plasma membrane for 4 h as demonstrated by surface immunofluorescence specific for each protein. Cells treated with glycoprotein HN-phosphatidylcholine recombinant vesicles initially bore glycoprotein HN on the surface but the protein eluted within 2 h. Surfaces of cells treated with glycoprotein F-phosphatidylcholine recombinant vesicles did not acquire the glycoprotein. Cells treated with glycoprotein HN-phosphatidylethanolamine: phosphatidylserine recombinant vesicles or glycoprotein F-phosphatidylethanolamine: phosphatidylserine recombinant vesicles in the presence of 5 mM Ca^{2+} acquired each protein for at least 2 h. Experiments showed that the acquired glycoproteins capped with antibody and that when glycoproteins HN and F were together on the surface they co-capped. Acquired viral glycoproteins did not co-cap with intrinsic H-2 glycoproteins.

Introduction

Paramyxovirus surface glycoproteins are important factors in evoking immune response to infection [1–4]. The glycoproteins are found in peplomers that project from the envelope of the virion and from the plasma membrane of the virus-infected cell. In Sendai virus, a parainfluenza virus, the glycoproteins are the HN protein [5] and the F protein [3]. In order to define the role and contribution of each glycoprotein in cell-mediated immune reactions we have developed methods for their insertion into the plasma membrane of target cells [6]. Purified glycoproteins HN and F, together and separately, were reconstituted with purified phospholipids, enabling the introduction of each independently. This approach bypassed the problems of de novo protein synthesis in cells infected with active virus and the presence of other viral components when the cells are treated with ultraviolet-inactivated virus or with reassembled vesicles using the native viral lipids [7].

In this report, we describe the interaction of glycoprotein-bearing recombinant vesicles with cell

* Present address: Department of Microbiology, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia.
** Present address: Department of Microbiology, UMDNJ-Rutgers Medical School, Piscataway, NJ 08854, U.S.A.
*** To whom correspondence should be addressed at: 218 Sherman Hall, State University of New York at Buffalo, Buffalo, NY 14214, U.S.A.

Abbreviations: CNBr, cyanogen bromide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

surfaces and the subsequent behavior of the glycoproteins after insertion.

Materials and Methods

Virus. The Cantell strain of Sendai virus was grown in the allantoic cavity of 10-day-old embryonated eggs. The allantoic fluid was harvested and clarified by centrifugation at $3000 \times g$ for 30 min at 4°C. The virus was pelleted at $60\,000 \times g$ for 2 h at 4°C. Further purification was carried out by sedimenting the virus to equilibrium through a 15–60%, w/w sucrose gradient as described by Haywood [8]. The protein concentration as determined by the method of Bradford [9], was adjusted to 1 mg/ml in 10 mM phosphate buffer (pH 7.2) after removal of the sucrose by dialysis.

Preparation of HN and F glycoproteins. The virus particles were disrupted by adding the nonionic detergent β-D-octylglucoside (Calbiochem, San Diego, CA) to a final concentration of 1% (33 mM). The detergent/virus suspension was mixed gently and incubated at room temperature for 1 h. Centrifugation was carried out at $100\,000 \times g$ for 30 min at 4°C to remove intact virus and other nonsolubilized components [7]. The supernatant contained glycoproteins HN and F, viral lipids and β-D-octylglucoside.

Preparation of antisera. Preliminary separation of glycoprotein HN from glycoprotein F was carried out with some modifications according to the method of Urata and Seto [10] for HN and Hale et al. [11] for F, using DEAE-Bio-Gel A and hydroxyapatite columns (Bio-Rad Laboratories, Richmond, CA), respectively. β-D-Octylglucoside was used to equilibrate the column material and removed from the separated HN and F glycoprotein preparations by dialysis. The ion-exchange preparations were used to immunize rabbits. The antisera were heat-inactivated at 56°C for 30 min. Monospecificity was achieved by absorption on glycoprotein-bearing beads as described below.

Preparation of immunoaffinity columns. In order to produce monospecific antisera, immunoabsorbants were made by coupling the ion-exchange preparations of glycoprotein HN or F separately to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). Anti-HN and anti-F sera were absorbed several times on glycoprotein F-Sepharose and glycoprotein HN-Sepharose columns, respectively, to insure monospecificity. The monospecific antisera were precipitated with ammonium sulfate and the precipitate redissolved in phosphate buffer (pH 7.2). After passage over Sephadex G-200, the protein concentration was adjusted to 200 μg/ml. Anti-HN and anti-F were coupled to CNBr-activated Sepharose. Glycoproteins HN and F were separated by running the supernatant from the β-D-octylglucoside disruption of virions through the anti-HN-Sepharose column. The effluent was then run through the anti-F-Sepharose column. In both cases, the glycoprotein molecules were eluted by 3 M KSCN (pH 7.2). KSCN was removed by dialysis against phosphate buffer (pH 7.2).

Constitution of glycoproteins into recombinant vesicles. Phosphatidylcholine (GIBCO, Grand Island, NY) or a 1:1 mixture of phosphatidylethanolamine and phosphatidylserine (Avanti Biochemicals, Birmingham, AL) were used to reconstitute HN, F, or both glycoproteins into recombinant vesicles. The recombinant vesicles were prepared by adding β-D-octylglucoside and lipids in chloroform to a clean glass-tube. In some experiments, 10 μCi of [^{3}H]triolein in ethanol was added for each 2 mg phospholipid. The mixture was dried under a stream of N_2. The glycoproteins in phosphate buffer (pH 7.2) were added and mixed until the residue was dissolved. The final proportions by weight of β-D-octylglucoside/lipid/protein were 8:2:1. The mixture was dialyzed against a 500-fold volume of phosphate buffer (pH 7.2) at 4°C for 36 h. Nitrogen gas was bubbled through the buffer and the buffer exchanged every 12 h. Less than 0.1 mM of β-D-octylglucoside remained after dialysis as determined by the anthrone test [7]. The retained material was layered on a 10–45%, w/w sucrose gradient in phosphate-buffered saline, (pH 7.2) in the case of glycoprotein HN/F-phosphatidylcholine recombinant vesicles or a 0–45% for the other recombinants and centrifuged to equilibrium at $60\,000 \times g$ for 18 h at 4°C. Fractions were collected and dialyzed against phosphate buffer (pH 7.2) in the case of phosphatidylcholine recombinant vesicles or 0.01 M Tris (pH 7.4)/5 mM $CaCl_2$/0.1 mM dithiothreitol in the case of phosphatidylethanolamine : phosphatidylserine recombinant vesicles.

All buffers were flushed with N_2. 1-ml fractions were collected and characterized by measurement of radioactivity, protein concentration and biologic activities. The fractions containing the recombinant vesicles were pooled and the protein concentration adjusted to 100 μg/ml.

Measurement of biological activities of glycoproteins. The HN glycoprotein has both hemagglutinating and neuraminidase activities [5]. Hemagglutination and neuraminidase activity of virus and recombinant vesicles were measured by methods previously described [7]. The method of Aminoff [12] was used to measure released *N*-acetylneuraminic acid in the neuraminidase activity assay.

Hemolysis by Sendai virus is a function of the F glycoprotein [13]. Measurement of hemolytic activity was carried out by the method of Hsu et al. [14].

SDS-polyacrylamide gel electrophoresis. Discontinuous SDS-polyacrylamide gel electrophoresis was performed by the modified Laemmli procedure described by Smith et al. [15] using 0.75 mm thick slab gels. The total polyacrylamide, which contained 2.7% of the cross-linking bisacrylamide, was 10% for the separating gel and 3% for the stacking gel, and both contained 0.067% of each of ammonium persulfate and tetramethylenediamine. 20-25 μg of each sample was mixed with digestion mixture containing Bromophenol blue as a tracking dye and loaded in 100 μl volume. The gels were run at 30 mA per gel until the dye was approx. 0.5 cm from the bottom. The gels were stained by either Coomassie blue or by the silver procedure as described by Merril et al. [16].

Western blotting

SDS-polyacrylamide gel electrophoresis was performed using purified Sendai virus and ovalbumin. The separated proteins were transferred to nitrocellulose sheets (Bio-Rad Laboratories, Richmond, CA) as described by Towbin et al. [17]. Antisera in a 1:50 dilution were incubated with the sheets. Reaction with transferred proteins was detected by the method of Burnette [18] using ^{125}I-labeled protein A and exposing the treated sheets to Kodak XR film at -70°C overnight.

Cell lines. P815 mastocytoma tumor cells from DBA/2 mice and EL4 lymphoma cells from C57BL/6 mice were provided by Dr. Ichiro Nakamura (Department of Pathology, SUNY at Buffalo). They were maintained by weekly transfer of 10^7 ascites cells in mice of the strain of origin for 4 weeks and then grown as a stationary cell suspension in RPMI-1640 (GIBCO, Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum, 25 mM Hepes buffer. 25 mM $NaHCO_3$ and 25 μg/ml gentamycin. The pH of the medium was 7.4, and the cells were passaged every other day.

Surface immunofluorescence of cells treated with glycoprotein-bearing recombinant vesicles. Monospecific rabbit antibodies to glycoprotein HN and to glycoprotein F were labeled with fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate, respectively. Direct immunofluorescence assay was then used to detect Sendai virus glycoproteins on the P815 and EL-4 cells. 5 or 10 μg of protein in recombinant vesicles were incubated with $5 \cdot 10^5$ cells, in their exponential phase of growth, at 0°C for 15 min and transferred to 37°C for 45 min or at 0°C for 1 h. The cells were washed twice with Hanks' balanced salt solution and fluorescein-conjugated anti-HN or rhodamine-conjugated anti-F were added and the cells incubated an additional hour at 0°C. A sample at 0°C was taken and the cells washed twice with Hanks' balanced salt solution containing 0.25% NaN_3. The rest of the cells were washed twice with Hanks' balanced salt solution and transferred to 37°C. At various times, they were sampled, washed twice with Hanks' balanced salt solution containing 0.25% NaN_3, fixed for 15 min at 0°C with 1% paraformaldehyde in 0.1 M $KHPO_4$ (pH 7.4) and mounted on slides. They were examined by a Leitz Ortholux 2 incident fluorescence microscope. The degree of fluorescence was graded as 4^+ when the fluorescence was intense and observed on more than 90% of the cells in the field to 1^+ where the fluorescence was faint and observed on fewer than 25% of the cells in the field. Negative reactions ($-$) were recorded when fewer than 1% of cells showed fluorescence.

Interaction between HN and F glycoproteins on the surface of cells. Glycoprotein HN/F-phosphatidylcholine recombinant vesicles (10 μg) or a mixture of glycoprotein HN-phosphatidylethanolamine : phosphatidylserine recombinant vesicles and glycoprotein F-phosphatidylethanolamine :

phosphatidylserine recombinant vesicles (5 μg each) was mixed with $5 \cdot 10^5$ cells, incubated at 0°C for 15 min, and transferred to 37°C for 45 min. The cells were washed twice with Hanks' balanced salt solution, incubated with either fluorescein-conjugated anti-HN or rhodamine-conjugated anti-F at 0°C for 1 h, washed twice, and transferred to 37°C for the designated time. After that, they were washed twice with Hanks' balanced salt solution containing 0.25% NaN_3 and either rhodamine-conjugated anti-F or fluorescein-conjugated anti-HN was added for 1 h at 0°C. Cells were washed twice with Hanks' balanced salt solution containing 0.25% NaN_3, fixed, mounted and examined as described above.

Interaction between viral glycoproteins and glycoproteins of the major histocompatibility complex. 5 μg of glycoprotein HN-phosphatidylethanolamine : phosphatidylserine recombinant vesicles or glycoprotein F-phosphatidylethanolamine : phosphatidylserine recombinant vesicles were incubated with $5 \cdot 10^5$ cells at 0°C and 37°C and washed as described above. Fluorescein-conjugated anti-HN or rhodamine-conjugated anti-F was added at 0°C for 1 h. Cells were washed twice and transferred to 37°C for the designated time, after which they were washed twice with Hanks' balanced salt solution containing 0.25% NaN_3 and incubated with monoclonal anti-H-$2D^d$ or H-$2D^b$ (Bionetics, Charleston, SC) was added at 0°C for 1 h. Cells were again washed with Hanks' balanced salt solution containing 0.25% NaN_3 and fluorescein-conjugated or rhodamine-conjugated anti-mouse IgG (Cappel Labs., Cochranville, PA) was added at 0°C for 1 h. Finally, cells were washed twice with Hanks' balanced salt solution containing 0.25% NaN_3, fixed, mounted and examined as described above.

Results

Monospecificity of antisera

The antisera raised by inoculation of rabbits with DEAE-Bio-Gel-isolated glycoprotein HN and with hydroxyapatite-isolated glycoprotein F were found to be cross-reacting (i.e., the anti-F serum contained anti-HN antibody and the anti-HN serum contained anti-F) We absorbed the sera extensively with Sepharose-bound antigens; glycoprotein HN was used to absorb the anti-F serum and glycoprotein F to absorb the anti-HN serum. Fig. 1 is a Western blot using the antisera run against polyacrylamide gel electrophoresis separations of whole virus (and ovalbumin in the case of the anti-F serum). Additional tests of the antisera showed the anti-F antiserum did not inhibit hemagglutination or neuraminidase activity nor did anti-HN inhibit hemolysis (data not shown).

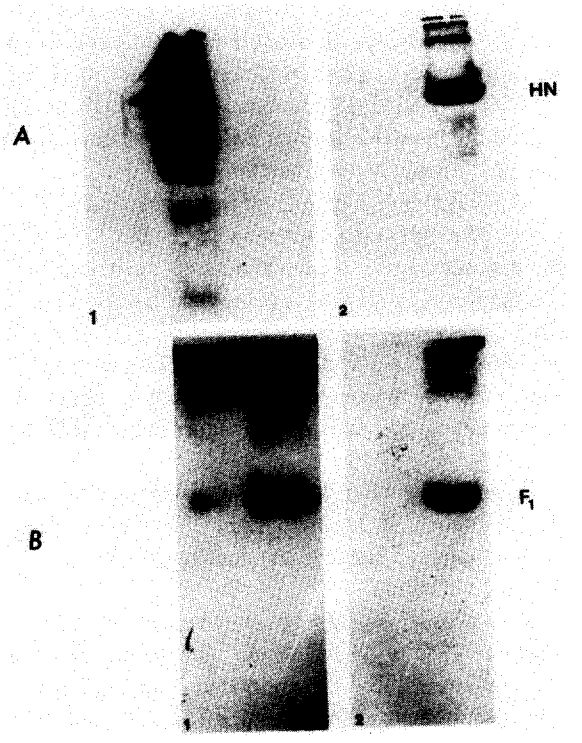

Fig. 1. (Panel A) Western blot of anti-HN serum before (1) and after (2) absorption with glycoprotein F-Sepharose run against whole Sendai virus. (Panel B) Anti-F before (1) and after (2) absorption with glycoprotein HN-Sepharose run against ovalbumin and whole Sendai virus.

The absorbed antisera were used in the immunoaffinity procedures for the purification of glycoproteins HN and F and, after conjugation with fluorchromes, for the localization of the glycoproteins on cell surfaces.

Immunoaffinity purification of glycoproteins HN and F

Supernatant fluids containing glycoproteins HN and F from virions disrupted with 33 mM β-D-oc-

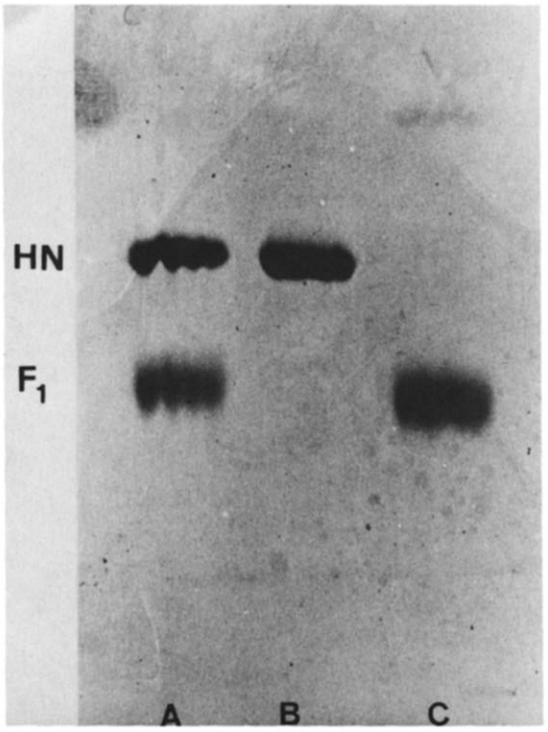

Fig. 2. Silver-stained SDS-polyacrylamide gel electrophoresis preparation of (A) supernatant from β-D-octylglucoside-disrupted virions, (B) 3 M KSCN eluate from anti-HN-Sepharose column and (C) 3 M KSCN eluate from anti-F-Sepharose column.

tylglucoside were applied to columns of Sepharose to which anti-HN or anti-F had been covalently bound. The supernatants contained between 300 and 400 μg/ml of total protein. 1–3 ml of supernatant was applied to the anti-HN column containing 3 ml of gel to which 1.8 mg of antibody had been bound. The effluent from the anti-HN column was applied to the anti-F gel which carried 2.1 mg of antibody in 3 ml. After washing with 10 mM PO_4 buffer containing 33 mM β-D-octylglucoside, the columns were treated with 3 ml of 3 M KSCN to elute the affinity-bound glycoproteins. Eluates were dialyzed against phosphate buffer (pH 7.2) and β-D-octylglucoside was added to the retained material to a final concentration of 33 mM. These procedures allowed the purification of approx. 130 μg of glycoprotein HN and 170 μg of glycoprotein F from 1 mg of purified virus. Fig. 2 is a polyacrylamide gel electrophoresis preparation of the supernatant and the eluates from the anti-HN and anti-F columns stained by the silver method. The figure illustrates the high degree of purity obtained by the immunoaffinity method.

Gradient isolation of protein-bearing recombinant vesicles

Recombinant vesicles were prepared by the detergent dialysis method using a 2 : 1 ratio of phospholipid to protein in 40 mM β-D-octylglucoside. Trace amounts of the triolein labeled with ^3H were incorporated with the phospholipid. After centrifugation on sucrose gradients, visible bands were found floating between densities of 1.07 and 1.055 g/ml. The gradients were fractionated and each fraction was measured for protein content and radioactivity. When appropriate, neuraminidase and hemolysis activities also were measured. Fig. 3 shows the results of typical experiment in which glycoproteins HN, F and HN and F together were incorporated into phosphatidylcholine recombinant vesicles. In all cases, the [^3H]triolein label of the lipid coincided with the proteins and activity markers in the fraction containing the visible band. In the case of glycoprotein HN/F-phosphatidylcholine recombinant vesicle 27.5% of total protein

TABLE I

HEMOLYZING ACTIVITY OF GLYCOPROTEIN F-PHOSPHATIDYLCHOLINE RECOMBINANT VESICLES

Hemolyzing activity was determined by incubating samples with 0.5% human erythrocyte suspension as described in the text. Released hemoglobulin was measured by absorbance at 545 nm. HN glycoprotein purified by affinity chromatography on Sepharose 4B bearing anti-HN antibody. Eluates were dialysed against phosphate buffer (pH 7.2) to remove KSCN and β-D-octylglucoside.

Sample	A_{545}
Glycoprotein F-phosphatidylcholine recombinant vesicles	0.000
Glycoprotein F-phosphatidylcholine recombinant vesicles + purified glycoprotein HN (micelles)	0.720
Purified glycoprotein F (micelles)	0.000
Purified glycoprotein HN (micelles)	0.000
7.5 mM β-D-octylglucoside in phosphate-buffered saline	0.000
Sendai virus	0.613
Phosphatidylcholine recombinant vesicles	0.006

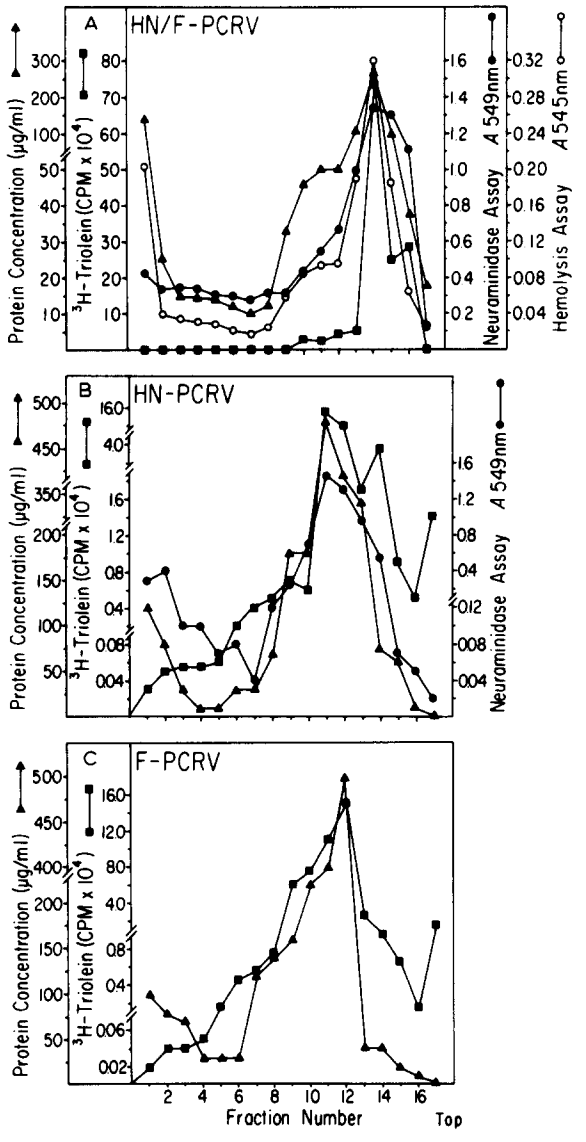

Fig. 3. Analysis of fractions from sucrose gradients glycoprotein HN/F-phosphatidylcholine recombinant vesicles (10–45%), glycoprotein HN-phosphatidylcholine recombinant vesicles and glycoprotein F-phosphatidylcholine recombinant vesicles (0–45%) measured for protein concentration μg/ml (▲), [^{3}H]triolein, cpm × 10^4 (■), neuraminidase activity, A_{549nm} (●) and hemolytic activity A_{545nm} (○). Panel A, Glycoprotein HN/F-phosphatidylcholine recombinant vesicles (HN/F-PCRV); panel B, glycoprotein HN-phosphatidylcholine recombinant vesicles (HN-PCRV); and panel C, glycoprotein F-phosphatidylcholine recombinant vesicles (F-PCRV).

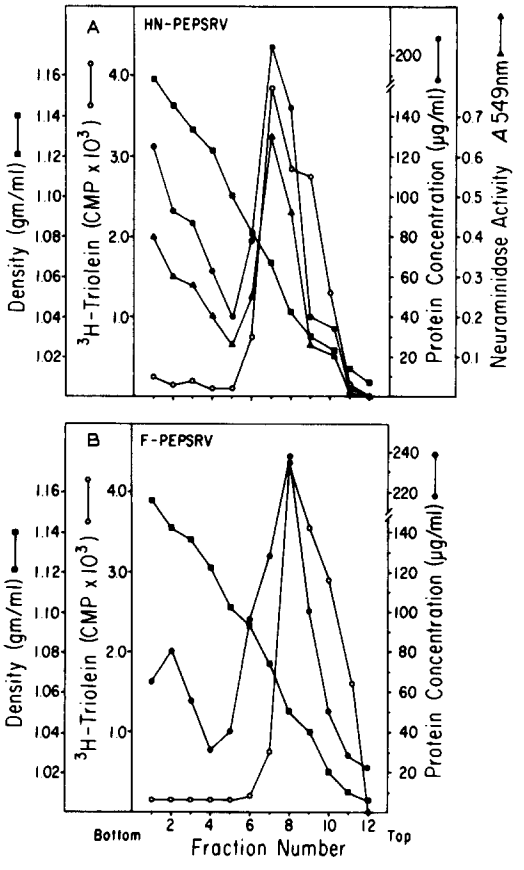

Fig. 4. Analysis of fractions from 0–45% sucrose gradients through which glycoprotein HN-phosphatidylethanolamine: phosphatidylserine recombinant vesicles (HN-PEPSRV) (panel A) and glycoprotein F-phosphatidylethanolamine: phosphatidylserine recombinant vesicles (F-PEPSRV) (panel B) were centrifuged 60000 × g, 4°C, for 18 h and measured for density g/ml (■), [^{3}H]triolein cpm × 10^3 (○), protein μg/ml (●), and neuraminidase activity A_{549nm} (▲).

was incorporated in this fraction while the glycoprotein HN-phosphatidylcholine recombinant vesicle peak contained 24% of total protein and the glycoprotein F-phosphatidylcholine recombinant vesicle incorporated 25%.

As shown in Fig. 3, the neuraminidase activity of the HN protein was demonstrable. The hemagglutination activity (although not shown) was similarly present. The hemolytic activity of glycoprotein F-bearing recombinants was only demonstrable by the addition of glycoprotein HN as shown in Table I.

Fig. 4 shows the results of an analysis of gradients on which glycoprotein HN-phosphatidylethanolamine : phosphatidylserine recombinant vesicles and glycoprotein F-phosphatidylethanolamine : phosphatidylserine recombinant vesicles

TABLE II

SURFACE FLUORESCENCE OF P815 CELLS TREATED WITH GLYCOPROTEIN-BEARING PHOSPHATIDYLCHOLINE RECOMBINANT VESICLES

Stained with	P815 cells treated with recombinant vesicles containing:								
	Glycoprotein HN-phosphatidylcholine			Glycoprotein F-phosphatidylcholine			Glycoprotein HN/F-phosphatidylcholine		
Incubated at:	0°C	37°C	37°C	0°C	37°C	37°C	0°C	37°C	37°C
for:	1 h	1 h	2 h	1 h	2 h	1 h	1 h	2 h	4 h
Anti-HN	+++ [a]	+	−	−	n.d.	n.d.	++++	+++	+++
Anti-F	−	n.d.	n.d.	−	−	−	++++	+++	+++

[a] Degree of fluorescence as described in Materials and Methods.

were sedimented. The glycoprotein HN-phosphatidylethanolamine: phosphatidylserine recombinant vesicles formed a band at a density of 1.068 g/ml that contained 21% of the total protein and the highest ^3H counts and neuraminidase activity. Glycoprotein F-phosphatidylethanolamine: phosphatidylserine recombinant vesicles floated at a density 1.050 g/ml as indicated by a band that contained 24% of the original protein and the highest radioactivity.

Interaction of phosphatidylcholine recombinant vesicles with cell surfaces

5–10 μg (protein weight) of glycoprotein HN/F-phosphatidylcholine recombinant vesicles, glycoprotein HN-phosphatidylcholine recombinant vesicles or glycoprotein F-phosphatidylcholine recombinant vesicles were incubated with $5 \cdot 10^5$ P815 or EL-4 cells at 0°C for 1 h. Cells were then washed and incubated with 100 μl of a 1 : 20 dilution of fluorescein-conjugated anti-HN or rhodamine-conjugated anti-F for an additional hour at 0°C and transferred to 37°C for various periods. Cells were sampled and fixed with 1% paraformaldehyde, mounted, and examined. The results of such an experiment is shown in Table II. Cells incubated with glycoprotein HN/F-phosphatidylcholine recombinant vesicles bore both glycoproteins on their surfaces for periods up to 4 h of incubation at 37°C. In contrast, cells incubated with glycoprotein HN-phosphatidylcholine recombinant vesicles did not retain glycoprotein HN. After incubation at 0°C, the cell surfaces were brightly stained; however, fluorescence diminished after 1 h at 37°C and was completely lost after 2 h at 37°C. Cells treated with glycopro-

TABLE III

ELUTION OF NEURAMINIDASE ACTIVITY FROM CELL SURFACES

P815 cells treated with recombinant vesicles containing:	Incubated					
	0°C, 1 h		37°C, 1 h		37°C, 2 h	
	cells	medium	cells	medium	cells	medium
Glycoprotein F-phosphatidylcholine + purified glycoprotein HN (micelles) [a]	0.326 [b,c]	0.012	0.102	0.241	0.011	0.317
Glycoprotein F-phosphatidylcholine + glycoprotein HN-phosphatidylcholine	0.303	0.056	0.114	0.209	0.016	0.322
Glycoprotein HN-phosphatidylcholine	0.325	0.080	0.207	0.133	0.014	0.307

[a] Not incorporated into recombinant vesicles.

[b] Absorbance at 549 nm (A_{549}).

[c] 5 μg glycoprotein HN (micelles) gave an A_{549} value of 0.353 in the absence of cells.

TABLE IV

EFFECT ON GLYCOPROTEIN HN ON THE INCORPORATION OF GLYCOPROTEIN F

Stained with	P815 cells treated with recombinant vesicles containing:						
	Glycoprotein F-phosphatidylcholine	Glycoprotein F-phosphatidylcholine + glycoprotein HN (micelles)			Glycoprotein F-phosphatidylcholine + glycoprotein HN-phosphatidylcholine		
Incubated at:	0°C	0°C	37°C	37°C	0°C	37°C	37°C
for:	1 h	1 h	1 h	1 h	1 h	1 h	2 h
Anti-HN	–	+++ [a]	+	–	+++	–	–
Anti-F	–	++++	+++	+++	++++	+++	+++

[a] Degree of fluorescence as described in Materials and Methods.

tein F-phosphatidylcholine recombinant vesicles did not show any staining with the anti-F conjugate.

Elution of glycoprotein HN-phosphatidylcholine recombinant vesicles

The disappearance of glycoprotein HN staining from the cell surfaces was interpreted to be the consequence of elution resulting from neuraminidase activity of the vesicles. We conducted an experiment to confirm this interpretation. Cells treated with glycoprotein HN-phosphatidylcholine recombinant vesicles were incubated as before but in this case, without the antibody. Neuraminidase activity was measured on the cell surface and in the incubation medium. The results are shown in Table III. Neuraminidase activity was first found most abundantly on the surfaces, however as incubation continued at 37°C, the greater portion was found in the medium until almost all activity was lost from the cells.

Glycoprotein HN-mediated insertion of glycoprotein F

The ability of glycoprotein F-bearing vesicles to affect hemolysis has been shown to be dependent upon the presence of a viral HN glycoprotein as shown in Table II or lectin as shown by Hsu et al. [14]. We, therefore, studied the effect of glycoprotein HN on the interaction of glycoprotein F-phosphatidylcholine recombinant vesicles with the cell surfaces. Glycoprotein HN alone or glycoprotein HN-phosphatidylcholine recombinant vesicles were mixed with glycoprotein F-phosphatidylcholine recombinant vesicles and incubated with cells at 0°C, and then after washing, incubated at 37°C. the glycoprotein HN alone was prepared by affinity chromatography and dialyzed against phosphate buffer (pH 7.2) to remove KSCN and β-D-octylglucoside. Table IV shows that glycoprotein HN and HN-phosphatidylcholine recombinant vesicles facilitated the interaction of glycoprotein F-phosphatidylcholine recombinant vesicles with cell surfaces and that the F glycoprotein remained on the cell while HN glycoprotein eluted. In a similar experiment, [^{3}H]triolein in the glycoprotein F-phosphatidylcholine recombinant vesicles was shown to become associated with the cells and did not partition into the medium (data not shown).

Interaction of phosphatidylethanolamine : phosphatidylserine recombinant vesicles with cell surfaces

Glycoprotein HN-phosphatidylethanolamine : phosphatidylserine recombinant vesicles and glycoprotein F-phosphatidylethanolamine: phosphatidylserine recombinant vesicles were incubated with $5 \cdot 10^5$ P815 or EL-4 cells in the presence of 5 mM $CaCl_2$ at 0°C and 37°C, washed, stained with conjugated antibody, and further incubated at 0°C for 1 h and at 37°C for 2 h. Table V shows the results of fluorescence tests with these cells. Both glycoproteins were present on the cell surfaces up to 2 h. We also explored the influence of Ca^{2+} on the interaction of the phosphatidylcholine recombinant vesicles with P815 cells. The results are shown in Table VI. The calcium ions appeared to inhibit the initial interaction of glycoprotein HN-phosphatidylcholine recombinant vesicles with the cell surface while the interaction of the glycoprotein F-phosphatidylcholine recombinant vesicles was facilitated. On the other hand, in the absence of Ca^{2+} there was a strong initial interaction of

TABLE V

SURFACE FLUORESCENCE OF P815 CELLS TREATED WITH GLYCOPROTEIN-BEARING PHOSPHATIDYLETHANOLAMINE: PHOSPHATIDYLSERINE RECOMBINANT VESICLES

Stained with	P815 cells treated with recombinant vesicles					
	Glycoprotein HN-phosphatidylethanolamine: phosphatidylserine			Glycoprotein F-phosphatidylethanolamine: phosphatidylserine		
	0°C 1 h	37°C 1 h	37°C 2 h	0°C 1 h	37°C 1 h	37°C 2 h
Anti-HN	++++ [a]	+++	+++	–	n.d.	n.d.
Anti-F	–	n.d.	n.d.	++++	+++	+++

[a] Degree of fluorescence as described in Materials and Methods.

TABLE VI

EFFECT OF CALCIUM AND RECOMBINANT VESICLE COMPOSITION ON THE INTERACTION OF GLYCOPROTEINS HN AND F WITH THE SURFACE OF P815 CELLS

Treated with recombinant vesicles containing	P815 cells incubated					
	0°C, 1 h		37°C, 1 h		37°C, 2 h	
	Ca^{2+} present [a]	Ca^{2+} absent	Ca^{2+} present	Ca^{2+} absent	Ca^{2+} present	Ca^{2+} absent
Glycoprotein HN-phosphatidylcholine [b]	+ [c]	++++	+	++	–	–
Glycoprotein-HN-phosphatidylethanolamine: phosphatidylserine	++++	++	+++	+	+++	–
Glycoprotein F-phosphatidylcholine [d]	++	–	++	–	+	–
Glycoprotein F-phosphatidylethanolamine: phosphatidylserine	++++	–	+++	–	+++	–

[a] 5 mM $CaCl_2$ in 10 mM Tris (pH 7.2).
[b] Glycoprotein HN-treated cells stained with fluorescein-conjugated anti-HN.
[c] Degree of fluorescence as described in Materials and Methods.
[d] Glycoprotein F-treated cells stained with rhodamine-conjugated anti-F.

glycoprotein HN-phosphatidylcholine recombinant vesicles with the cells that later eluted from the surface. Glycoprotein HN-phosphatidylethanolamine : phosphatidylserine recombinant vesicles did not affect as strong a fluorescence nor did the vesicles transfer the glycoprotein to the cell, as the fluorescence was lost by 2 h at 37°C.

Membrane mobility of glycoproteins HN and F

Experiments were conducted to determine the behavior of the glycoproteins on the surface of the cells. Cells were treated with glycoprotein HN/F-phosphatidylcholine recombinant vesicles or equal amounts of glycoprotein HN-phosphatidylethanolamine :phosphatidylserine recombinant vesicles and glycoprotein F-phosphatidylethanolamine : phosphatidylserine recombinant vesicles for 15 min at 0°C and shifted to 37°C for 45 min. Cells were washed, incubated at 0°C for 1 h with fluorescein-conjugated-anti-HN, washed again, and transferred to 37°C for 2 h. After washing with Hanks' balanced salt solution containing 0.25% NaN_3, rhodamine-conjugated-anti-F was added at 0°C for 1 h. Cells were then fixed and examined. The two glycoproteins were found to form patches and caps on the cells. Both glycoproteins were always found to be distributed in the same pattern and staining was always coincident.

A similar experiment was conducted to show the interaction between glycoproteins HN and H-2 or F and H-2 in which anti-HN or anti-F was added first, then the monoclonal anti-H-2 was

added. The localization of anti-H-2 antibodies was investigated by adding a second antibody that was either fluorescein-conjugated or rhodamine-conjugated labeled and was directed against mouse IgG. The results of these experiments demonstrated independence of the viral glycoproteins from the glycoprotein H-2 antigens. No comigration of the proteins was seen.

Discussion

Previous studies [19–22] have shown that the surface of cells can be modified by interaction with whole Sendai virions or vesicles formed from detergent-released glycoproteins and the native viral lipids. Several of these studies used detergents such as Triton X-100 and Nonidet P-40 that are very difficult to remove from the medium. Reconstituted vesicles, although biologically active undoubtedly incorporated some detergent. The present investigation used two modifications of previous approaches to reconstituting of Sendai glycoproteins into vesicles. We used the non-ionic detergent β-D-octylglucoside which has a high critical micellar concentration and can be readily removed by dialysis. The amount of β-D-octylglucoside present after dialysis was less than 0.1 mM as determined by the anthrone test. This figure may in fact be high since the test material also contained glycoproteins. In addition, we used immunoaffinity methods of the purification of the glycoproteins. By this process, we were not only able to achieve high levels of protein purity but were also able to remove over 99% of the native viral lipid (data not shown). Thus, the procedure offers the advantage of reducing contaminating lipids, detergents and proteins from the study system.

Isolation of recombinant vesicles on sucrose gradients insured homogeneity of the preparations used for the experiments with cells. In contrast to results with vesicles formed with native viral lipids [7], these experiments employing purified phosphatidylcholine and phosphatidylethanolamine: phosphatidylserine produced very uniform recombinant vesicles and indicated in the analysis of gradients. The lipid marker, protein content and biological activities of the recombinant vesicles were confined to very discrete bands in the gradients.

The interaction of phosphatidylcholine recombinant vesicles with cell surfaces was studied under conditions of temperature that separated the attachment of the vesicles from subsequent fusion of the vesicles with the plasma membranes.

It has been established that the HN glycoprotein functions to attach the Sendai virion while the F glycoprotein mediates fusion. Hsu et al. [14] showed that glycoprotein F alone in recombinant vesicles was unable to affect hemolysis but could accomplish it if a means of cell attachment was provided. As we have demonstrated in the present study, when glycoproteins HN and F are in the same vesicles, the fusion event results in the incorporation of both glycoproteins HN and F into the membrane. However, only glycoprotein F is incorporated when glycoproteins HN and F are in separate recombinant vesicles or the HN glycoprotein is present in micellar form after removal of β-D-octylglucoside. The lectin-assisted hemolytic activity of glycoprotein F vesicles has been explained on the assumption that the lectin provides the cell attachment function allowing expression of the fusion potential of glycoprotein F. In the present experiments, such a model does not seem to be adequate. Glycoprotein HN is not in the vesicle with glycoprotein F and it is not introduced into the plasma membrane, indeed it elutes from the surface apparently after destruction of receptor by neuraminidase activity. Ozawa et al. [23] reported experiments that tended to implicate the need for an active neuraminidase as well as a means of cell attachment to allow the expression of glycoprotein F. Similar implications come from the findings of Huang et al. [24] and Heath et al. [25]. An interesting aspect of our results is that the HN glycoprotein is apparently not inserted into the F glycoprotein-bearing phosphatidylcholine vesicle and yet affects close enough contact to allow fusion. The surface fluorescence staining of cells treated with glycoprotein F-phosphatidylcholine recombinant vesicles and glycoprotein HN or HN-phosphatidylcholine recombinant vesicles showed coincident distribution of the HN and F glycoproteins after 1 h at 0°C. After 1 h at 37°C, the glycoprotein HN-specific fluorescence was considerably diminished and yet was still coincident with the glycoprotein F distribution. At 2 h there was only glycoprotein F-specific staining. These observa-

tions suggests a physical association of glycoproteins HN and F without the insertion of HN into the lipid bilayer of the vesicle.

Our results seem at variance with those reported by Peterhans et al. [26] who observed a direct adsorption of glycoprotein F-phosphatidylcholine recombinant vesicles to mouse spleen cells measured by chemiluminescence and confirmed by immunoferritin electron microscopic techniques. The glycoprotein F-bearing recombinant vesicles in this study did not associate with erythrocyte surfaces and yet were present on spleen cells after 60 min at 4°C. There was no evidence of fusion in their electron micrographs nor mention of it in the report. The differences in our findings and theirs may be the result of using different cell types.

The ability of phosphatidylethanolamine: phosphatidylserine recombinant vesicles to fuse with the plasma membrane and introduce integral glycoproteins was described by Engelhard et al. [27] and Correa-Freire et al. [28] in studies with HLA glycoproteins. These investigations documented the insertion and subsequent functioning as targets for cytotoxic T-lymphocytes of the human histocompatibility antigens. The role of Ca^{2+} in the interaction of pure phospholipid vesicles, specifically phosphatidylserine, with cell surfaces has been attributed by Ohki [29] to an increase in surface tension resulting in binding of the vesicle to the surface followed by fusion. The case with the phosphatidylethanolamine: phosphatidylserine recombinant vesicles and phosphatidylcholine recombinant vesicles containing glycoproteins is quite different. We observed an inhibition of glycoprotein HN-phosphatidylcholine recombinant vesicles binding in the presence of Ca^{2+} while the glycoprotein HN-phosphatidylethanolamine: phosphatidylserine recombinant vesicles were not only bound but the HN glycoprotein apparently integrated into the plasma membrane. These same vesicles behaved differently in the absence of Ca^{2+} in that glycoprotein HN eluted from the surface without integration into the plasma membrane. Glycoprotein F-phosphatidylcholine recombinant vesicles on the other hand interacted with cell surfaces in the presence of Ca^{2+} while they did not in its absence. Clearly, the type of glycoprotein and the nature of the phospholipids are important factors in determining the role that divalent cations play in liposome-cell interactions.

The behavior of the viral glycoproteins after introduction into the membrane of cell resembles that of glycoproteins appearing as a result of virus infection [30,31]. Glycoproteins HN and F co-cap under the influence of antibody while they remain independent of the intrinsic H-2 glycoproteins. In other work [6], we have shown that the individual glycoproteins can function as target antigens of specifically sensitized cytotoxic T-lymphocytes.

Acknowledgements

These investigations were supported by funds from the Saudi Arabian Educational Mission to the U.S. and a Research Competition Award from the School of Medicine, State University of New York at Buffalo.

References

1 Blanden, R.V. (1974) Transplant. Rev. 19, 56–88
2 Choppin, P.W. and Scheid, A. (1980) Rev. Infect. Dis. 2, 40–61
3 Homma, M. and Ohuchi, M. (1973) J. Virol. 12, 1457–1465
4 Merz, D.C., Scheid, A. and Choppin, P.W. (1980) J. Exp. Med. 151, 275–288
5 Scheid, A., Caliguiri, L.A., Compans, R.W. and Choppin, P.W. (1972) Virology 50, 674–652
6 Al-Adahl, M.N., Nakamura, I. and Flanagan, T.D. (1985) J. Virol. 54, 53–57
7 Abidi, T.F. and Flanagan, T.D. (1984) J. Virol. 50, 380–386
8 Haywood, A.M. (1974) J. Mol. Biol. 83, 427–436
9 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
10 Urata, D.M. and Seto, J.T. (1975/76) Intervirology 6, 108–114
11 Hale, A.H., Lyles, D.S. and Fan, D.P. (1980) J. Immunol. 124, 724–731
12 Aminoff, D. (1961) Biochem. J. 81, 384–392
13 Scheid, A. and Choppin, P.W. (1974) Virology 57, 475–490
14 Hsu, M., Scheid, A. and Choppin, P. (1979) Virology 95, 476–491
15 Smith, G.H., Stubbs, G.W. and Litman, J.B. (1975) Exp. Eye Res. 20, 211–217
16 Merril, C.R., Goldman, D. and Van Keuren, M.L. (1982) Electrophoresis 3, 17–23
17 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354
18 Burnette, W.N. (1981) Anal. Biochem. 112, 195–203
19 Finberg, R., Mescher, M. and Burakoff, S.J. (1978) J. Exp. Med. 148, 1620–1627
20 Hale, A.H., Ruebush, M.J., Lyles, D.S. and Harris, D.T. (1980) Proc. Natl. Acad. Sci. USA 77, 6105–6108

21 Koszinowski, U., Gething, M.J. and Waterfield, M. (1977) Nature (Lond.) 267, 160–163
22 Sugamura, K., Shimizu, K., Zarling, D.A. and Bach, F.W. (1977) Nature (Lond.) 270, 251–253
23 Ozawa, M., Asano, A. and Okada, Y. (1979) Virology 99, 197–202
24 Huang, R.T.C., Whan, K., Klenk, H.D. and Rott, R. (1979) Virology 97, 212–217
25 Heath, T.D., Martin, F.J. and Macher, B.A. (1983) Exp. Cell Res. 149, 163–175
26 Peterhans, E., Baechi, T. and Yewdell, J. (1983) Virology 128, 366–376
27 Englehard, V.H., Powers, G.A., Moore, L.C., Holterman, M.J. and Correa-Freire, M.C. (1984) J. Immunol. 132, 76–80
28 Correa-Freire, M.C., Powers, G.A. and Englehard, V.H. (1984) J. Immunol. 132, 69–75
29 Ohki, S. (1982) Biochim. Biophys. Acta 689, 1–11
30 Buchmeier, M.J., Welsh, R.M., Dulko, F.J. and Oldstone, M.B.A. (1980) Adv. Immunol. 30, 225–331
31 Hackett, C.J. and Askonas, B.A. (1982) Immunology 45, 431–438