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SURFACE PROPERTIES OF SENDAI VIRUS ENVELOPE

TANVEER F. ABIDI and PHILIP L. YEAGLE

Department of Biochemistry, SUNY/Buffalo School of Medicine, Buffalo, NY 14214 (U.S.A.)

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Surface properties of Sendai virus envelope membrane have been measured, using both biological and biophysical techniques. Both normal and trypsin-treated virus were studied. SDS gel electrophoresis showed cleavage of the F protein exclusively by trypsin. The major activity change was observed in the hemolysing activity which is an expression of F protein. Hemolysis was reduced to less than 10% of its value for intact virus. ^{31}P nuclear magnetic resonance studies of the envelope surface of the native virus showed a highly restricted phospholipid headgroup environment. Interestingly, this restriction was relieved by treatment with trypsin. Thus these data suggest a role of the F protein of Sendai virus in tightly organizing the surface of the viral envelope membrane.

Introduction

Sendai virus is a member of Paramyxoviridae. It is an enveloped virus with a negative strand RNA. The envelope is composed of a lipid bilayer which is acquired from the host plasma membrane. Two viral glycoproteins project through the outer layer of the envelope in the form of spikes, as visualized in electron micrographs. One spike type consists of a glycoprotein which has hemagglutinating and neuraminidase activities and is designated HN. The other type of spike is composed of glycoprotein F and has cell fusing and hemolytic activities. These glycoproteins can be selectively removed by using different proteases [15,19]. Underlying the inner monolayer of the envelope is a non-glycosylated membrane protein, M.

Membranes of many viruses such as vesicular stomatitis, influenza, Sindbis, and Semiliki Forest virus have been studied extensively with spin labels and electron spin resonance (ESR) and with fluorescence depolarization [2,9–13]. Both techniques probe the hydrophobic interior of the envelope membrane. These techniques indicate that the interior of the viral membranes is considerably more rigid than the host cell plasma membrane from which the virus buds. Proteolytic cleavage of the surface glycoproteins does not make the membrane interiors any less rigid. It was concluded that the hydrophobic portions of these glycoproteins which are intramembraneous and are likely not removed by proteolytic cleavage are responsible for the rigidity of the membranes.

Very little information is available for the membrane surface. Virtually the only method available for studying the membrane surface without perturbing it is with ^{31}P nuclear magnetic resonance (NMR). This technique is sensitive to the viral envelope membrane surface because it measures the properties of the phosphate of the phospholipids, which is located in the lipid polar headgroup and therefore is found on the membrane surface. Only one such study is available. Moore et al. [14] examined the behavior of the surface of the vesicular stomatitis envelope membrane. It was found

Abbreviations: ESR, electron spin resonance; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate.

that the surface of the membrane is much more rigid than for normal plasma membranes. In contrast to the above studies, removal of the glycoproteins did relieve the motional restrictions and produce a much less rigid membrane surface than found in the intact virus. Therefore, it can be hypothesized that the carbohydrate-bearing extramembraneous portion of the glycoprotein was responsible for the perturbations of membrane surface structure observed in the intact virus.

In this study, the above suggestions are pursued in the Sendai virus system. ^{31}P-NMR studies show that the surface is rigid in the intact virus, but that this property is manifest in a different manner and with greater severity than in vesicular stomatitis virus. This rigidity can be at least partially relived by selective cleavage of the F protein with trypsin. This trypsin cleavage of F protein is correlated with loss of hemolysing activity.

Materials and Methods

Sendai virus strain cantell (received from Dr. K. Chadha, Roswell Park Memorial Institute, Buffalo, NY) was grown in the allantoic cavity of 10-day-old embryonated chicken eggs. The eggs were inoculated with 0.5 hemagglutinating units and incubated at 37°C. Allantoic fluid was harvested after 72 h, and cellular debris was removed by centrifugation at 3000 × *g* for 30 min at 4°C in a Sorvall RC-5B centrifuge using a SS 34 rotor. The allantoic fluid, which had a hemagglutinating titer of 1024 or greater was sedimented by centrifugation at 46 000 × *g* for 2 h at 4°C. The pelleted virus was resuspended in 20 mM Tris buffer (pH 7.5) containing 100 mM NaCl and 1 mM EDTA. The virus was purified by centrifugation to equilibrium through a linear 15%–60% sucrose gradient according to the procedure of Haywood [7]. Purified virus was kept at −30°C. Nitrogen gas was used to purge all the buffers and preparations to prevent oxidation of the lipids.

Treatment of virus with trypsin. The purified Sendai virus suspension in 20 mM Tris buffer (pH 7.5) was centrifuged at 46 000 × *g* for 1 h and then resuspended in the same buffer to give a concentration of 5 mg/ml (protein). Crystalline trypsin (50 μg) from bovine pancreas (Sigma Chemical Co.) was added to 1 ml of virus preparation. The mixture was incubated at 37°C for 1 h. After incubation trypsin inhibitor (Sigma Chemical Co.) was added to stop proteolysis. The trypsin treated virus was stored at −30°C.

Protein determination. Protein concentration was determined by the procedure of Bradford [4] using a commercial dye reagent (Bio-Rad Laboratories). Bovine gamma globulin was used as a standard.

Hemolysis assay. Hemolytic activity of purified intact Sendai virus and trypsin-treated Sendai virus was determined by a modified procedure of Chu and Morgan [5]. Virus samples were diluted in phosphate-buffered saline (pH 7.2) to a volume of 0.5 ml in 12 × 75 mm glass tubes. To each tube was added 0.5 ml of a chicken erythrocyte suspension (1% in phosphate-buffered saline). The mixture was kept at 4°C for 45 min and then incubated at 37°C for 1 h with shaking every 10 min. Tubes were chilled and centrifuged at 1500 rpm in a clinical centrifuge for 5 min. The hemoglobin released by hemolysis was measured by its absorbance at 540 nm in a Gilford 250 spectrophotometer. The percent of cells hemolysed was obtained by dividing the absorbance at 540 nm for each sample by the total possible absorbance. The latter value is obtained from the absorbance at 540 nm from the same number of cells following complete lysis in distilled water and 2 min sonication in a bath sonicator.

Hemagglutination titeration. Serial 2-fold dilutions of allantoic fluid, and purified intact and trypsin-treated Sendai virus were made in 0.5 ml of phosphate-buffered saline (pH 7.2). To each tube was added 0.5 ml of chicken erythrocytes (0.5% suspension in phosphate-buffered saline). The samples were incubated at 4°C for 1 h. A positive hemagglutinin response was recognized by the formation of the characteristic pattern on the bottom of the tube. Titers were recorded as the reciprocal of the highest dilution giving a positive response and were expressed as hemagglutinating units/ml.

Neuraminidase assay. Neuraminidase activity was assayed by the procedure of Aminoff [2] using thiobarbituric acid. The samples were diluted in a series of 2-fold dilutions in phosphate-buffered saline to a volume of 0.1 ml. Fetuin from fetal bovine serum (Calbiochem. San Diego, CA) was used as substrate. To each sample was added 1 mg

of fetuin in 0.1 ml of 0.2 M sodium acetate buffer (pH 5.0). Neuraminidase from *Vibrio cholera* was used as a standard.

Lipid extraction from Sendai virus. Lipids from 6 ml of purified Sendai virus (1.5 mg/ml protein) were extracted with 24 ml of chloroform/methanol (2 : 1, v/v). The lower organic phase was removed and dried under a stream of nitrogen gas.

Formation of liposomes. Lipids were dried out of organic solvent under a stream of nitrogen and then pumped dry under a vacuum to make a thin film on the glass vessel. Buffer (20 mM Tris (pH 7.5)/100 mM NaCl/1 mM EDTA) was added and the sample was then vortexed vigorously until all the lipid was suspended as a milky suspension. For NMR measurements the sample was loaded in a 10 mm tube and flooded with argon gas.

SDS-polyacrylamide gel electrophoresis. The proteins of purified intact and trypsin-treated Sendai virus were analyzed by electrophoresis according to the procedure of Laemmli [9] as modified by Smith et al. [23]. A discontinuous system was employed using an 8% running gel at pH 6.8 and a 3% stacking gel at pH 8.8. Electrophoresis was carried out in tube gels in a water jacketed Buchler electrophoresis apparatus maintained at 10°C with a Fisher refrigerated circulating water bath. The gels were stained with Coomassie blue and scanned on a Gilford gel scanner at 550 nm.

^{31}P-NMR measurements. ^{31}P-NMR spectra were obtained at 109 MHz on a JEOL FX 270 multinuclear Fourier transform spectrometer. Samples were run in 10 mm tubes and about 1 ml sample volume. Samples were maintained at 14°C throughout these experiments. A fully phase cycled chemical shift anisotropy echo sequence was used to obtain distortion-free spectra (kindly provided by Drs. I.C.P. Smith, A. Byrd, and M. Rance). No first order phase corrections were employed. The proton decoupler (9 kHz) was gated on during acquisition and off the remainder of the time to prevent sample heating, and to defeat any nuclear Overhauser effect [24]. Repetition rates of 1 s were used to obtain the spectra. Exponential multiplication of the free induction decay was used to enhance the spectra, and values were used that in each case caused linebroadening of substantially less that the width of the narrowest resonance in the spectrum. 2048 data points were obtained in the frequency domain, with a 50 kHz spectral width.

Results

Sendai virus preparation and trypsin treatment

The results for the hemolysing, hemagglutinating and neuraminidase activities for the native Sendai virus used in these experiments are presented in Table I. Also presented are the activities for the Sendai virus preparation treated with trypsin as described in Methods. Trypsin treatment has reduced the hemolysing activity to less than 10% of the intact virus, which indicates that the protein responsible for that activity, the glycoprotein F, is cleaved. Similar results were presented by Shimizu et al. [20], showing selective cleavage of F by trypsin. Trypsin treatment does not affect the neuraminidase activity [22]. There is, however, some reduction in the hemagglutinating activity by trypsin treatment, as reported previously [15]. As will be seen below, the electrophoresis patterns do not show any affect on HN, which carries both the latter two activities. As shown in Table II, at higher dilutions there is also a reduction in neuraminidase activity.

Fig. 1 shows the SDS-polyacrylamide disk gel electrophoresis of the native Sendai virus proteins. All the major viral proteins are represented and are labelled according to previous assignments [17,19,20].

Also shown in Fig. 1 is the SDS-polyacrylamide

TABLE I

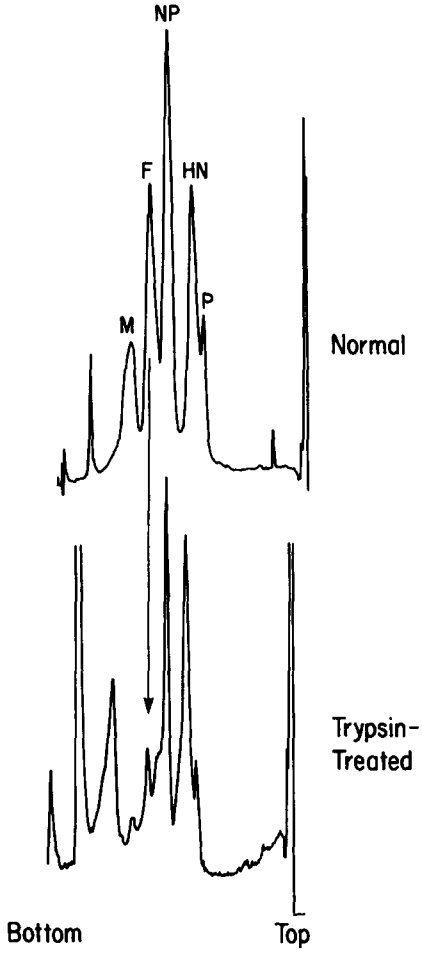
Results indicate the average of three experiments.

Virus	Percent hemolysis	Neuraminidase (units/mg)	Hemagglutination (units/mg)
Intact sendai virus	105	780	$5 \cdot 10^4$
Trypsinized Sendai virus	9.6	780	$2.5 \cdot 10^4$

TABLE II

Virus dilution	Intact virus			Trypsinized virus		
	%HE [a]	HA [b]	NAU [c]	%HE [a]	HA [b]	NAU [c]
1:5	105	+	44	9.6	+	44
1:10	105	+	40	4.8	+	40
1:20	102	+	32	2.0	+	28
1:40	102	+	29.6	0.2	+	23
1:80	101	+	19.8	0.00	+	13.8
1:160	101	+	15.2	0.00	+	13.7
1:320	96.2	+	n.d.		+	n.d.
1:640	79.8	+	n.d.		+	n.d.
1:1280	48.4	+	n.d.		+	n.d.
1:2560	28.4	+	n.d.		+	n.d.
1:5120	15.6	+	n.d.		±	n.d.
1:10240	7.8	−	n.d.		−	n.d.

[a] % hemolysis.
[b] Hemagglutination.
[c] Neuraminidase units. n.d., not determined.



gel electrophoresis of the reduced trypsin-treated Sendai virus. As can be seen, of the two surface glycoproteins HN and F, only the F protein has been affected by trypsin, as has been observed previously [15,21]. A quantitative analysis of the effect of trypsin treatment indicates that there is approximately a 70% reduction in intact F protein. When gels were run under non-reducing conditions, similar results were obtained (data not shown).

^{31}P-NMR measurements

^{31}P-NMR measurements were obtained as described in Methods. Fig. 2 shows the results. Fig. 2A shows the ^{31}P-NMR spectrum obtained from a dispersion of phosphatidylcholine bilayer membranes. These are multilamellar liposomes which consist of concentric bilayers of phospholipid. The resonance shape is characteristic of phospholipids in biological membranes [18]. It also has been observed for viral membranes [14]. It results from a partial motional averaging of the anisotropic chemical shift tensor characterizing the phosphate group. In general, the phosphate group exhibits a resonance position which is dependent

Fig. 1. SDS-polyacrylamide gel electrophoresis, densitometric scans of normal and trypsin-treated virus, performed as described in the text. The positions of the major proteins are marked.

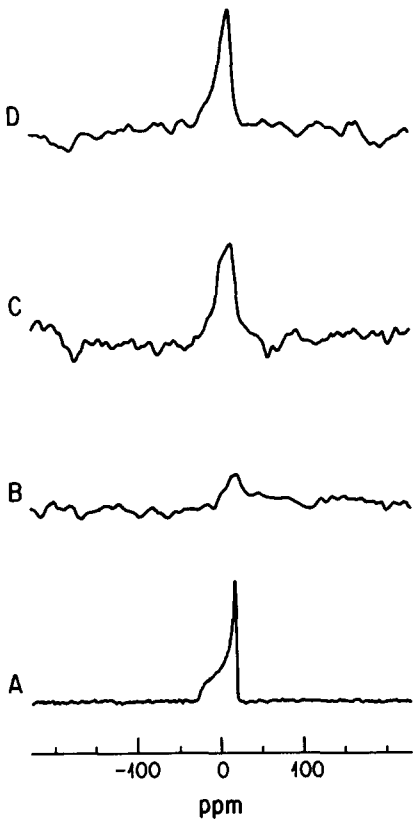


Fig. 2. 109 MHz ^{31}P-NMR spectra, taken as described in the text. (A) aqueous dispersion of 50 mg of egg phosphatidylcholine in 20 mM Tris/100 mM NaCl (pH 7.5). 2000 scans were obtained. Spectra were obtained at 14°C to stabilize the virus during the long accumulations required in the NMR experiments. (B) 5 mg (protein) of normal Sendai virus in the same buffer as (A), taken with 65000 scans. This sample contains 2 mg phospholipid. (C) the same sample as in (B), but treated with trypsin as described in the text, taken with 24000 scans. (D) total lipid extract from 10 mg of virus, reformed into bilayers as described in the text. 3000 scans were obtained for this spectrum.

upon orientation and can extend over 200 ppm. In the case of limited motional freedom some of the possible resonance positions are averaged by the motion. In the case of a membrane, the averaging is caused by rapid axial rotation of the phospholipid headgroup about an axis perpendicular to the membrane surface [18]. This is one of the few motions a phospholipid can readily undergo when in a membrane. The result is that only about 45 ppm of the original possible spectral width remains after motional averaging. Rapid axial rotation would directly lead to the characterisitc bilayer resonance shape observed.

Fig. 2B shows the ^{31}P-NMR spectrum from intact Sendai virus. This spectrum is remarkable for its lack of a defined shape. By comparison with Fig. 2A, apparently little or no bilayer-type spectrum appears. The same spectrum was obtained on a second independent preparation of virus. As shown by control experiments, and as in fact is evident from the data in Figs. 2C and D, the spectrum in Fig. 2B is taken under conditions where a prominent bilayer resonance should be observable, given the amount of phospholipid present in the sample. The fact that little or no such resonance is seen indicates that some unusual (compared to other membranes) phospholipid behavior is being observed.

Fig. 2C shows the spectrum for trypsin-treated virus. This represents the same sample as in Fig. 2B, after trypsin treatment. The remarkable result here is that a resonance which is characteristic of a phospholipid bilayer spectrum is now apparent. Sensitivity limitations prohibit an accurate measure of the absolute intensity of the spectrum. Thus it is not possible to determine whether all the viral phospholipids contribute to the spectrum observed, or only a portion of them. However, many more phospholipids contribute to the bilayer resonance in the trypsin-treated virus than in the normal virus.

Fig. 2D shows the kind of spectrum expected from the Sendai virus lipids when in a bilayer. This is a total lipid extract of the virus which is free of protein from the viral membrane. It should be noted that this spectrum is similar to that for the pure phosphatidylcholine shown in Fig. 2A.

Discussion

The main purpose of this study is to examine the properties of the membrane of this enveloped virus. The viral glycoproteins have most of their mass on the envelope membrane surface. These thoughts suggest that it is very important to measure directly what is occurring at the viral membrane surface. The technique chosen here to do this is ^{31}P-NMR. This is an excellent technique to use for membrane studies, as has been shown elsewhere [18], but to our knowledge, has only

been used in the study of one other virus, vesicular stomatitus virus [14]. The technique directly measures the behavior of the phosphates of the phospholipids in the membrane. It is a non-perturbing and non-destructive technique. No probes need by added to the system, since the ^{31}P nucleus, to which the technique is sensitive, is 100% naturally abundant. Because it measures the phospholipid phosphate behavior, ^{31}P-NMR is measuring the properties of the membrane surface in which the phospholipid phosphate resides. One additional factor must be considered. Since the technique is, in principle, sensitive to all the phosphorus-containing molecules, it may also reveal signals from the RNA. Two factors make the analysis more simple, however. First, the amount of phosphate in the RNA is substantially less than the amount of phosphate in the phospholipids [7]. Secondly, since the RNA is bound to protein in the nucleocapsid, and since it is a macromolecule, it will only be present in a very broad resonance, much broader (about 5-times broader) than that seen from a normal phospholipid bilayer [18]. Therefore if all the phospholipids would be by far the dominant resonance in the spectrum of the virus. This is in contrast to the results with the DNA containing virus, PM2. In PM2, the DNA makes an appreciable contribution to the ^{31}P-NMR resonance (Akutsu et al. [1]).

Therefore it is fascinating that in the ^{31}P-NMR spectrum of the intact Sendai virus, almost no resonance attributable to a phospholipid bilayer is present. This result does not necessarily imply that the viral envelope does not contain a phospholipid bilayer. However, it does indicate that the phospholipid headgroups are highly motionally restricted. No other biological membrane system studied by ^{31}P-NMR has exhibited such behavior. ^{31}P-NMR spectra characteristic of phospholipid bilayers always have been observed.

Severe motional restriction could produce the experimental result in at least two ways. (1) Motional restriction leads to strong dipolar interactions which produce much broader resonances than normally observed. (2) Motional restriction can reduce the effectiveness of averaging of the phosphate chemical shift tensor, leading to a greater expression of that tensor and a much broader resonance (up to 5-times broader). In each case, the motional restriction leads to a much broader resonance, which could, under the experimental conditions used here, produce the non-descript broad resonance of the virus seen in Fig. 2B.

The extent of the motional restriction is difficult to accurately describe, since the lineshape is not precisely defined. Some comparisons to known conditions may be useful. The spectra obtained from a membrane in a gel state do not produce resonances as broad as that seen here for the intact virus [18]. The resonance from dehydrated phospholipids is as broad as the resonance observed for the intact virus [18].

Therefore one must conclude that the intact Sendai virus membrane surface is quite rigid. A similar conclusion was reached in the case of vesicular stomatitus virus, though the motional restriction was manifest in a different manner, and did not appear to be as severe as observed here for Sendai virus [14].

In light of this discussion, the results obtained with the trypsin-treated virus are particularly interesting. As seen in Fig. 2C, trypsin treatment produces a ^{31}P-NMR spectrum that is typical of phospholipid bilayers. Apparently the trypsin treatment relieves some of the motional restriction inhibiting phospholipid movement in the intact virus. Since this removes the extramembraneous portion of the F glycoprotein, these results implicate the F protein in the motional restriction of the phospholipid headgroups in the intact virus. There may be some interaction between the carbohydrate-bearing part of the F protein and the surface of the viral envelope membrane.

Previous ESR studies with spin labels that sensed only the interior of the membrane showed a highly restricted environment that was not changed on trypsin treatment [9]. We have confirmed these results (Abidi and Yeagle, unpublished data). It may be that the intramembraneous portions of the glycoproteins are primarily responsible for the motional restriction of the membrane interior. This has been suggested previously [10].

These results also find an interesting parallel in the study of vesicular stomatitus virus [14]. Trypsin treatment of that virus removes the surface glycoprotein, G. Trypsin treatment also removes the motional restriction of the membrane surface present in the native vesicular stomatitus virus

[14]. Apparently in the case of enveloped virus, the surface glycoprotein plays a role in creating a rigid membrane surface.

Finally it is interesting to speculate that the creation by the viral glycoproteins of a rigid patch on the host cell membrane surface may be important in the process of viral budding.

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