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Effects of the 'fusion peptide' from measles virus on the structure of *N*-methyl dioleoylphosphatidylethanolamine membranes and their fusion with Sendai virus

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^{31}P nuclear magnetic resonance spectroscopy (^{31}P-NMR) was used to study phospholipid organization in hydrated preparations of *N*-methyl dioleoylphosphatidylethanolamine and a 'fusion peptide' with the sequence: FAGV-VLAGAALGVAAAAQI, which corresponds to the amino terminus of the F1 subunit of the membrane fusion protein of measles virus. These amino acids are believed to mediate syncytia formation, host-cell penetration and hemolysis by infectious virus. The presence of the peptide at 0.5 mole percent significantly facilitated the formation of isotropic ^{31}P resonances. The effects at 1 mole percent peptide were substantially enhanced over the effects observed at 0.5 mole percent, leading to a decrease in the onset temperature of the formation of the isotropic ^{31}P-NMR resonances by about 30 °C. The formation of such isotropic ^{31}P-NMR resonances has been previously associated with an increased rate of fusion of large unilamellar vesicles composed of *N*-methyl dioleoylphosphatidylethanolamine. Enhanced fusion of octadecyl rhodamine-labelled Sendai virus with *N*-methyl dioleoylphosphatidylethanolamine large unilamellar vesicles was observed when the 'fusion peptide' was incorporated into the large unilamellar vesicles.

The membranes of enveloped viruses contain proteins which promote membrane fusion. In Sendai and measles viruses this function is performed by homologous F_0 proteins. To be active in promoting membrane fusion, the F_0 protein must be proteolytically cleaved to form disulfide-linked F1 and F2 fragments. The N-terminus of the F1 subunit is particularly hydrophobic and is thought to play an important role in promoting membrane fusion because it can readily partition into membranes. Chemically modified small peptides with sequences corresponding to the N-terminus of F1 are markedly inhibitory to viral infection and viral-induced hemolysis [1]. These peptides were thought to compete with the virus for interaction with target membranes but may instead interfere with the action of the fusion polypeptide, F1. Measles virus variants which are resistant to the inhibitory oligopeptide contain mutations in a region away from the F1 amino terminus [2]. More recent work has shown that these peptides can alter the bulk properties of membranes by stabilizing bilayers against morphological rearrangements [3,4] and also inhibit membrane fusion in model systems [3,4].

The inhibitory peptides differ from the F1 protein, of course, being much shorter, with tripeptides approaching maximal activity, but in addition the most potent of these peptides have their amino terminal blocked with a carbobenzoxy group and contain D-amino acids. We have extended these studies with a much longer peptide of 19 amino acids which comprises the entire hydro-

Abbreviations: LUV, large unilamellar vesicles; *N*-methyl DOPE, *N*-methyl dioleoylphosphatidylethanolamine; NMR, nuclear magnetic resonance.

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phobic amino terminal segment of F1. This peptide has a free amino terminal and contains only L-amino acids. It is therefore a good model to study effects on membrane properties resulting from insertion of the amino terminal segment of the F1 protein into membranes. We have studied the effects of this peptide on the phase behavior and fusion rate of *N*-methyl dioleoylphosphatidylethanolamine (*N*-methyl DOPE). This phospholipid undergoes a thermotropic transition to give rise to structures which exhibit isotropic ^{31}P nuclear magnetic resonance (NMR) resonances. The presence of these isotropic resonances has been associated with increased rates of membrane fusion [5]. We find that our model 'fusion peptide' with the sequence FAGVVLAGAALGVAAAAQI promotes isotropic ^{31}P-NMR resonances and increases the rate of Sendai virus fusion to *N*-methyl DOPE large unilamellar vesicles (LUV). This sequence mimics the amino terminal of F1 from measles [6,7].

Materials and Methods

Materials

N-Methyl dioleoylphosphatidylethanolamine (*N*-methyl DOPE) was obtained from Avanti Polar Lipids, Birmingham, AL. Octadecylrhodamine B chloride (R_{18}) was obtained from Molecular Probes, Inc., Junction City, OR.

Peptide synthesis

The 'fusion peptide' of measles (FAGVVLAGAALGVAAAAQI) was chemically synthesized using an Applied Biosystems 430A solid phase peptide synthesizer. Peptide was desalted and purified by HPLC as previously described [8]. Rechromatography showed one peak on HPLC. The threonine residue at position 15 was converted to alanine to make the synthesis simpler. The amino acid analysis of the purified peptide agreed with the above sequence.

Vesicle preparation

Large unilamellar vesicles were prepared according to methods described previously [9] with further details as described subsequently [5]. The phospholipid and the peptide were co-solubilized in trifluoroacetic acid at room temperature in the indicated mole ratios. The trifluoroacetic acid was removed by evaporation under a stream of nitrogen gas followed by evaporation under high vacuum. The lipid mixture was hydrated for 3 h on ice, under N_2 in 100 mM NaCl, 10 mM glycine, 0.1 mM EDTA (pH 9.5). The lipid suspension was freeze-thawed five times and then extruded ten times through a polycarbonate membrane with 0.1 μm pores (Nuclepore Corp., Pleasanton, CA). Vesicles were stored on ice, under N_2 and were used within 2 days. Vesicles were characterized by negative stain transmission electron microscopy and by gel chromatography as a function of the number of extrusions. After 10 extrusions, no further improvement in homogeneity of vesicle size was seen. Also, no evidence of multilamellar vesicles was observed. According to the electron microscopy, the LUV ranged in size from 200 nm to 900 nm, with most LUV near 400 nm. Thin-layer chromatography of the *N*-methyl DOPE before and after this protocol for LUV showed no significant breakdown of the lipid by the trifluoroacetic acid or the extrusion.

Sendai virus preparation and labeling

Sendai virus (strain *cantell*) was grown in the chorioallantoic membrane of embryonated chicken eggs. The allantoic fluid was harvested 72 h post-infection and the virus was partially purified by centrifugation through a 30–60% sucrose density gradient. The amount of virus used in the fusion assays was determined by the number of μg of viral protein determined from a protein assay [10] Virus was labelled with octadecylrhodamine B chloride (R_{18}) as described previously [11]. Briefly, 20 nmol of R_{18} in 10 μl ethanol was added for each mg of viral protein in a total volume of 1 ml. The mixture was vortexed and allowed to incubate at room temperature for 1 h. Labelled virus was separated from unincorporated R_{18} by passing the incubation mixture over a Sephadex G-75 column and eluting with 100 mM NaCl, 10 mM glycine, 0.1 mM EDTA (pH 7.4).

Fusion assay

The R_{18} fusion assay for virus-vesicle fusion was carried out as described by Hoekstra [11]. *N*-Methyl DOPE LUV with and without the fusion peptide were diluted to 1 μmol/ml in 100 mM NaCl, 10 mM glycine (pH 9.5). A total volume of 1 ml was used for each assay. The LUV were first allowed to equilibrate to the appropriate temperature for 5 min. 25 μl of 2 M sodium acetate/acetic acid buffer and 50 μl of R_{18}-labelled virus were added simultaneously to the vesicles. Fluorescence was monitored with an excitation wavelength of 560 nm and an emission wavelength of 586 nm. The fluorescence intensity obtained without the addition of 2 M sodium acetate/acetic acid buffer was taken as baseline. 100% fluorescence was determined by adding 100 μl of 10% Triton X-100 to the vesicle/virus mixture. The initial rate of fusion was determined by measuring the slope of the trace of the increase in fluorescence with time after initiation of the fusion reaction, as close as possible to the time of initiation of fusion.

Membrane preparation for NMR

Multilamellar liposomes containing the measles peptide in the phospholipid bilayer were prepared in the following manner. The phospholipid and the peptide were co-solubilized in trifluoroacetic acid at room temperature in the indicated mole ratios. The trifluoroacetic

acid was removed by evaporation under a stream of nitrogen gas followed by evaporation under high vacuum. The material was then hydrated with 50 mM histidine, 1 mM EDTA (pH 7.4). The pH was measured to detect residual trifluoroacetic acid. In the case of a significant pH change, the pH was adjusted with NaOH. Following hydration, the membranes were subjected to three freeze-thaw cycles using liquid nitrogen. The membranes were then warmed to the starting temperature of the experiment. Control experiments were also performed using LUV of pure *N*-methyl DOPE. No difference in the phase behavior was observed, using ^{31}P-NMR, between the LUV and the multilamellar preparations [5]. The multilamellar preparation was used in the ^{31}P-NMR experiments on the measles peptide because of limited amounts of the peptide available and because the yield of material after the extrusions was too low for the ^{31}P-NMR experiments.

Nuclear magnetic resonance

^{31}P nuclear magnetic resonance (NMR) spectra were obtained with a Jeol FX270 Fourier transform spectrometer on a broad band probe in 10 mm tubes at the indicated temperatures. A fully phased cycle (32 pulse) Hahn echo was used with a 20 μs pulse spacing [12]. Gated proton decoupling (on only during acquisition) at a decoupling field of 9 kHz was employed to minimize sample heating. A 50 kHz spectral width was used. A delay time of 1 s was used between pulses. The only ^{31}P nuclei in the preparation were in the phospholipid component of these membranes.

Results

Multilamellar liposomes and large unilamellar vesicles (LUV) of *N*-methyl DOPE exhibited the same, complex phase behavior as a function of temperature [5]. ^{31}P-NMR provided a means of detecting all the known structures in this lipid system. Three different structures were observed: bilayer, hexagonal II, and structures that gave rise to an isotropic ^{31}P-NMR resonance. The hexagonal II structure has a characteristic ^{31}P-NMR powder pattern, is favored at high temperatures and is a non-bilayer structure of inverted 'tubes'. The isotropic ^{31}P NMR resonance arises from non-bilayer structures which are likely lipidic particles [13] or interlamellar attachments [14]. In cases where the lipid dispersion is recycled through the temperature region in which the isotropic resonances appear, cubic phase can form. However, in this study, the material was only heated once and cubic phase was not generally the dominant structure in these systems under such conditions as measured by X-ray diffraction [5]. The relative incidence of the structures which gave rise to the ^{31}P-NMR resonance was directly related to the fusion rate of LUV of *N*-methyl DOPE at pH 4.5 [5,15]. The bilayer structure exhibited a characteristic ^{31}P-NMR powder pattern that has been well described. The phospholipid used in this study was subjected to the same analysis described previously [16] and was found to behave similarly with respect to the onset temperatures for the formation of the various phospholipid structures (data not shown).

The purpose of this study was to determine the effect of incorporation into the phospholipid bilayer of a peptide mimicking the sequence of the 'fusion peptide' from the fusion protein of measles virus. The peptide used had the sequence: FAGVVLAGAALGVAAAAQ-I. This peptide was incorporated into multilamellar liposomes of *N*-methyl DOPE as described in Methods. These membranes were then examined with ^{31}P-NMR as a function of temperature. Fig. 1 shows the results obtained.

On the right hand side are the spectra from the control membranes, without any peptide. These data were obtained using phospholipid from the same lot that was used in all the other experiments reported here. In the middle of the figure are spectra from *N*-methyl DOPE membranes containing peptide at a mole ratio of 1 : 200 with respect to the phospholipid. At the lowest temperature shown, the spectrum exhibited two overlapping patterns. The majority of the phospholipid was in the bilayer structure. However, a small proportion of the lipid was in a structure promoting nearly isotropic motional averaging so that only a relatively narrow, isotropic ^{31}P-NMR resonance was observed. The proportion of the lipid in the isotropic structure increased as the temperature was increased. No isotropic ^{31}P-NMR resonance was seen in the pure phospholipid at these temperatures. Only at higher temperatures was the isotropic ^{31}P-NMR resonance observed in the pure phospholipid. For comparison, the ^{31}P-NMR spectrum of pure *N*-methyl DOPE at 45 °C contained a similar amount of isotropic resonance as the 1 : 200 (peptide/phospholipid) mixture exhibited at 30 °C.

Increasing the peptide to a 1 : 100 mole ratio (peptide/phospholipid) caused a further destabilization of the phospholipid bilayer. The isotropic ^{31}P-NMR resonance appeared at even lower temperatures at this higher peptide content, as can be seen in Fig. 1. Fig. 2 shows this relationship quantitatively.

Since the presence of the structures that gave rise to the isotropic ^{31}P-NMR resonance enhanced vesicle-vesicle fusion in the *N*-methyl DOPE LUV [4,5,15], we examined whether the increase in the resonance intensity of the isotropic ^{31}P-NMR resonance induced by the peptide was correlated with an increased rate of viral fusion with these LUV. The initial rate of fusion in control vesicles and in vesicles containing the peptide (initially incorporated at 1 mole percent during the vesicle preparation) was measured. Previous work revealed that Sendai virus fused with *N*-methyl DOPE

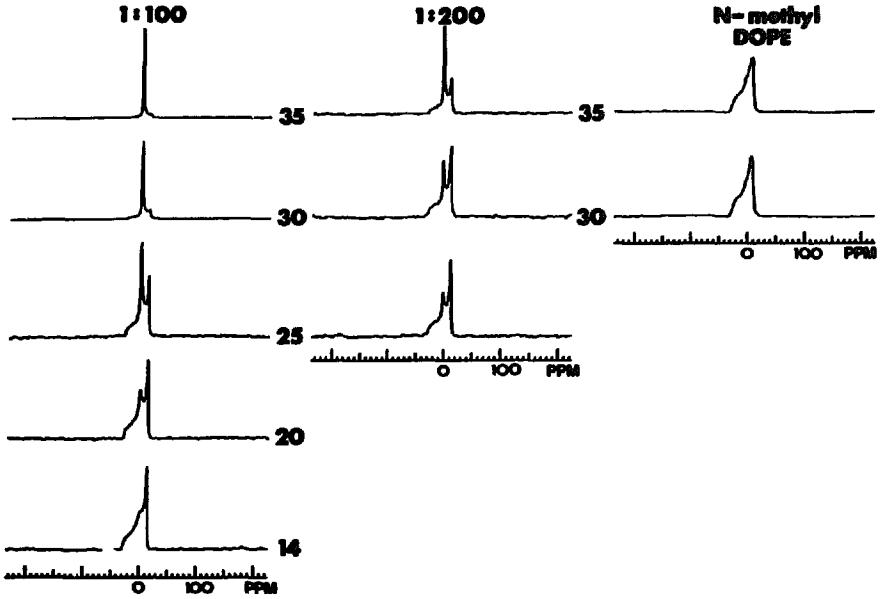

Fig. 1. 109 MHz ^{31}P-NMR spectra of *N*-methyl DOPE multilamellar liposomes, with and without the peptide from the measles fusion protein with the sequence: FAGVVLAGAALGVAAAAQI. The temperature (°C) and the molar ratio (peptide/phospholipid) are indicated. The right hand set of spectra are of 25 mg of pure *N*-methyl DOPE in 50 mM histidine, 1 mM EDTA (pH 7.4). Spectra were obtained with 20 000 transients and were Fourier transformed with 50 Hz of linebroadening.

LUV, without receptors for the virus [4]. Initial rates of Sendai virus fusion with *N*-methyl DOPE LUV increased with an increase in the intensity of the isotropic ^{31}P-NMR resonance. The presence of the peptide in the LUV appeared to enhance the initial rate of fusion in this system at all temperatures by about 30%. Table I shows the results.

It was not possible to test for the fusion of *N*-methyl DOPE LUV containing this peptide for two reasons. Contents mixing fusion assays rely on non-leaky vesicles and these vesicles proved to be leaky (data not shown). Lipid mixing fusion assays rely on fluorescent probes in the membrane which in this case perturb the phase behavior of the *N*-methyl DOPE (data not shown).

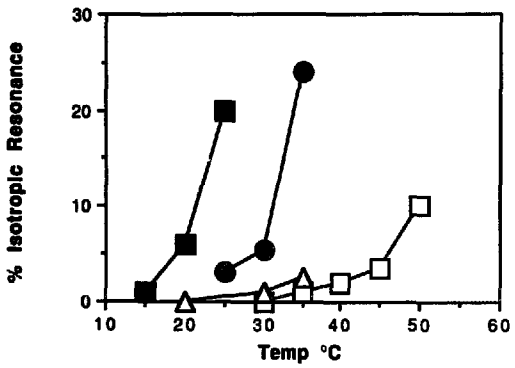

Fig. 2. Quantitative relationship of the percent of the total phospholipid contributing to the isotropic resonance in the 109 MHz ^{31}P-NMR spectra of *N*-methyl DOPE multilamellar liposomes, with and without the peptide from the measles fusion protein with the sequence FAGVVLAGAALGVAAAAQI. (□) pure *N*-methyl DOPE; (△) pure *N*-methyl DOPE prepared identically to the membranes containing the peptide; (●) 0.5 mole percent peptide; (■) 1 mole percent peptide.

Discussion

In previous studies, an increase in the intensity of the isotropic ^{31}P-NMR resonance in suspensions of *N*-methyl DOPE has been correlated with an increase in the rate of fusion of *N*-methyl DOPE LUV [5,15]. The isotropic ^{31}P resonance has also been shown to be related to certain non-lamellar structures observed as lipidic particles in freeze-fracture electron microscopy of these phospholipids [13]. Therefore it was suggested that these non-lamellar structures might be involved as an intermediate in the fusion event [5,15].

TABLE I

Effect of FAGVVLAGAALGVAAAAQI (0.5 mol% with respect to the lipids) on initial rate of fusion of Sendai virus with N-methyl DOPE LUV

Values show averages of two independent experiments.

Temp. (°C)	Fusion rate (%/min)	
	no peptide	with peptide
20	0.14	0.16
25	0.16	0.27
30	0.25	0.36
35	0.36	0.5
40	0.57	0.79
45	0.73	1.0

The data in this report revealed a facilitation of the formation of the isotropic ^{31}P-NMR resonance by a peptide with the same sequence as the 'fusogenic' peptide of measles virus. When this peptide was incorporated into the *N*-methyl DOPE membranes, a significant increase in the intensity of the isotropic ^{31}P resonance was observed at all temperatures. An analogous effect was observed from incorporation of a relatively hydrophobic signal peptide into phospholipid bilayers [17].

An increase in the rate of Sendai virus fusion with the *N*-methyl DOPE LUV containing the measles virus peptide was also observed at all temperatures measured.

Recently it was reported that the fusion protein of Sendai virus penetrated the target membrane during fusion [18]. The portion of the fusion protein penetrating the target membrane was not described; however after cleaving the fusion protein in two, the subunit containing the 'fusion' peptide was the F1 implicated in target membrane penetration.

On the basis of these data, one could hypothesize that the fusion protein penetrated the target membrane with the 'fusogenic' peptide, perturbed the bilayer structure, and produced localized defects in the bilayer. Local defects in the form of interlamellar attachments (ILA), which may be similar to the structures formed in the present study, have been implicated in membrane fusion in vesicle fusion studies with *N*-methyl DOPE LUV and LUV made of phosphatidylcholine/phosphatidylethanolamine mixtures [14].

Although these data are in qualitative agreement with this specific mechanism for viral fusion, there is a lack of quantitative correlation between peptide-induced isotropic ^{31}P resonance formation and the rates of peptide-induced viral fusion. The large increase in the prevalence of the isotropic ^{31}P-NMR resonance due to the measles peptide apparently produced only a modest increase in the rate of fusion. Furthermore, both a signal sequence [17] and here a 'fusion peptide' produce an increase in the isotropic ^{31}P-NMR resonance from phospholipid bilayers. Thus the influence of hydrophobic peptides on the morphology of phospholipids may not be specific. Therefore one would be led to the suggestion that the role of the hydrophobic peptide of the viral fusion protein is not yet adequately described.

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