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Inhibition of Sendai Virus Fusion with Phospholipid Vesicles and Human Erythrocyte Membranes by Hydrophobic Peptides[1]

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Hydrophobic di- and tripeptides which are capable of inhibiting enveloped virus infection of cells are also capable of inhibiting at least three different types of membrane fusion events. Large unilamellar vesicles (LUV) of *N*-methyl dioleoylphosphatidylethanolamine (*N*-methyl DOPE), containing encapsulated 1-aminonaphthalene-3,6,8-trisulfonic acid (ANTS) and/or *p*-xylene bis(pyridinium bromide) (DPX), were formed by extrusion. Vesicle fusion (contents mixing) and leakage were then monitored with the ANTS/DPX fluorescence assay. Sendai virus fusion with lipid vesicles and Sendai virus fusion with human erythrocyte membranes were measured by following the relief of fluorescence quenching of virus labeled with octadecylrhodamine B chloride (R_{18}), a lipid mixing assay for fusion. This study found that the effectiveness of the peptides carbobenzoxy-L-Phe-L-Phe (Z-L-Phe-L-Phe), Z-L-Phe, Z-D-Phe, and Z-Gly-L-Phe-L-Phe in inhibiting *N*-methyl DOPE LUV fusion or fusion of virus with *N*-methyl DOPE LUV also paralleled their reported ability to block viral infectivity. Furthermore, Z-D-Phe-L-PheGly and Z-Gly-L-Phe inhibited Sendai virus fusion with human erythrocyte membranes with the same relative potency with which they inhibited vesicle–vesicle and virus–vesicle fusion. The evidence suggests a mechanism by which these peptides exert their inhibition of plaque formation by enveloped viruses. This class of inhibitors apparently acts by inhibiting fusion of the viral envelope with the target cell membrane, thereby preventing viral infection. The physical pathway by which these peptides inhibit membrane fusion was investigated. ^{31}P nuclear magnetic resonance (NMR) of proposed intermediates in the pathway for membrane fusion in LUV revealed that the potent fusion inhibitor Z-D-Phe-L-PheGly selectively altered the structure (or dynamics) of the hypothesized fusion intermediates and that the poor inhibitor Z-Gly-L-Phe did not. One possible interpretation of these ^{31}P NMR results was that the inhibitory peptide stabilized a membrane structure with a large radius of curvature, when the fusion pathway demanded a membrane defect with a small radius of curvature. This hypothesis was tested by determining the influence of an inhibitory and a noninhibitory peptide on the formation of membraneous structures with small radii of curvature, through ultrasonic irradiation of phospholipid dispersions. The inhibitory peptide prevented the formation of membrane structures with small radii of curvature, while the noninhibitory peptide did not prevent the formation of such structures. These data taken collectively suggest that the peptides that inhibit the fusion of enveloped viruses could act, at least in part, by interfering with the formation of intermediates in membrane fusion (possibly punctate membrane defects induced by the virus) that force lipids into structures with small radii of curvature.

INTRODUCTION

Enveloped viruses possess an outer limiting membrane containing glycoproteins responsible for recognition of the target cell and mediation of the fusion between the viral envelope and the target cell plasma membrane. Fusion of the viral envelope with the plasma membrane or the endocytic vesicle membrane of a target cell allows the entry of the viral genome and initiation of replication. Studies of inhibition of viral fusion may reveal details about the mechanism by which enveloped viruses gain entry to cells. Inhibition of the fusion event may also prove to be a viable strategy for preventing viral infection of cells.

Many enveloped viruses, including Sendai, measles, influenza, SV5 (Richardson *et al.*, 1980), STLV-3, RSV, HIV-1, and HIV-2 (Gallaher, 1987), have fusion proteins which must be proteolytically cleaved to generate a fusion competent virus. After cleavage, new, highly hydrophobic N-termini are generated on the fusion proteins. The amino acid sequences of these new N-termini contain regions of homology, particularly within the first 20 or so amino acids, which are highly hydrophobic. The sequence *N*-Phe-X-Gly in particular, where X is any hydrophobic amino acid, is common to many of them (Gallaher, 1987). It has been hypothesized that this highly hydrophobic N-terminal domain can insert itself into the membrane of the target cell and thereby initiate the fusion process (Novick and

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Hoekstra 1988; Daniels *et al.,* 1985; Doms *et al.,* 1985; Skehel *et al.,* 1982). In the case of Sendai, the envelope glycoprotein, F, is thought to be the fusion protein (Scheid and Choppin, 1974; Hsu *et al.,* 1979; Nakanishi *et al.,* 1982).

Viruses must possess an active fusion protein in order to complete the infection process. For many enveloped viruses the final step in the fusion pathway occurs at acidic pH within endosomes in the interior of the cell (Skehel *et al.,* 1982). In the case of Sendai, however, fusion occurs at the plasma membrane of the cell at neutral pH (Haywood, 1988).

Richardson *et al.* (1980) have shown that some oligopeptides, with sequences similar to those of the hydrophobic N-termini of viral fusion proteins, were capable of inhibiting measles and Sendai virus fusion with target cells. The mechanism of this inhibition was not clear. Richardson and Choppin (1983) presented evidence suggesting that the inhibitory peptides, in particular Z-D-Phe-L-PheGly,[3] bound to the cell membrane but did not bind to the envelope of measles virus. However, a second group (Asano and Asano, 1985), working with another paramyxovirus (Sendai), observed a decrease in the hemolytic activity of the virus when preincubated with Z-D-Phe-L-PheGly, opening up the possibility that the peptide may have been acting on the virus in addition to any effect on the target cell membrane. A recent study (Hull *et al.,* 1987) of a measles virus mutant which was resistant to inhibition by Z-D-Phe-L-PheGly found that the mutant fusion protein was altered at a position in the amino acid sequence far from the hydrophobic N-terminus. This suggested that a mechanism of inhibition other than competition for a common receptor was utilized by the peptide.

Recent work (Kelsey *et al.,* 1990) has shown that Z-D-Phe-L-PheGly inhibited the fusion of large unilamellar vesicles (LUV) of *N*-methyl dioleoylphosphatidylethanolamine (*N*-methyl DOPE LUV) and fusion between Sendai virus and *N*-methyl DOPE LUV. The effectiveness of Z-D-Phe-L-PheGly in these relatively simple model systems suggested that it may exert its inhibitory influence through some alteration of the stability or fusogenic potential of the lipid bilayer. The present study revealed that the peptides Z-L-Phe-L-Phe, Z-L-Phe, Z-D-Phe, and Z-Gly-L-Phe-L-Phe had the same relative potency in inhibiting *N*-methyl DOPE LUV fusion and Sendai virus fusion with *N*-methyl DOPE LUV as in inhibiting viral infection of cultured cells (Richardson *et al.,* 1980). Sendai viral fusion with human erythrocyte ghosts was also inhibited by Z-Gly-L-Phe-L-Phe. Thus the mechanism of action of these peptides appeared to be inhibition of the viral fusion event, thereby blocking enveloped virus infection of target cells. This evidence was consistent with the possibility that the inhibitory oligopeptides blocked diverse membrane fusion events by a common mechanism. The mechanism of inhibition of fusion by these peptides was examined in this study. The accumulated evidence suggested (but did not prove) that the peptides that inhibit the fusion of enveloped viruses could act, in part, by interfering with the formation of intermediates in membrane fusion (possibly induced punctate membrane defects) that contained small radii of curvature.

[3] Abbreviations used: *N*-methyl DOPE, *N*-methyl dioleoylphosphatidylethanolamine; LUV, large unilamellar vesicles; ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; DPX, *p*-xylylene bis(pyridinium bromide); R_{18}, octadecylrhodamine B chloride; Z-D-Phe-L-PheGly, carbobenzoxy-D-Phenylalanyl-L-Phenylalanyl-Glycine; Z-L-Phe-L-Phe, carbobenzoxy-L-Phenylalanyl-L-Phenylalanine; Z-L-Phe, carbobenzoxy-L-Phenylalanine; Z-D-Phe, carbobenzoxy-D-Phenylalanine; Z-L-Phe-L-Tyr, carbobenzoxy-L-Phenylalanyl-L-Tyrosine; Z-Gly-L-Phe, carbobenzoxy-Glycyl-L-Phenylalanyl-L-Phenylalanine; Z-Gly-L-Phe, carbobenzoxy-Glycyl-L-Phenylalanine; ^{31}P NMR, ^{31}P nuclear magnetic resonance; EDTA, ethylenediamine tetraacetic acid; PC phosphatidylcholine; PBS, phosphate-buffered saline.

MATERIALS AND METHODS

N-methyl DOPE was obtained from Avanti Polar Lipids (Birmingham, AL). Octadecylrhodamine B chloride (R_{18}), 1-aminonaphthalene-3,6,8-trisulfonic acid (disodium salt) (ANTS), and *p*-xylene bis(pyridinium bromide) (DPX) were from Molecular Probes, Inc. (Junction City, OR). Carbobenzoxy-D-Phe-L-PheGly (Z-D-Phe-L-PheGly), carbobenzoxy-D-Phe (Z-D-Phe), and carbobenzoxy-L-Phe (Z-L-Phe) were purchased from Sigma. Erythrocytes were isolated from fresh whole human blood.

Peptide synthesis and derivatization

L-Phe-L-Phe and Gly-L-Phe-L-Phe were synthesized at the SUNY Buffalo Microsequencing Lab by the solid phase method of Tam *et al.* (1983). An N-terminal carbobenzoxy moiety was added to these peptides by the method of Bodanszky (1984). A solution of the peptide to be derivatized in 3 ml water and 2 ml 5 *N* NaOH was stirred on ice. Benzyl chloroformate (1.1 moles per mole of peptide) and 5.5 ml 2 *N* NaOH were added, alternately, in 10 portions while the reaction mixture was being stirred at 10°. Additions were completed within 90 min. The reaction mixture was stirred an additional 30 min at 25° after which the pH was adjusted to 10.0 with 1 *N* NaOH and the solution was extracted three times with 2 vol of diethyl ether. The aqueous layer was acidified to pH 3.0 with HCl and extracted three times with 2 vol of diethyl ether. Solvent was removed by lyophilization and the crude product was pu-

rified by reverse-phase HPLC using an Altex Ultrasphere C_{18} column. The products were eluted over a period of 1 hr with a linear gradient of 15–100% acetonitrile in 0.1% trifluoroacetic acid. Z-L-Phe-L-Phe eluted at approximately 61% acetonitrile and Z-Gly-L-Phe-L-Phe eluted at approximately 65% acetonitrile.

Vesicle preparation

Large unilamellar vesicles encapsulating ANTS, DPX, or ANTS/DPX were prepared according to methods described by Szoka *et al.* (1980) with further details described by Ellens *et al.* (1989). *N*-methyl DOPE was hydrated for 3 hr on ice, under N_2, in 25 m*M* ANTS, 45 m*M* NaCl, 10 m*M* glycine, pH 9.5, or 90 m*M* DPX, 10 m*M* glycine, pH 9.5, or 12.5 m*M* ANTS, 45 m*M* DPX, 22.5 m*M* NaCl, 10 m*M* glycine, pH 9.5. The lipid suspension was next subjected to five freeze–thaw cycles followed by 10 extrusions through a polycarbonate membrane with 0.1-μm pores (Nucleopore Corp., Pleasanton, CA). Encapsulated material was separated from unencapsulated material on a Sephadex G-50 column (Pharmacia) with 100 m*M* NaCl, 10 m*M* glycine, 0.1 m*M* EDTA, pH 9.5, used as the elution buffer. Vesicles were stored on ice, under N_2, and were used within 2 to 3 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 measurements of the electron micrographs, the LUV ranged in size from 200 to 900 nm, with most LUV near 400 nm. Encapsulation volume of the LUV was found to be approximately 12 μl/μmol of phospholipid. For the NMR experiments where a peptide was present, the appropriate amount of peptide in methanol was added to the suspension of LUV following extrusion. The final concentration of methanol was 2.5% (v/v). The LUV were then concentrated by centrifugation at 35,000 rpm for 45 min in a Beckman ultracentrifuge using a Ti50 rotor. The supernatant was decanted and the pellet was resuspended in approximately 1 ml. The pH was lowered to 4.5 with 25 μl of 2 *M* sodium acetate/acetic acid buffer.

Sendai virus preparation and labeling

Sendai virus was grown in the chorioallantoic membrane of embryonated chicken eggs. The allantoic fluid was harvested 72 hr postinfection 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 quantified by the number of micrograms of viral protein determined by the dye binding assay (Bradford, 1976). Virus was labeled with octadecylrhodamine B chloride (R_{18}) as described by Hoekstra *et al.* (1984). Briefly, 20 nmol of R_{18} in 10 μl ethanol was added for each milligram of viral protein in a total volume of 1 ml. The mixture was vortexed and allowed to incubate at room temperature for 1 hr. Labeled virus was separated from unincorporated R_{18} by passing the incubation mixture over a Sephadex G-75 column and eluting with 100 m*M* NaCl, 10 m*M* glycine, 0.1 m*M* EDTA, pH 7.4.

Preparation of human erythrocyte ghosts

Human erythrocyte ghosts were prepared by the method of Clague *et al.* (1990) except that resealing was done at 20°. Fresh whole human blood was washed three times in phosphate-buffered saline (PBS), which consists of 137 m*M* NaCl, 2.7 m*M* KCl, 8.1 m*M* Na_2HPO_4, 1.5 m*M* KH_2HPO_4, pH 7.4. Erythrocytes were lysed at 4° in 20 vol of 10 m*M* Tris, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 0.1 m*M* EGTA, 0.1% BSA, pH 7.4. After 2 min, isotonicity was restored by addition of 1/10 vol of hypertonic buffer consisting of 150 m*M* Na_2HPO_4, 50 m*M* KH_2HPO_4, 1.22 *M* NaCl, 30 m*M* KCl, 1 m*M* $CaCl_2$, and 1 m*M* $MgCl_2$. The cells were stirred at 20° for 40 min followed by three washes in PBS.

Fusion and leakage assays

All fluorescence measurements were made on an SLM 8000D fluorimeter. The ANTS/DPX fusion and leakage assays were carried out as described by Ellens *et al.* (1985). Vesicles contained either 25 m*M* ANTS and 45 m*M* NaCl, or 90 m*M* DPX, or 12.5 m*M* ANTS, 45 m*M* DPX, and 22.5 m*M* NaCl. Fluorescence intensity was monitored with an excitation wavelength of 380 nm and an emission wavelength of 510 nm. All assays were carried out in a total volume of 1 ml. The final lipid concentration was 0.4 μmol/ml for both fusion and leakage assays. Fusion or leakage was initiated by lowering the pH from 9.5 to 4.5 with 25 μl of 2 *M* sodium acetate/acetic acid buffer. For fusion assays a 9:1 molar ratio of DPX-containing LUV to ANTS-containing LUV was used. Fluorescence quenching due to contents mixing (resulting in an ANTS–DPX complex with reduced quantum yield) reflected the rate of LUV fusion. For vesicle–vesicle fusion assays, baseline fluorescence was taken to be the level obtained with the shutters of the fluorimeter closed and 100% fluorescence was taken to be the initial fluorescence intensity before lowering the pH. The initial rate of fusion was then calculated from the slope of the fluorescence decay curve during the period (1–2 min) immediately following the initiation of fusion. Leakage was measured

by dequenching of fluorescence due to leakage and dilution (and dissociation) of the ANTS–DPX complex.

The R_{18} fusion assay for virus–vesicle fusion was carried out as described by Hoekstra *et al.* (1984). A stock suspension of *N*-methyl DOPE LUV in 100 m*M* NaCl, 10 m*M* glycine, pH 9.5, was used, and 0.4 μmol (approximately 50 μl) of these LUV was added to PBS such that the combined volume was 975 μl. Where appropriate, 25 μl methanol with or without peptide was injected into the suspension of LUV. 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, and then 50 μl of R_{18}-labeled virus was added to the vesicles to initiate the assay. Fluorescence was monitored with an excitation wavelength of 560 nm and an emission wavelength of 586 nm. The fluorescence intensity obtained at zero time was taken as baseline. One hundred percent fluorescence was determined by adding 25 μl of 10% Triton X-100 to the vesicle/virus mixture. The final pH of the assay mixture was found to be between 7.6 and 7.8. Unless otherwise noted, the R_{18} fusion assay for virus–erythrocyte fusion was carried out in the same manner as the virus–vesicle assays except that erythrocyte ghosts in PBS (0.674 μmol lipid) were used instead of LUV. The assays were carried out at 37° unless otherwise indicated.

Other assays

Phosphate was determined by the method of Bartlett (1959).

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) chemical shift anisotropy (CSA) echo was used with a 40-μsec echo (Rance and Byrd, 1983). Gated proton decoupling (on only during acquisition) at a decoupling field of 9 kHz was employed to eliminate sample heating. A 50-kHz spectral width was used, with 50 Hz of linebroadening in the Fourier transformation. A delay time of 1 sec was used between pulses. The only ^{31}P nuclei in the preparation were in the phospholipid component of these membranes.

^{1}H NMR spectra were obtained on the same instrument at 270 MHz in 5-mm tubes using 16-k data points in the time domain and a presaturation of the HDO resonance. A 2700-Hz spectral width was used with no broadening in the Fourier transformation.

Sonication

Sonication was performed with a Branson W350 probe sonicator with an ice bath. First, multilamellar liposomes containing the peptide in the phospholipid bilayer were prepared in the following manner. Egg phosphatidylcholine and Z-D-Phe-L-PheGly were cosolubilized in chloroform–methanol (2:1) at room temperature in the indicated mole ratios. The solvent was removed by evaporation under a stream of nitrogen gas followed by evaporation under high vacuum overnight. The material was then hydrated in D_2O with 50 m*M* NaCl, sealed under nitrogen gas, and vortexed vigorously. The membranes were then sonicated with 5-min sonications followed by a 1-min rest period in which the light scattering of the sample was determined as effective absorbance at 400 nm.

Determination of fraction of peptides in aqueous phase

Z-D-Phe-L-PheGly or Z-Gly-L-Phe (0.1 μmol) was added in methanol to 0.4 μmol *N*-methyl DOPE LUV in a total volume of 1 ml. The final concentration of methanol was 2.5% (v/v). The absorbance at 215 nm ($A_{215\ nm}$) was taken. To correct for light scattering, the total absorbance of the peptide was determined as follows: $A_{215\ nm}$ for 0.4 μmol *N*-methyl DOPE LUV alone was measured and this value was subtracted from the value obtained in the presence of peptide. The number obtained was considered to represent 100% of the peptide. To determine the fraction in the aqueous phase, the LUV were pelleted by centrifugation at 35000 rpm for 30 min in a Beckman Ti50 rotor. The $A_{215\ nm}$ of the resulting supernatant was measured and compared with the value obtained for 100% peptide above to arrive at the percentage of peptide in the aqueous phase. As a control, the same amount of peptide was injected into the same buffer without LUV and subjected to the same centrifugation protocol. Of the peptide injected, more than 85% remained in the supernatant.

RESULTS

Effect of peptides on *N*-methyl DOPE LUV fusion

One of the goals of the work presented here was to better determine the extent to which the ability of hydrophobic antimeasles peptides to inhibit membrane fusion is similar among different fusion systems. Therefore, the effect of several peptides (examined by Richardson *et al.*, 1980 for their ability to inhibit viral plaque formation) on vesicle–vesicle fusion with *N*-methyl DOPE vesicles was examined. A correlation between the initial rate of fusion of *N*-methyl DOPE vesi-

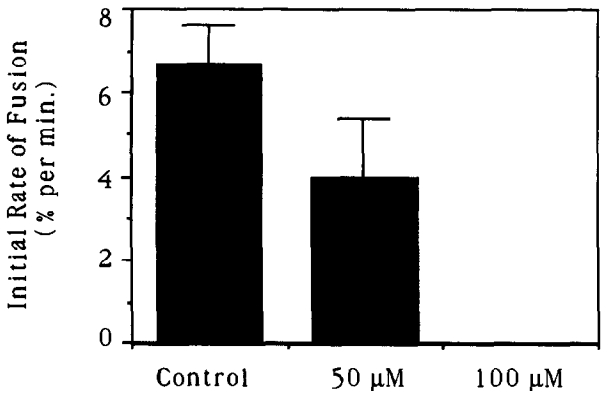

FIG. 1. Initial rate of *N*-methyl DOPE LUV fusion as measured by the ANTS/DPX contents mixing assay. A 9:1 molar ratio of DPX-containing LUV to ANTS-containing LUV was used in all assays. The concentration of lipid was 0.4 μmol/ml. Methanol (2.5% v/v) was included in the control experiment because Z-L-Phe-L-Phe had to be added in methanol due to its hydrophobic nature. Z-L-Phe-L-Phe was included in some assays as indicated below each bar. No fusion was observed at 100 μ*M* Z-L-Phe-L-Phe. Results shown are the averages of three separate experiments. Error bars represent one standard deviation.

cles and the appearance of isotropic ^{31}P NMR resonances superimposed on the ^{31}P NMR bilayer powder patterns that reflect the phase behavior of the lipid was previously reported (Ellens *et al.*, 1989; Kelsey *et al.*, 1990). A marked increase in the initial rate of fusion of *N*-methyl DOPE vesicles was observed around 35°–40° (Kelsey *et al.*, 1990), quantitatively similar to data published previously (Ellens *et al.*, 1989).

Shown in Fig. 1 is the effect of Z-L-Phe-L-Phe on the initial rate of fusion of *N*-methyl DOPE vesicles as measured by the ANTS/DPX contents mixing fusion assay described under Materials and Methods. Half maximal inhibition of fusion was found to occur at approximately 50 μ*M* Z-L-Phe-L-Phe. The peptides Z-L-Phe, Z-D-Phe, and Z-Gly-L-Phe-L-Phe were also assayed for their ability to inhibit fusion. A small inhibitory activity was sometimes observed at 200 μ*M* Z-L-Phe or Z-D-Phe. No inhibitory activity was observed for Z-Gly-L-Phe-L-Phe at concentrations up to 200 μ*M* in any experiments performed. The hydrophobicity of these peptides necessitated adding them to the assay mixture in a small amount of methanol. Control experiments showed that methanol had only small effects on initial rates of fusion or on the temperature at which an isotropic resonance appeared in ^{31}P NMR spectra of these vesicles (see below). The relative ability of these peptides to inhibit vesicle–vesicle fusion was Z-L-Phe-L-Phe ≫ Z-L-Phe ≅ Z-D-Phe > Z-Gly-L-Phe-L-Phe. This was the same relative potency with which these peptides were found to inhibit infectivity of Sendai and measles viruses (Richardson *et al.*, 1980).

Effect of peptides on fusion of Sendai virus with *N*-methyl DOPE LUV

In order to further characterize the effect of the peptides used in the vesicle–vesicle fusion assays above, their ability to inhibit fusion of Sendai virus with *N*-methyl DOPE LUV was assessed. Z-L-Phe-L-Phe was found to be inhibitory as shown in Fig. 2. Fusion was 50% inhibited at approximately 200 μ*M* Z-L-Phe-L-Phe. Z-L-Phe-L-Phe is therefore less effective in inhibiting virus–vesicle fusion than Z-D-Phe-L-PheGly (Kelsey *et al.*, 1990). Z-L-Phe, Z-D-Phe, and Z-Gly-L-Phe-L-Phe were also assayed for their ability to inhibit Sendai fusion with *N*-methyl DOPE LUV and none were found to be effective at concentrations up to 200 μ*M*. This parallels the ability of these peptides to inhibit viral plaque formation as described by Richardson *et al.* (1980), i.e., an order of potency with Z-D-Phe-L-PheGly > Z-L-Phe-L-Phe ≫ Z-L-Phe, Z-D-Phe, and Z-Gly-L-Phe-L-Phe.

Effect of peptides on fusion of Sendai virus with human erythrocyte ghosts

The question of whether or not peptides assayed for their ability to inhibit vesicle–vesicle or virus–vesicle fusion exhibit similar activities with respect to viral fusion with a biological membrane has been examined. Figure 3 shows the effect of varying concentrations of Z-D-Phe-L-PheGly on Sendai virus fusion with human erythrocyte ghosts. Ghosts were preincubated at 37° for 5 min in the presence or absence of Z-D-Phe-L-PheGly. R_{18}-labeled virus was then added to the assay mixture and fusion was measured as an increase in fluorescence as a function of time. The results show that significant inhibition first occurs at 100–200 μ*M* Z-D-Phe-L-PheGly. This is consistent with results obtained by Asano and Asano (1985), using an hemolysis assay. An identical experiment using 200 μ*M* Z-Gly-L-

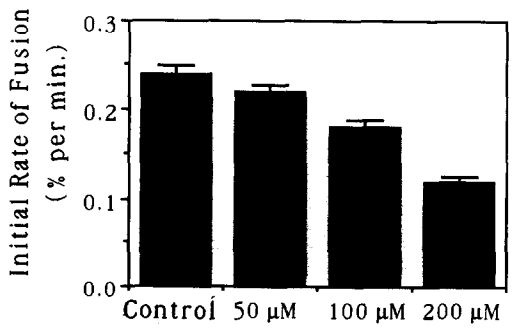

FIG. 2. Initial rate of fusion of R_{18}-labeled Sendai virus with *N*-methyl DOPE LUV at 37°. The assays were performed as described under Materials and Methods and contained the indicated concentrations of Z-L-Phe-L-Phe. The results presented are the averages of three experiments. Error bars represent one standard deviation.

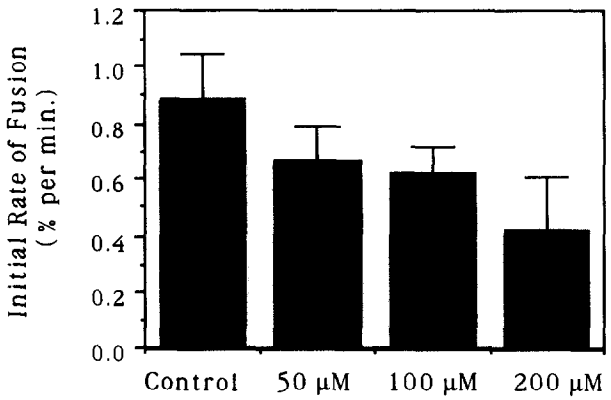

FIG. 3. Effect of Z-D-Phe-L-PheGly on the initial rate of fusion of Sendai virus with human erythrocyte ghosts resealed in the presence of 0.1% bovine serum albumin. The experiments were performed as described under Materials and Methods. In the control experiments methanol (2.5%) was included in each assay. Z-D-Phe-L-PheGly, when present in the assay mixture, was added in methanol to give a final concentration of peptide that is indicated beneath each bar. The results represent the average of three experiments. Error bars are one standard deviation.

Phe showed no inhibition of Sendai fusion with erythrocyte ghosts.

In the above experiments erythrocyte ghosts were exposed to the hydrophobic peptides prior to the addition of virus. In order to test the possibility that a different order of addition would result in a different level of inhibitory activity, virus was prebound to erythrocytes using a low temperature incubation. The ability of Z-D-Phe-L-PheGly to inhibit virus–erythrocyte fusion was abolished if R_{18}-labeled virus was incubated with erythrocyte ghosts for 15 min on ice, and fusion was initiated by injecting the virus–ghost mixture into buffer containing 200 *μM* Z-D-Phe-L-PheGly that was prewarmed to 37°.

Quantitative comparison between peptide inhibition of viral infection of cells and peptide inhibition of LUV fusion

Table 1 shows a quantitative comparison of the dose dependence of the effects of Z-D-Phe-L-PheGly on vesicle fusion, virus–vesicle fusion, and virus–erythrocyte fusion. These data show a sensitivity of each of the fusions to a similar concentration of Z-D-Phe-L-PheGly. Table 2 shows the concentration of peptide at which 50% inhibition of fusion was achieved for all the peptides used in this study and our previous study (Kelsey *et al.*, 1990).

The absolute concentrations for inhibition of LUV fusion and virus fusion with LUV were substantially higher than for inhibition of measles plaque formation (Richardson *et al.*, 1980). However, this quantitative difference can be at least partially understood in terms of partitioning of the hydrophobic inhibitory peptides into the relevant membranes. The data in this report indicated that the site of action was the membranes involved in the fusion event. Therefore, just as in the case of anesthetics, the crucial factor was how much of the inhibitory peptide entered the membrane. The LUV fusion experiments in this study required much greater amounts of membranes in the assay than are found in terms of plasma membranes of cells targetted by enveloped virus in a plaque assay. Therefore, to achieve the same amount of peptide in the membrane (relative to the phospholipid content of the membrane, for example), higher levels of peptide were required in the experiments involving the greater amounts of membranes. For that reason, the relative potency was emphasized in this discussion, rather than the absolute potency, for comparison of systems with significantly different membrane content.

Studies on the mechanism of action

Several experiments were designed to explore by what mechanism these peptides may inhibit membrane fusion. These studies were limited to the pure phospholipid systems, because present technology does not permit such experimentation on the viral or cellular systems.

We first examined the partitioning of these peptides into phospholipid bilayers to determine whether the site of action was the membrane. The partition coefficient was estimated as described under Materials and Methods after addition of peptide in methanol to performed LUV, and it was found to be similar for all peptides used in this study. At pH 9.5 approximately 65% of the peptides were in the aqueous phase. At pH 4.5 this was reduced to about 45% in the aqueous phase. These results show that differential partitioning into the lipid bilayer was not responsible for the different inhibitory activities of Z-D-Phe-L-PheGly and Z-Gly-L-Phe.

Next we examined the partitioning of Z-D-Phe-L-PheGly by first incorporating it into the lipid in organic solvent. A lipid film containing the peptide and the lipid was hydrated in D_2O and sonicated as described for an experiment below. ^1H NMR showed no evidence for peptide in solution in these experiments (that which was in the membrane had resonances too broad to be readily observed due to the motional restrictions on the peptide from the lipid bilayer), so most of the peptide must have been incorporated into the membrane in this protocol, in contrast to the protocol involving addition to the aqueous phase in methanol.

These experiments suggested that, as one would expect, the hydrophobicity of the peptides drove them into the membrane. However, the method of addition

TABLE 1

DOSE RESPONSE OF INHIBITION BY Z-D-Phe-L-Phe-Gly OF MEMBRANE FUSION OF *N*-METHYL DOPE LUV, OF SENDAI WITH *N*-METHYL DOPE LUV, OF SENDAI WITH ERYTHROCYTE GHOSTS, AND THE LINEBROADENING OF THE ISOTROPIC ^{31}P NMR RESONANCE AND THE INHIBITION OF FORMATION OF SMALL UNILAMELLAR VESICLES BY ULTRASONIC IRRADIATION

[ZPPG] μM	Vesicle–vesicle[a]	Sendai-vesicle[a]	Sendai-RBC[a]	^{31}P linewidth[b]	SUV formation[c]
0	100	100	100	1.9	100
25	101	—	—	2.2	73
50	95	92	76	7.5	18
100	28	75	72	13.8	0
200	0	50	49	—	—

[a] Initial rate of fusion, percentage of control.
[b] Linewidth of isotropic ^{31}P NMR resonance, determined as described in text, in ppm.
[c] Extent of formation of SUV of PC by sonication; percentage of control reduction in light scattering.

of the peptides necessitated by the fusion experiments (mimicking the original plaque inhibition assays) at the concentrations required here left some of the peptide in suspension, outside the membrane. Therefore the potency of the peptides at inhibiting fusion was effectively double that reported above.

The correlation between the appearance of isotropic ^{31}P NMR resonances in LUV preparations of *N*-methyl DOPE and the rate of fusion of these vesicles was described previously (Ellens *et al.*, 1989). Based on such correlations and other data, it was suggested that the isotropic ^{31}P NMR resonances may arise from structures that are themselves involved as intermediates in membrane fusion or may reflect structures that are involved in membrane fusion (Ellens *et al.*, 1989). We therefore undertook to measure the effect of Z-D-Phe-L-PheGly on these isotropic ^{31}P NMR resonances.

TABLE 2

INHIBITION OF FUSION BY HYDROPHOBIC PEPTIDES OF *N*-METHYL DOPE LUV, OF SENDAI WITH *N*-METHYL DOPE LUV, AND OF SENDAI WITH ERYTHROCYTE GHOSTS: CONCENTRATION OF ADDED PEPTIDE WHICH CAUSED 50% INHIBITION

Peptide	Vesicle–vesicle	Virus–vesicle	Virus–RBC ghost
Z-D-Phe-L-PheGly	100 μM	200 μM	200 μM
Z-L-Phe-L-Phe	200 μM	200 μM	N.D.
Z-L-PheGly	200 μM	>200 μM	N.D.
Z-L-Phe-L-Tyr	200 μM	>200 μM	N.D.
Z-L-Phe	>200 μM	>200 μM	N.D.
Z-D-Phe	>200 μM	>200 μM	N.D.
Z-Gly-L-Phe	DNI	DNI	DNI
Z-Gly-L-Phe-L-Phe	DNI	N.D.	N.D.

Note. ">" at 200 μM inhibition was observed but 50% inhibition was not achieved.
DNI, no inhibition detected up to 200 μM.
N.D., not determined.

Effects of Z-D-Phe-L-PheGly and Z-Gly-L-Phe on a putative intermediate in LUV fusion

Kelsey *et al.* (1990) observed that a known inhibitor of viral plaque formation, Z-D-Phe-L-PheGly, inhibited fusion of *N*-methyl DOPE LUV when added to a concentration of 100 μM. It has also been shown (Ellens *et al.*, 1989; Kelsey *et al.*, 1990) that increased rates of leakage and fusion of *N*-methyl DOPE LUV are correlated with the appearance of an isotropic resonance (I_s) in ^{31}P NMR spectra. In the present work, the effect of Z-D-Phe-L-PheGly on I_s was examined. ^{31}P NMR spectra of *N*-methyl DOPE LUV are shown in Fig. 4. In the absence of Z-D-Phe-L-PheGly, I_s first appeared at approximately 35–40° as a sharp peak in agreement with the results of Gagne *et al.* (1985) and Ellens *et al.* (1989). Peptide was then added to LUV with the same protocol as in all the fusion studies. As the concentration of added Z-D-Phe-L-PheGly was increased from 25 to 100 μM, I_s became progressively broader. The isotropic resonance may have increased in relative intensity, compared to the bilayer powder pattern at high peptide concentrations. The ^{31}P bilayer powder pattern was also altered, possibly due to a change in phospholipid headgroup dynamics in the presence of the peptide. It was inferred that a lipid bilayer was actually present at 100 μM Z-D-Phe-L-PheGly on the basis of the results of Kelsey *et al.* (1990), who showed that leakage of vesicle contents was relatively slow in LUV treated in this manner. Any loss of bilayer integrity would be expected to result in massive leakage of vesicle contents. The inhibition of leakage indicates that an intact bilayer was present.

Kelsey *et al.* (1990) have shown that a poor inhibitor of viral plaque formation, Z-Gly-L-Phe, is not capable of inhibiting fusion of *N*-methyl DOPE when present at concentrations of 200 μM or less. The effect of Z-Gly-L-Phe on the formation of I_s in LUV prepared exactly as in

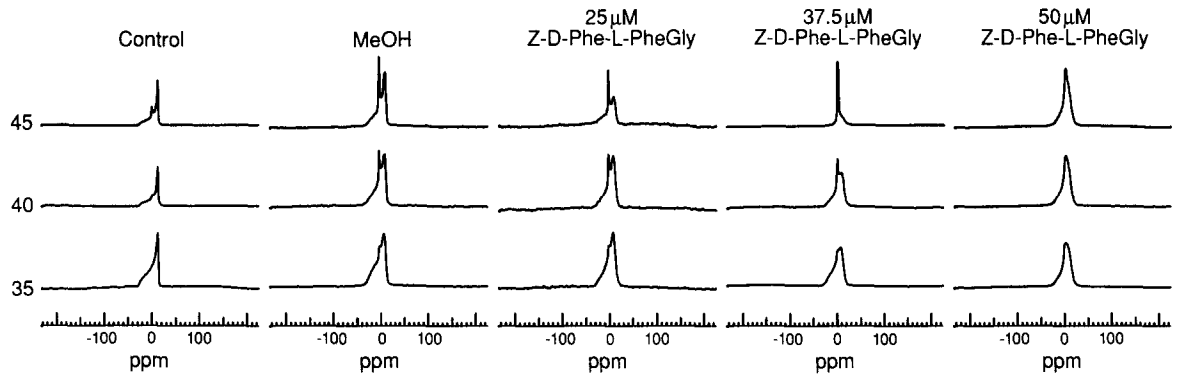

FIG. 4. Representative ^{31}P NMR spectra of *N*-methyl DOPE LUV are shown. Z-D-Phe-L-PheGly was added in methanol as described under Materials and Methods at the concentrations indicated above each group of spectra. For each spectrum 60,000 transients were collected at the temperature indicated on the left side of the figure.

Kelsey *et al.* (1990) was examined by ^{31}P NMR. The results are shown in Fig. 5. In contrast with the effects of Z-D-Phe-L-PheGly described above, Z-Gly-L-Phe did not broaden I_s at concentrations of 50 μM or less. A slight broadening of 1–2 ppm was observed at 100 μM Z-Gly-L-Phe. Thus, Z-Gly-L-Phe is much less effective than Z-D-Phe-L-PheGly in altering I_s.

In order to examine quantitatively I_s in isolation, the spectrum of *N*-methyl DOPE LUV at pH 9.5 (i.e., unfused LUV), 40°, was subtracted from the spectra of identical LUV at pH 4.5 that were treated with various concentrations of Z-D-Phe-L-PheGly. This procedure was used to attempt to remove the ^{31}P powder pattern for the bilayer from underneath the isotropic resonance. The resulting difference spectrum predominantly displayed the isotropic resonance of interest, although because of modest changes in the ^{31}P powder pattern of the bilayer the subtraction did not show zero intensity in all regions of the spectrum outside the isotropic resonance. Nevertheless, this subtraction did allow a relatively accurate quantitative determination of the linewidth of the isotropic resonance.

Normally, the isotropic ^{31}P NMR resonance in question has a linewidth of about 1.5 ppm. Z-D-Phe-L-PheGly caused a broadening of these resonances when added to LUV in methanol, mimicking the protocol for addition of peptide in the fusion experiments. In particular, 50 μM Z-D-Phe-L-PheGly (8:1 phospholipid:peptide mole ratio) caused a fourfold broadening and 100 μM Z-D-Phe-L-PheGly (4:1 phospholipid:peptide mole ratio) caused an eightfold broadening of the isotropic resonance. This was the same concentration of Z-D-Phe-L-PheGly at which inhibition of *N*-methyl DOPE LUV leakage and fusion has been observed (Kelsey *et al.*, 1990). Figure 6 shows the measured linewidth as a function of added Z-D-Phe-L-PheGly and Z-Gly-Phe. Table 1 shows a quantitative comparison between the inhibition of membrane fusion and the broadening of the ^{31}P NMR isotropic resonance for Z-D-Phe-L-PheGly.

Experiments above revealed that only a portion of the added peptide entered the membrane in these experiments. The potency of these peptides was therefore about double the above values.

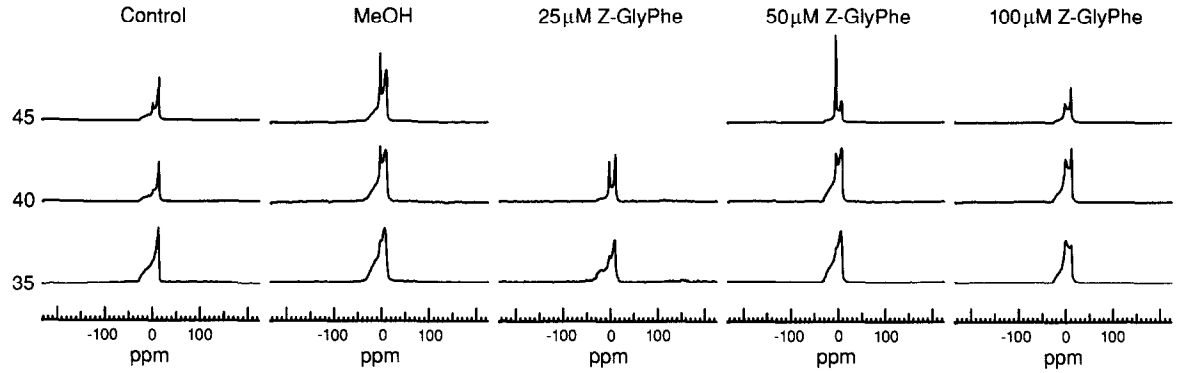

FIG. 5. Representative ^{31}P NMR spectra of *N*-methyl DOPE LUV are shown. Z-Gly-L-Phe was added in methanol as described under Materials and Methods at the concentrations indicated above each group of spectra. For each spectrum 60,000 transients were collected at the temperature indicated on the left side of the figure.

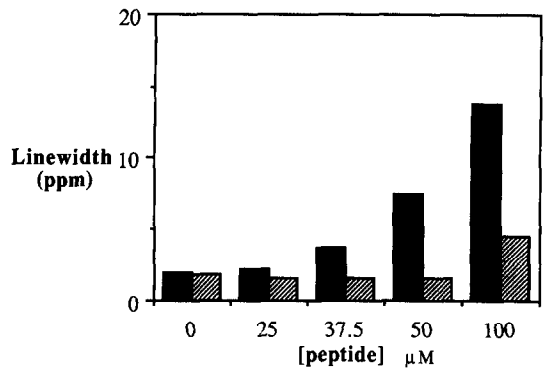

FIG. 6. The linewidth of the isotropic peak in the ^{31}P NMR spectra of *N*-methyl DOPE LUV in the presence of various concentrations of Z-D-Phe-L-PheGly and Z-Gly-Phe is shown. The values were obtained as follows: The ^{31}P NMR spectrum of *N*-methyl DOPE LUV at pH 9.5 was subtracted from the spectrum of *N*-methyl DOPE LUV at pH 4.5 (± the concentration of peptide shown below each bar). Only the isotropic resonance remained after this subtraction and the linewidth was measured directly. The black bars represent the data in the presence of Z-D-Phe-L-PheGly and the hatched bars represent the data from Z-Gly-Phe.

Effects of Z-D-Phe-L-PheGly and Z-Gly-L-Phe on formation of membrane structures with small radii of curvature

One possible interpretation of the ^{31}P NMR data invoked an alteration in the structure that gave rise to the isotropic ^{31}P NMR resonance. The isotropic ^{31}P NMR resonance in the *N*-methyl DOPE LUV was likely generated by structures in the membrane with small radii of curvature. The broadening of the isotropic ^{31}P NMR resonance was consistent with an increase in that radius of curvature. Therefore, we examined the effect of the peptide Z-D-Phe-L-PheGly on the formation of structures of small radii of curvature. Z-D-Phe-L-PheGly was chosen as the most potent inhibitor of fusion among the peptides that were examined in this study. Sonicated phospholipid vesicles were chosen for examination because these structures had a radius of curvature small enough to produce an isotropic resonance of similar linewidth to that observed in the *N*-methyl DOPE LUV (Yeagle *et al.*, 1975). The procedure described under Materials and Methods was employed. Egg phosphatidylcholine was chosen for sonication because PE and its derivatives did not sonicate at neutral pH due to poor headgroup hydration and because the physical properties of egg phosphatidylcholine-sonicated vesicles were very well characterized (Huang, 1969). The normal product from such sonication (of pure egg phosphatidylcholine) was formation of a suspension that was nearly clear from a suspension that was at first highly turbid (due to the large multilamellar liposomes). Figure 7 shows quantitatively the reduction in light scattering observed when small radii of curvature were achieved through sonication. As also shown in Fig. 7, in the presence of the Z-D-Phe-L-PheGly at a 4:1 mole ratio with the phospholipid (i.e., at a ratio that caused substantial inhibition of fusion and caused a very strong broadening of the isotropic ^{31}P NMR resonance), the light scattering of the suspension did not significantly change upon sonication for a period much longer than that needed to completely clarify a suspension of the same lipid without the peptide. Thus it was not possible to form small vesicles by sonication when Z-D-Phe-L-PheGly was incorporated into the membrane. The noninhibitory peptide, Z-Gly-Phe, had no significant effect (see Fig. 7). Table 1 shows a quantitative comparison among the inhibition of fusion, the broadening of the isotropic ^{31}P NMR resonance, and the inhibition of formation of small vesicles through sonication.

DISCUSSION

This study explores the mechanism by which certain hydrophobic peptides inhibit viral infection. The peptides Z-L-Phe-L-Phe, Z-L-Phe, Z-D-Phe, and Z-Gly-L-Phe-L-Phe were assayed for their ability to inhibit membrane fusion, an essential step in enveloped virus infection. In all fusion assays the results showed that the relative sequence of effectiveness at inhibiting membrane fusion was Z-L-Phe-L-Phe ≫ Z-L-Phe, Z-D-Phe > Z-Gly-L-Phe-L-Phe. This was the same order of effectiveness these same peptides showed at inhibition of enveloped virus infection of target cells (Richardson *et al.*, 1980). It was also shown previously that Z-D-Phe-L-PheGly was capable of inhibiting the fusion induced by myelin basic protein using a lipid mixing assay (Epand *et al.*, 1987). These results implied that the block in

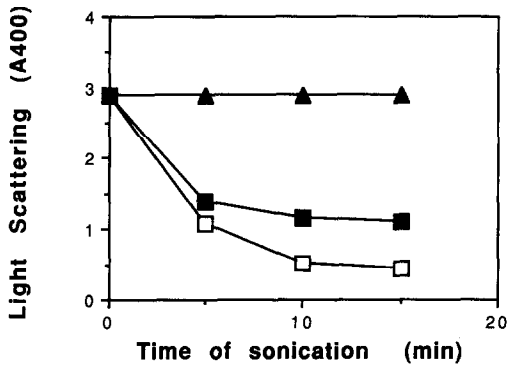

FIG. 7. Effect of sonication time on the light scattering at 400 nm from dispersions of egg phosphatidylcholine in the presence of hydrophobic peptides. (□) pure egg phosphatidylcholine; (▲) addition of Z-D-Phe-L-PheGly in a 1:4 mole ratio (peptide/phospholipid); (■) addition of Z-Gly-L-Phe in a 1:4 mole ratio (peptide/phospholipid).

viral infection by Z-D-Phe-L-PheGly was the result of inhibition of enveloped virus fusion with the target cell. It is interesting to speculate that the inhibition of fusion in the virus–*N*-methyl DOPE LUV fusion or even in the more complex virus–cell fusion may involve some step or steps in common with the mechanism of simple *N*-methyl DOPE LUV fusion.

Ideally, the same fusion assay would have been used to study fusion in all three systems examined in the current work. This was not possible due to the presence of two obstacles. First, since neither ANTS nor DPX could be easily encapsulated within the Sendai virion or within erythrocyte ghosts, the ANTS/DPX contents mixing assay could not be used to study virus–vesicle fusion or virus–erythrocyte fusion. Second, the incorporation of R_{18} into *N*-methyl DOPE LUV seriously disrupted the phase behavior of the LUV as assessed by ^{31}P NMR (Kelsey *et al.*, 1990). Therefore the R_{18} lipid mixing assay could not be used to measure vesicle–vesicle fusion.

Vesicle–vesicle fusion was the simplest of the fusion systems examined here. A brief summary of the current state of knowledge of the pathways by which these vesicles can fuse is therefore in order.

Two kinds of fusion pathways have been described in model membrane fusion studies. One utilized a mechanism involving calcium and phosphatidylserine (or cardiolipin) which may have involved a gel (dehydrated) phase formation by the calcium–phosphatidylserine complex (Papahadjopoulos *et al.*, 1977; Hoekstra, 1982; Silvius and Gagne 1984; Leventis *et al.*, 1986). Other investigators ruled out this pathway for virus fusion (Stegmann *et al.*, 1985).

The second pathway was suggested to involve "isotropic" structures, I_s, identified in ^{31}P NMR spectra of lipid dispersions, arising from lipidic particles (Hui *et al.*, 1981c), as intermediates in membrane fusion (Gagne *et al.*, 1985; Ellens *et al.*, 1986, 1989; Siegel *et al.*, 1989a). This pathway was relevant to *N*-methyl DOPE LUV fusion (Gagne *et al.*, 1985; Ellens *et al.*, 1984, 1986, 1989; Siegel, 1987; Siegel *et al.*, 1989a, b; Verkleij *et al.*, 1980; Verkleij, 1984). I_s and its relationship to membrane fusion have been characterized by a number of methods including fusion assays, ^{31}P NMR, differential scanning calorimetry, freeze fracture electron microscopy, and time resolved X-ray diffraction (Ellens *et al.*, 1989). In addition, interlamellar attachments were observed in cryotransmission electron micrographs under conditions where I_s appears in ^{31}P NMR spectra (Siegel *et al.*, 1989b), as well as in cross fractures in freeze fracture electron microscopy (Hui *et al.*, 1981a, b).

Ellens *et al.* (1989), and subsequently Kelsey *et al.* (1990), observed that the formation of I_s in *N*-methyl DOPE LUV was strongly correlated with an increase in vesicle leakage and fusion. A marked increase in fusion and leakage rates was observed at about 35°. This was also the temperature at which I_s appeared in ^{31}P NMR spectra of these LUV (Ellens *et al.*, 1989; Kelsey *et al.*, 1990). On the basis of these observations Ellens *et al.* (1989) suggested that the pathway of fusion in this lipid vesicle system included these nonlamellar structures as intermediates. Such nonlamellar structures may have a role in virus–*N*-methyl DOPE LUV fusion, too. Kelsey *et al.* (1990) found that Sendai virus fused with pure *N*-methyl DOPE LUV but not with pure egg PC LUV (in the absence of receptors for HN). *N*-methyl DOPE LUV were capable of forming I_s, whereas egg PC LUV were not.

Fusion between Sendai virus and a biological membrane is a more complex process than either simple *N*-methyl DOPE LUV fusion or fusion between Sendai virus and LUV. However, the present work, building upon that of Kelsey *et al.* (1990), showed that the relative order of effectiveness of peptides in inhibiting fusion in all the systems examined (vesicle–vesicle fusion, virus–vesicle fusion, and virus–erythrocyte membrane fusion) was Z-D-Phe-L-PheGly > Z-L-Phe-L-Phe > Z-L-Phe-L-Tyr $\gg$ Z-L-Phe $\cong$ Z-D-Phe > Z-Gly-L-Phe-L-Phe $\cong$ Z-Gly-L-Phe. This order of potency was the same as reported by Richardson *et al.* (1980) for inhibition of viral plaque formation by measles virus among others.

The structural basis of the process of inhibition by these peptides remained to be understood. One question that could be asked was whether the peptides have a direct effect on the site of fusion, or whether they perturb the lipid bilayer with which they interact in a general manner which in turn affected membrane fusion.

The first part of this question was whether there was a general effect of the inhibitory peptides on the lipid bilayer. Due to the amphipathic structure of these inhibitory peptides, they likely oriented in the surface of the lipid bilayer with the charged carboxyl terminal facing the aqueous phase and the hydrophobic remainder of the inhibitor inserted into the hydrophobic interior of the membrane. Therefore ^{31}P NMR, which probed the state of the membrane surface, provided a sensitive means to study the effects of these peptides (Yeagle, 1990). Up to a mole ratio of 4:1 (*N*-methyl DOPE:peptide, which significantly inhibited vesicle–vesicle fusion), there was little evidence for significant change in the observed ^{31}P NMR powder pattern arising from the phospholipid bilayer in these systems for the two peptides examined. Therefore a general perturbation of the surface of the membrane was unlikely, since the ^{31}P powder pattern provides some sensitivity to the bilayer surface properties.

The second part of this question was whether the peptide directly affected the fusion site. It appeared from the data in this report that the inhibitory peptide must have access to the fusion site. This was illustrated by the fact that preincubating virus with erythrocyte ghosts for 15 min on ice eliminated the ability of Z-D-Phe-L-PheGly to inhibit fusion. The preincubation on ice allowed the virus to bind to the ghosts but not to fuse with them. An increase in temperature to 37° at the end of the preincubation period initiated the fusion process. It was possible that allowing the binding event to occur prior to Z-D-Phe-L-PheGly addition brought the virion into such close proximity to the target membrane that Z-D-Phe-L-PheGly was excluded from the microdomain where fusion was initiated by steric hindrance of the viral envelope glycoproteins. In the experiments where the virus was not preincubated with the erythrocyte ghosts, Z-D-Phe-L-PheGly had access to all areas of the target membrane surface prior to the binding of the virus and, as a result, was effective in blocking viral fusion.

The next mechanistic question was what aspect of the fusion process did the inhibitory peptides affect. ^{31}P NMR provided a sensitivity to a putative intermediate in the fusion process of the *N*-methyl DOPE LUV as described in detail elsewhere (Ellens *et al.,* 1989). This simple membrane fusion system offered an opportunity for study of the mechanism of action of these inhibitory peptides not available in the more complex viral fusions studied here due to technical limitations. However, the similarity in relative potency of the inhibitory peptides among all the fusion systems tested in these studies encouraged exploration of the mechanism of inhibition in the simplest of these membrane fusion systems.

The data in this report revealed that the most potent of the inhibitory peptides, Z-D-Phe-L-PheGly, had a strong affect on the structure of the putative fusion intermediates in *N*-methyl DOPE LUV fusion, as indicated by the broadening of the isotropic ^{31}P NMR resonance. Therefore the target of the inhibitory peptide may be a putative fusion intermediate in the lipid of the involved membranes. In addition it was reported previously that Z-D-Phe-L-PheGly increased the transition temperature of phosphatidylethanolamine from the lamellar to the hexagonal II phase (Epand, 1986). We have focused here on the isotropic structures since they appear in the temperature range where fusion is also observed, whereas in this *N*-methyl DOPE system, the transition temperature to the hexagonal II phase occurs at a temperature much higher than the temperatures at which membrane fusion can be measured.

The most straightforward interpretation of the increase in the ^{31}P NMR linewidth was an increase in the effective radius of curvature of the structure giving rise to that resonance. This interpretation was supported by the observations of increasing ^{31}P linewidths with increasing size for small vesicles (Burnell *et al.,* 1980). The increase in linewidth was a direct result of the reduction in the effectiveness of the motional averaging of chemical shift anisotropy and dipolar contributions to that linewidth when the radius of curvature became larger and the individual phospholipid required more time to randomly sample an appreciable distribution of orientations with respect to the applied magnetic field by lateral diffusion on the surface of the vesicle (Yeagle, 1990). Therefore, we propose as the most simple, but not the only, interpretation that the inhibitory peptides increase the radius of curvature of a putative intermediate in the membrane fusion process.

Another possibility that could not be ruled out was an effect on the dynamics of the structures giving rise to the isotropic ^{31}P NMR resonance. An increased diffusion rate between the isotropic structure and the lipid bilayer could have led to such a broadening of the ^{31}P NMR resonance and the modest perturbation of the ^{31}P bilayer powder pattern. Or a decreased diffusion rate within the isotropic structure could also have led to an increase in the resonance width. How such alterations in dynamics would lead to a decrease in the observed rate of membrane fusion was not clear, however. Therefore, this discussion concentrated on the hypothesis involving radii of curvature since that hypothesis related directly to important elements of the pathway of membrane fusion (see below).

At this point, the data have already suggested an answer to the next question of the structural basis of the inhibition by the inhibitory peptides. The ^{31}P NMR data suggested an increase in the radius of curvature of the putative fusion intermediates in the *N*-methyl DOPE LUV. This concept was tested further. Phospholipids such as phosphatidylcholine are known to readily form small unilamellar vesicles when subjected to ultrasonication (*N*-methyl DOPE was not usable in this experiment because it would not sonicate into small vesicles at neutral pH). The vesicles that formed had a small enough radius of curvature to exhibit a ^{31}P NMR resonance that is nearly identical in linewidth to the isotropic ^{31}P NMR resonance in the *N*-methyl DOPE LUV (Hutton *et al.,* 1977). It should be pointed out that the structures in the LUV that gave rise to the isotropic ^{31}P NMR resonances need not be of the size of the sonicated vesicles or vesicular at all; only that they formed structures that covered about one quadrant of

a sphere and had a similar radius of curvature to the sonicated vesicles for that portion of a sphere.

In the sonication experiment it was observed that small vesicles would not readily form with the inhibitory peptide, Z-D-Phe-L-PheGly, in the membrane, under conditions adequate to form the small vesicles with pure phospholipid. Small vesicles formed by sonication of egg phosphatidylcholine were characterized previously. They exhibited a small radius of curvature, about 105 Å (Huang, 1969). This observation could be interpreted to support the hypothesis suggested above that the inhibitory peptide, Z-D-Phe-L-PheGly, when incorporated into a membrane, prevented the formation of structures of small radius of curvature.

The last question that could be addressed in this discussion was what role small radii of curvature might play in membrane fusion. It was argued elsewhere that small radii of curvature were required to facilitate the fusion of two membranes (Siegel 1986, 1987; Siegel *et al.,* 1989a). Virtually all structural intermediates that have been suggested for the membrane fusion event have involved some portion of the membrane in a structure with a small radius of curvature. Even models of the fusion promoted by fusion proteins of enveloped viruses have suggested a role for a similar intermediate (Daniels *et al.,* 1985b; Doms *et al.,* 1985; Skehel *et al.,* 1982). Therefore the ability of Z-D-Phe-L-PheGly to inhibit the formation of structures with small radii of curvature would be expected, on the basis of the present state of knowledge of membrane fusion, to inhibit the fusion event.

How these fusion inhibitory peptides may inhibit the formation of structures in the membrane with small radii of curvature is under further investigation in this laboratory.

SUMMARY

The following conclusions could be suggested based on the data in this report.

1. Hydrophobic antimeasles peptides inhibited vesicle–vesicle membrane fusion, Sendai virus–vesicle fusion, and Sendai virus–erythrocyte fusion with the same relative potency as inhibition of measles plaque formation.
2. The inhibition of measles plaque formation likely occurred through inhibition of virus fusion with the target cell membrane.
3. The mechanism of inhibition of membrane fusion by these antiviral peptides most likely involved inhibition of structures with small radii of curvature formed from membrane lipids at the site of contact (between two membranes destined to fuse) as an integral part of the membrane fusion event.

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