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Peptide Models for the Membrane Destabilizing Actions of Viral Fusion Proteins

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SYNOPSIS

The fusion of enveloped viruses to target membranes is promoted by certain viral fusion proteins. However, many other proteins and peptides stabilize bilayer membranes and inhibit membrane fusion. We have evaluated some characteristics of the interaction of peptides that are models of segments of measles and influenza fusion proteins with membranes. Our results indicate that these models of the fusogenic domains of viral fusion proteins promote conversion of model membrane bilayers to nonbilayer phases. This is opposite to the effects of peptides and proteins that inhibit viral fusion. A peptide model for the fusion segment of the HA protein of influenza increased membrane leakage as well as promoted the formation of nonbilayer phases upon acidification from pH 7–5. We analyze the gross conformational features of the peptides, and speculate on how these conformational features relate to the structures of the intact proteins and to their role in promoting membrane fusion.

INTRODUCTION

In order to be infectious, viruses must possess a mechanism allowing their nucleic acid to pass through the plasma membrane of the target cells. Two such mechanisms have been described for enveloped viruses, both requiring membrane fusion. One path is by direct fusion of the viral membrane with the plasma membrane of the target cell, while the other path requires prior endocytosis of the virus through coated pits, followed by fusion of the viral membrane with an endosomal membrane of the host cell. The latter mechanism requires a lowering of the pH, while the former can take place at physiological pH. In the present study, we have chosen measles virus as an example of a virus that fuses to the plasma membrane and influenza virus as one requiring endosomal acidification.

There has been much work attempting to elucidate the mechanism of viral fusion to target membranes. Through experiments of proteolytic cleavage, mutagenesis, and reconstitution of viral proteins into virosomes, it is well established that the F protein of measles and the HA protein of influenza are required for membrane fusion. What is less well understood is the segment(s) of these proteins required for membrane fusion and the mechanism of this protein-mediated fusion event. For both measles and influenza virus, hydrolysis of a single peptide bond in the fusion protein is required for the virus to become fusogenic. This has implicated the segment around the cleaved bond as being important for fusion activity. In the case of measles virus, the newly generated amino terminus that resides on the F1 fragment of the fusion protein has a sequence of about 20 hydrophobic amino acids. This hydrophobic segment may partition into membranes as a result of the proteolytic cleavage. This possibility is supported by the finding that the F1 subunit of the Sendai fusion protein, a protein homologous to the F1 subunit of measles,[1] is specifically labeled by a hydrophobic photoaffinity reagent present in the target membrane.[2] In the case of influenza virus, the

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amino terminal segment of the HA2 fragment of the membrane fusion protein has a sequence that could form an amphipathic helix. This segment is also labeled by a hydrophobic photoaffinity reagent.[3] The labeling pattern is suggestive of the formation of an amphipathic helix in the membrane. This is supported by several studies on model peptides with sequences corresponding to this region of the protein.[4-6]

HYDROPHOBIC PEPTIDES

Several years ago, it was suggested that hydrophobic peptides could play an important role in membrane fusion.[7] It has also been suggested that nonbilayer phases are important as intermediates in membrane fusion.[8] Whether or not such intermediates are required for fusion, it has been demonstrated that substances having an increase in their hydrophobic volume compared to their solvated polar groups will both lower the bilayer to hexagonal phase transition temperature in model membranes as well as promote membrane fusion. In the case of peptides, a particular problem arises because polar amide groups linking the amino acid residues are distributed through the entire molecule, preventing the formation of a purely hydrophobic domain. A number of these amide groups can become desolvated without a great cost in energy only if they form intramolecular hydrogen bonds. A common structural motif of membrane-inserted peptides and proteins is the α-helix. However, transmembrane α-helices generally stabilize membrane bilayers[9] and would be expected to inhibit fusion. Also surface-seeking amphipathic helices such as melittin[10] or the apolipoprotein A-I (R. Epand, Anantharamaiah, and Segrest, unpublished results) are also bilayer stabilizers unless they can induce charge neutralization of anionic phospholipids, resulting in a decrease in headgroup volume.[10] Two other ways in which "cylindrical-shaped" structures such as α- or β-helices can promote nonbilayer phases are by inserting into the membrane at an angle to the bilayer normal or by having a structure that places large amino acid side chains close to the center of the bilayer. From analysis of the spatial segregation of hydrophobic and hydrophilic residues, it has been suggested that a number of viral fusion proteins insert into membranes as an α-helix oriented at an angle of about 50°–60° from the bilayer normal, resulting in destabilization of the bilayer.[11] The "cone-shaped" structure is achieved with gramicidin by the presence of a bulky Trp residue near the center of the bilayer. This Trp is essential for inducing the hexagonal phase[12] and is believed to be important for stabilizing the self-association of this peptide in a $\beta^{6,3}$ conformation with an overall cone shape.[13]

We studied the properties of a 19 amino acid hydrophobic peptide with the sequence FAGVVLAGAALGVAAAAQI. This sequence corresponds to that of the amino terminus of the F1 subunit of the measles fusion protein, except that a threonine residue at position 15 was converted to alanine to make the synthesis simpler. The resulting peptide is sparsely soluble in a variety of both polar and nonpolar solvents. We were able to prepare films of the peptide mixed with dielaidoylphosphatidylethanolamine (DEPE) from solution in trifluoroacetic acid (TFA). Precautions were taken to immediately evaporate the solvent while maintaining the solution cooled in an ice bath. When a film was made from pure DEPE and reconstituted in pH 7.4 piperazine-*N,N'*-bis[2-ethanesulfonic acid] (PIPES) buffer (20 μM), 0.15 M NaCl, 1 mM EDTA, and 0.02 mg/mL NaN_3, its phase transition behavior was found to be indistinguishable from films made in the usual way from chloroform/methanol solutions.[14] In the presence of a high mol fraction of 0.23 peptide, the bilayer to hexagonal phase transition temperature was lowered 1.5°C. This is a relatively modest amount, but it is in direction opposition to that observed for many other peptides and proteins.[10,15] Because of the weak tendency of the peptide to be solvated, it is probably not uniformly distributed in the membrane and therefore may not have as great an affect on the phase behavior of the lipid. The limited solubility of the peptide also prevents testing the effect of solvent on the reconstitution of the peptide into lipid. In the case of gramicidin, it was found that the effects of the peptide on lipid properties were highly dependent on the solvent and thus the conformation of the peptide at the time it was incorporated into membranes.[16] When the hydrophobic measles peptide was incorporated into egg phosphatidylcholine (PC) from TFA, followed by hydration in water and sonication, CD studies showed that the peptide adopted a conformation other than the α-helix (Figure 1). A 23-residue hydrophobic peptide with a sequence corresponding to the N-terminus of the gp 41 protein of HIV has been studied.[17] This peptide can form α-helical structures when bound to anionic lipids at low concentration, but folds into a β conformation at lower lipid : peptide ratios or in the presence of zwitterionic lipid. Thus the conformational properties of hydrophobic peptides may depend on the conditions. Further studies are required to understand how these conformational features correlate with rates of membrane fusion.

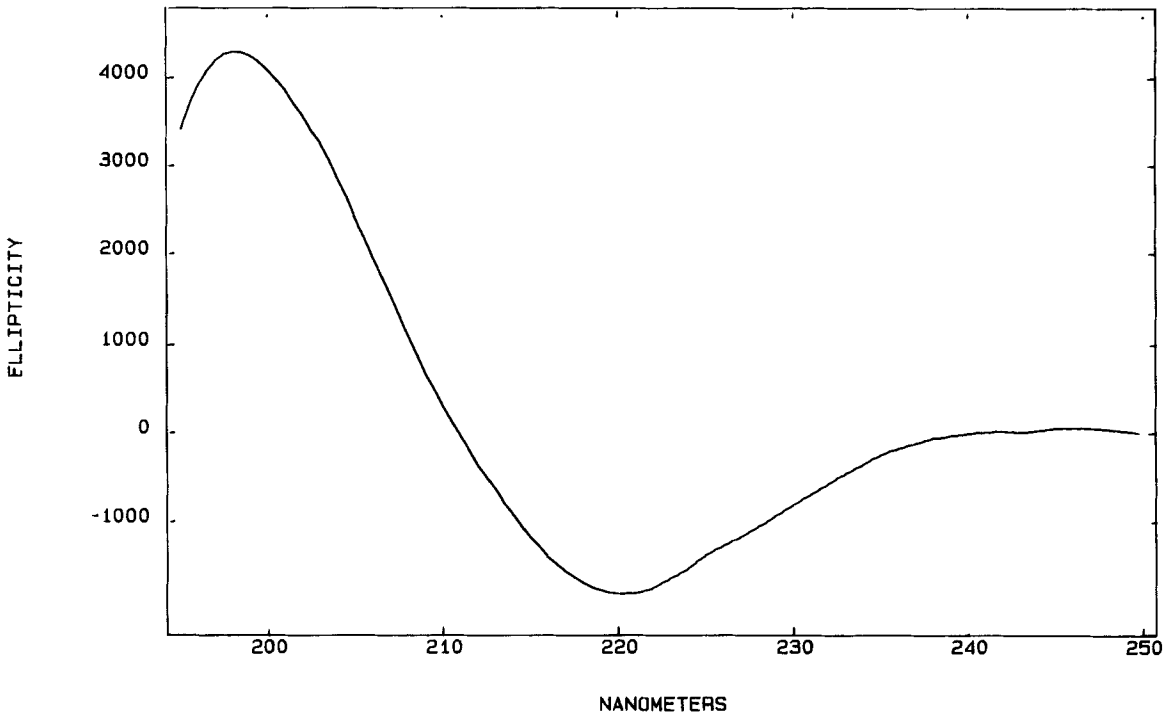

Figure 1. Circular dichroism of measles fusion peptide in the presence of egg-phosphatidylcholine (PC) at a lipid to peptide ratio of 6. The peptide was incorporated into PC from TFA, then this solvent was evaporated under argon while the solution was maintained in an ice bath. After lyophilization, the mixture was hydrated with 10^{-2} M Na_2HPO_4, pH7.4, and sonicated. C.D. spectra was measured in an Aviv Model 61DS solid-state instrument (Aviv Associates, Lakewood, NJ) using a 1 mm cell maintained at 25°C. Scans were corrected for the buffer baseline and multiplied by a constant to convert observed ellipticity to mean residue ellipticity, which is reported here. Secondary structure estimated by curve-fitting showed 73% beta structure.

The effect of this 19 amino acid hydrophobic peptide on the properties of N-methyl-dioleoyl-phosphatidylethanolamine has also been studied.[18] This phospholipid can form stable cubic phases as well as the hexagonal phase.[19] At temperatures below the bilayer to cubic transition, the phospholipid undergoes rearrangements to a structure that gives rise to narrow ^{31}P-nmr resonance lines, indicative of motional averaging of the chemical shift anisotropy.[20] The presence of these isotropic resonances has been associated with increased rates of membrane fusion.[21] We have found that this hydrophobic peptide increases the amount of isotropic signal in a preparation of N-methyl-dioleoylphosphatidylethanolamine, even when the peptide is present at less than a 0.01 peptide/lipid molar ratio.[18] The presence of the peptide in vesicles of N-methyl-dioleoylphosphatidylethanolamine also increases the rate of fusion of Sendai virus as measured by dilution of the octadecyl-rhodamine probe from the virus.[18]

AMPHIPATHIC PEPTIDE

As a model for the fusogenic sequence of the HA protein of influenza hemagglutinin, we used the peptide GLFGAIAGFIENGWEGMIDG-amide, which corresponds to the sequence of the amino terminus of the HA-2 fragment for the X31F/68 strain of influenza. This peptide, unlike the model for the measles fusogenic sequence, has a number of hydrophilic residues. Among these hydrophilic residues are three containing carboxyl groups in their side chains. Upon acidification, protonation of these carboxyl groups would make the peptide less hydrophilic. This may result in pH-dependent effects of the peptide that mimic those of the intact virus. It has already been shown that the peptide exhibits an acid-induced blue shift of tryptophan fluorescence and increased secondary structure at low pH, and it induces a pH-dependent increased rate of membrane fusion as measured by lipid mixing.[4]

We wished to determine how this peptide affected lipid polymorphism. Addition of this peptide to dipalmitoleoylphosphatidylethanolamine at pH 7.4 caused an increase in the bilayer to hexagonal phase transition temperature (T_H). However, at pH 5.0, the opposite effect on T_H was observed, with T_H decreasing upon the addition of peptide (Figure 2). The phospholipid used was chosen because of its low T_H so as to avoid thermal denaturation of a folded helical form of the peptide. The peptide was mixed with the lipid in chloroform/methanol (2 : 1 *v, v*), a solvent in which the peptide was not completely soluble. As a consequence, nonuniform mixing of peptide and lipid during sample preparation may contribute to reducing the effect of the peptide on lipid polymorphism. What is clear, however, is that the peptide has marked and opposite effects on lipid polymorphism at neutral and at acidic pH. At neutral pH, we believe it acts as a surface-seeking helix to stabilize the bilayer, while at acidic pH the peptide enters the bilayer more deeply and destabilizes the bilayer arrangement with respect to the hexagonal phase.

We determined the extent to which changes in membrane properties induced by the influenza virus or by this model peptide are dependent on the nature of the lipid composition of the membrane. Influenza virus induces almost no leakage of aqueous contents

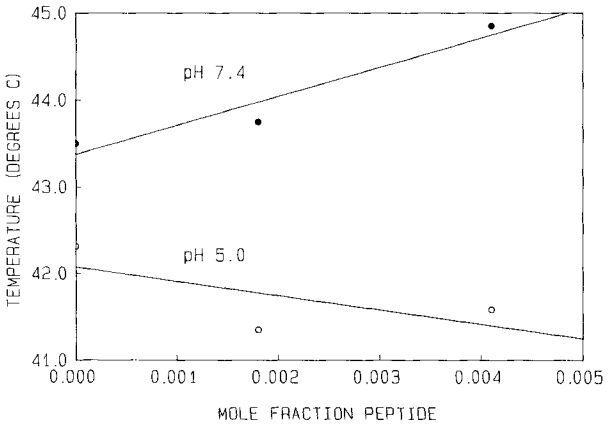

Figure 2. Dependence of T_H of dipalmitoleoylphosphatidylethanolamine on the mol fraction of influenza fusion peptide at pH 5.0 (○) and at pH 7.4 (●). Buffers were 20 m*M* PIPES, 1 m*M* EDTA, 0.15*M* NaCl, 0.002% NaN_3, pH 7.4, or 10 m*M* citric acid, 0.15*M* NaCl, pH 5.0. Heating scans were made in a MC-2 high-sensitivity calorimeter (Microcal Co., Amherst, MA), at a scan rate of 45 K/h, as described previously.[14] Lines are drawn only to indicate the opposite trends at the two pH values. The data are insufficient to determine the functional dependence of T_H on the mol fraction of peptide added.

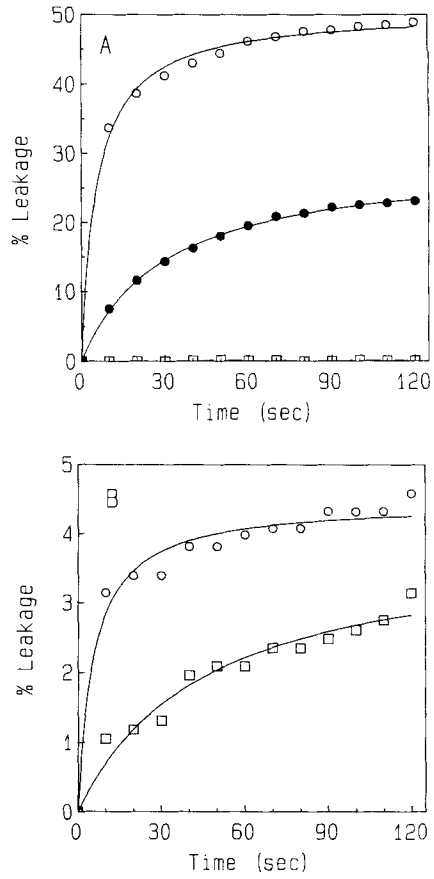

Figure 3. Calcein leakage from PC and PE liposomes at pH 7.4 and 5.0 induced by the influenza fusion peptide and influenza virus. (A) HA peptide-induced leakage of calcein from egg PC/5 mol % ganglioside G_{D1a} LUVs at pH 7.4 (●) and pH 5.0 (○), and HA peptide-induced leakage from PE (made by transphosphatidylation from egg PC)/5 mol % G_{D1a} LUV's at pH 5.0 (□). (B) Influenza virus-induced leakage of calcein at pH 5.0 from egg PC/5 mol % G_{D1a} LUVs (○) and PE/5 mol % G_{D1a} LUVs (□).

from liposomes at neutral pH, yet causes a small but detectable leakage at acidic pH (Figure 3). The leakage is greater from PC vesicles than from vesicles of phosphatidylethanolamine (PE), made by transphosphatidylation of PC. There are some qualitative similarities between the pH and lipid dependence of the leakage caused by the peptide and the virus. In both cases, PC vesicles show more leakage than PE and leakage is promoted by low pH. Beyond this, however, there are marked differences between the peptide and the intact virus. The difference in leakage between PC and PE is much greater for the peptide than the virus, the relative increase in leakage rate with acid is greater for the virus than for the peptide. The lipid dependence is

Table I Insertion of Model Fusogenic Peptide from the HA2 Fragment of Influenza

	Condition					
	pH 7.4			pH 5.0		
Criterion of Insertion	Buffer	PC	PE	Buffer	PC	PE
$[\theta]_{222}$[a] (deg cm^2/dmol)	−2720	−12180	−7200	−6520	−23100	−20600
λ_{em}[b] (nm)	355	350	348	355	330	333

[a] Mean residue ellipticity of peptide. Larger negative values correspond to increased secondary structure and imply greater peptide insertion into the membrane, but a precise quantitative comparison may not be valid because of possible light scattering artifacts.
[b] Wavelength of maximum Trp emission. Lower wavelength implies greater membrane penetration.

opposite to what one would predict based on hexagonal phase propensity, since PE is much more prone to form the hexagonal phase than PC and formation of the hexagonal phase will cause leakage of internal contents. Opposing the nonbilayer-forming tendency of PE is the rigid interlipid, hydrogen-bonded, dehydrated head-group region of PE. This may inhibit the entry of the fusion peptide or the fusogenic sequence of the viral hemaglutinin protein into the membrane. In the case of the model peptide, this may contribute to, but is not likely to be the sole cause of, the lower susceptibility of PE to peptide-induced leakage. The peptide can insert into PE, although not quite as deeply as into PC (Table I).

USES AND LIMITATIONS OF PEPTIDE MODELS FOR VIRAL FUSION

Many features of the intact virus are not present in the simple system of a peptide segment of a viral fusion protein. Unlike the intact virus, the fusogenic peptides do not bind to specific "receptor" sites on the membrane. Furthermore, insertion of the fusogenic peptide into the membrane does not cross-link two membranes. In comparison, insertion of a segment of a viral fusion protein into a target membrane cross-links the target and viral membranes, since another segment of the fusion protein is already imbedded in the viral membrane. The physical properties of the viral membrane that fuses with the target membrane are likely to be different from that of a model system of peptide-induced fusion of two identical liposomes. In addition, oligomerization of viral fusion proteins is believed to play an essential role in their mechanism of action. As a result, several fusogenic segments can act in a coordinated fashion. Such cooperativity is, of course, unlikely in a simple peptide model. Finally, the conformation adapted by the isolated peptide segment may not be the same as the conformation of that segment when present in the intact protein. Additional caveats are required for the measles peptide since a Thr residue has been substituted by an Ala and the COOH end of the peptide has a negative charge. These changes in hydrophobicity of specific groups may alter the orientation of the peptide with respect to the bilayer normal.[11] Thus, it is unlikely that the model peptides will be capable of reproducing the specific and efficient fusion of the intact virus. There clearly are also regions outside of the fusogenic domains, whose mutation can destroy the fusogenic capacity of the intact virus (e.g., Ref. 22).

Despite all of these caveats, model fusogenic peptides can still provide information about some of the early events that trigger viral fusion. The molecular nature of these steps may be more difficult to unravel using the more complex system of an intact virus. In the examples presented in the present paper, a common element is that conditions and peptides that destabilize membrane bilayers can accelerate the rate of membrane fusion. This may also explain the opposite phenomenon, i.e., that a number of agents that raise T_H are inhibitors of membrane fusion and in some cases these substances exhibit antiviral activity.[15] Further work is required to determine how sensitive the membrane effects of model viral fusion peptides are to changes in the sequence or amino acid composition of the peptide.

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