A molecular model for membrane fusion based on solution studies of an amphiphilic peptide from HIV gp41

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Abstract

The mechanism of protein-mediated membrane fusion and lysis has been investigated by solution-state studies of the effects of peptides on liposomes. A peptide (SI) corresponding to a highly amphiphilic C-terminal segment from the envelope protein (gp41) of the human immunodeficiency virus (HIV) was synthesized and tested for its ability to cause lipid membranes to fuse together (fusion) or to break open (lysis). These effects were compared to those produced by the lytic and fusogenic peptide from bee venom, melittin. Other properties studied included the changes in visible absorbance and mean particle size, and the secondary structure of peptides as judged by CD spectroscopy. Taken together, the observations suggest that protein-mediated membrane fusion is dependent not only on hydrophobic and electrostatic forces but also on the spatial arrangement of the amino acid residues to form an amphiphilic structure that promotes the mixing of the lipids between membranes. A speculative molecular model is proposed for membrane fusion by α -helical peptides, and its relationship to the forces involved in protein-membrane interactions is discussed.

Keywords: human immunodeficiency virus; lipid binding; membrane fusion; protein-membrane interactions

The envelope glycoprotein (gp160) of HIV directs the infection of cells that express the CD4 receptor. The processes that gp160 mediates include (1) recognition and binding to the CD4 receptor (Kowalski et al., 1987; Lasky et al., 1987); (2) attachment and fusion of the viral membrane to the target cell with the subsequent release of the viral genome into the cell (McCune et al., 1988); and (3) formation of syncytia by cell-cell fusion (Lifson et al., 1986). Interruption of any or all of these functions would stop the pathogenicity of the virus and thus, hinder the

spread of AIDS. The present state of studies aimed at characterizing the structure and function of gp160 with the goal of designing drugs or vaccines has been summarized by Bolognesi (1990).

The gp160 glycoprotein originates as a monomeric precursor from spliced mRNA coded for by the retroviral *env* gene. Synthesis of the gp160 monomer (Allan et al., 1985; Veronese et al., 1985) takes place on membrane-bound polyribosomes, and the precursor remains anchored to the membrane as the first glycosylation steps occur. Further glycosylation takes place in the golgi region, followed by transport of a fraction of the molecules to the cell surface membrane to await virion assembly (Willey et al., 1988). Between these two events, the protein forms oligomers (Earl et al., 1990) and undergoes cleavage (McClure et al., 1988; Willey et al., 1988) to produce the surface glycoprotein gp120 and the transmembrane glycoprotein gp41, both of which are oligomeric (Pinter et al., 1989; Thomas et al., 1991).

Although cellular recognition appears to be a primary

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Abbreviations: HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; PBS, phosphate buffered saline; EM, electron microscopy; TFA, trifluoroacetic acid; DPPC, dipalmitoylphosphatidylcholine; DOPS, dioleoylphosphatidylserine; NBD-PE, 7-nitro-benzoxadiazole phosphatidylethanolamine; RH-PE, lissamine rhodamine phosphatidylethanolamine; Chol, cholesterol; PAM, OCH₂-phenylacetamidomethyl; BHA, 4-methylbenzhydrylamine; HPLC, high performance liquid chromatography; UPA, ultrafine particle analyzer; polylysine, poly-L-lysine.

function of the gp120 portion of gp160 (Kowalski et al., 1987; Lasky et al., 1987), the gp41 portion appears to control viral membrane interactions such as fusion, virion assembly, and anchoring of the envelope proteins (Kowalski et al., 1987; McCune et al., 1988). Analysis of the primary amino acid sequence of gp41 reveals several segments that are hydrophobic or highly amphiphilic (Muesing et al., 1985; Modrow et al., 1987; Eisenberg & Wesson, 1990). Such segments have been proposed to play major roles in mediating membrane interactions (Kaiser & Kézdy, 1984; Lucy, 1984; Hoekstra, 1990). For example, hydrophobic segments near the N-terminus of many viral surface glycoproteins, such as the hemagglutinin (HA2) coat protein of the influenza virus, have been implicated in the fusion process between viruses and cells (Doms et al., 1985; Gething et al., 1986). HIV gp41 also has a hydrophobic N-terminal segment that may potentially be the fusion site (Kielian & Jungerwirth, 1990; White, 1990). In addition to the possible N-terminal fusion segment, gp41 has an unusually long C-terminal sequence common only to a few other lentiviruses. This sequence contains a segment that has been shown to potentially be among the most highly amphiphilic α -helices known (Eisenberg & Wesson, 1990). The function of this portion of gp41 is unknown, but amino acid replacements or deletions in this region affect T-cell killing (Fisher et al., 1986) and HIV infectivity and replication (Kowalski et al., 1987; Lee et al., 1989). This evidence suggests that the amphiphilic segment of HIV gp41 is important to its life cycle.

Viral assembly and infectivity depend to a large extent on the interactions of the viral envelope proteins with their host membranes. The mechanisms of such processes are not yet well understood but have been postulated to be mediated by either amphiphilic or hydrophobic segments (Kaiser & Kézdy, 1984; Lucy, 1984; Hoekstra, 1990). Numerous studies conducted on amphiphilic synthetic peptides and naturally occurring small proteins show that amphiphilic structures promote membrane activity (Anantharamaiah et al., 1985; Hong & Vacquier, 1986; Subbarao et al., 1987). In this study, a peptide corresponding to the C-terminal segment of HIV gp41 has been synthesized, and its *in vitro* membrane association properties have been examined.

Results

Peptides studied

Four peptides were selected for study (Table 1) for their possible lytic or fusogenic effects on liposomes. Peptide SI represents the C-terminal sequence of HIV gp41 identified by its unusually high amphiphilicity (Eisenberg & Wesson, 1990). Figure 1 illustrates the potential for SI to form an amphiphilic α -helix using the helical wheel diagrams of Schiffer and Edmundson (1967). Also shown is a scrambled sequence (SIscr) of SI in which the potential

Fig. 1. Helical wheel (Schiffer & Edmundson, 1967) diagram of the highly amphiphilic segment identified by Eisenberg and Wesson (1990) from the C-terminus of HIV gp41 (SI) and of the scrambled sequence (SIscr). The diagrams were drawn with the program WHEEL kindly supplied by J. Segrest (Segrest et al., 1990). **A:** Peptide SI, showing a strong segregation of the charged from the hydrophobic side chains. **B:** Peptide SIscr, with the amphiphilicity destroyed.

amphiphilicity has been destroyed. The other two peptides studied were melittin, the major component from bee venom, chosen as a model amphiphilic peptide that is water soluble and associates with membranes, and polylysine, chosen as a nonlytic positively charged control

Table 1. Sequences of peptides studied with their hydrophobic moments (μ_H) and their average hydrophobicities ($\langle H \rangle$)

Peptide	Residues	Sequence	μ_H^a	$\langle H \rangle^b$
SI ^c	827-850	DRVIEVVQGAYRAIRHIPPRIQGG	1.08	-0.74
SIscr	1-24	DVRIRRVGAYRAQIHIPRIEVQGG	0.36	-0.59
Polylysine	—	(KKK...) _n ^d	—	-1.50
Melittin ^c	1-26	GIGAVLKVLTTGLPALISWIKRKRQQ	0.57	0.25

^a μ_H is the maximum value calculated from Equation 1 using an 11-residue moving window.

^b $\langle H \rangle$ is calculated using values from the Eisenberg consensus scale (Eisenberg et al., 1982b).

^c Muesing et al. (1985).

^d $n \approx 22$.

^e Habermann and Jentsch (1967).

peptide. The largest hydrophobic moment of each segment is listed in Table 1. SI has the potential for being even more amphiphilic than melittin, which is itself generally considered to be an extremely amphiphilic peptide (Eisenberg et al., 1982a).

Fusion and lysis of liposomes by peptides

Fusion was monitored by changes in the resonance energy transfer between fluorescent probes in liposomal membranes as peptides were added. Lysis was monitored by the increase in fluorescence of fluorexon dye when pep-

tides were added to solutions of liposomes. The effects of the four peptides on the fusion and lysis of liposomes at different lipid/peptide ratios are displayed in Figures 2 and 3. SI was very active in disrupting the DPPC:DOPS liposomes, but had little effect on the uncharged DPPC:Chol vesicles at 25 °C. In contrast, the scrambled SIscr peptide did not fuse or lyse either the DPPC:DOPS or the DPPC:Chol liposomes. As expected, melittin, the bee venom peptide widely known (Dempsey, 1990) for its lytic and fusogenic properties, was very efficient in disrupting both the negatively charged and the neutral lipid vesicles. Positively charged polylysine was not observed

Fig. 2. Fusion of liposomes by peptides as measured by resonance energy transfer (Struck et al., 1981). The % fusion observed at different lipid-to-peptide ratios (Ri) is shown for DPPC:DOPS (A) and DPPC:Chol (B) liposomes in PBS. Samples were prepared by mixing different concentrations of peptides with 0.1 mg/mL liposomes and then incubating for 15 min at 25 °C. Fluorescence changes were monitored at 535 nm with an excitation wavelength of 470 nm. —, SI; ---, melittin; ·····, SIscr; - · - ·, polylysine.

Fig. 3. Lysis of liposomes by peptides as monitored by the release of encapsulated fluorexon dye. As the dye leaks out of the liposomes, it becomes diluted and fluoresces. The % lysis observed at different lipid-to-peptide ratios (Ri) is shown for DPPC:DOPS (A) and DPPC:Chol (B) liposomes in PBS. Samples were prepared by mixing different concentrations of peptides with 0.1 mg/mL liposomes and incubating for 15 min at 25 °C. Fluorescence changes were monitored at 545 nm with excitation at 490 nm. —, SI; ---, melittin; ·····, SIscr; - · - ·, polylysine.

to lyse or fuse either type of liposome. Comparison with Table 1 suggests that amphiphilic peptides promote the fusion and lysis of lipid bilayers.

Visible absorbance and particle size studies

All peptides were discovered to have some activity with liposomes as judged by the increase in absorbance at 400 nm and changes in the size of the liposomes. Figure 4 shows the changes in the absorbance of liposome samples at different concentrations of peptides, and Figure 5 displays the mean particle size associated with those absorbance changes. In the presence of the neutral DPPC:Chol liposomes, only melittin caused a significant increase in absorbance and mean particle size. However, in the presence of the negatively charged liposomes, absorbance and particle size changes were observed to occur with all peptides. Polylysine caused the greatest increase in absorbance, whereas SI and SIscr caused moderate increases. Consistent with the absorbance results, the polylysine liposome samples reached a mean particle size greater than 1 μm at higher lipid-to-peptide ratios (>500) than any other peptide tested. SI, in the presence of the negative liposomes, also attained a mean particle size greater than 1 μm , but at lipid/peptide ratios near 100.

Fig. 4. Detection of aggregation and fusion, by observing absorbance changes at 400 nm of liposomes mixed with peptides. The absorbance at different lipid-to-peptide ratios (Ri) is shown for DPPC:DOPS (A) and DPPC:Chol (B) liposomes in PBS. Samples were prepared by adding different concentrations of peptides with 1 mg/mL liposomes and incubating for 15 min at 25 °C. Shown are the absorbance changes in the peptide/vesicle mixtures corrected against the liposome background absorbance without peptide. —, SI; ---, melittin; — — —, SIscr; ····, polylysine.

Fig. 5. Mean particle size in peptide-liposome mixtures as judged by laser light scattering. The change in the mean particle size at different lipid-to-peptide ratios (Ri) is displayed for DPPC:DOPS (A) and DPPC:Chol (B) liposomes in PBS. Samples were prepared by mixing different concentrations of peptides with 1 mg/mL liposomes followed by incubation for 15 min at 25 °C. The size distribution was measured on a Microtrac UPA. —, SI; ---, melittin; — — —, SIscr; ····, polylysine.

SIscr caused significant increases in the size of the negatively charged liposomes, but the mean particle size did not reach 1 μm at the conditions tested. Melittin increased the absorbance at high lipid/peptide ratios (>100) but, interestingly, had a decrease in absorbance below lipid/peptide ratios of 100. Increasing absorbance suggests that the liposomes are aggregating or fusing, whereas a decreasing absorbance is often interpreted as a reduction in size of the liposomes, perhaps by micelle formation. In agreement with the absorbance results the changes in the mean particle size (Fig. 5) of the liposomes also increased or decreased in parallel with the absorbance changes. Because of the pronounced effects of polylysine with the negatively charged liposomes, these results indicate that electrostatic forces strongly influence the interactions of peptides with lipid bilayers.

Circular dichroism studies

The SI peptide was identified (Eisenberg & Wesson, 1990) by its unusually large hydrophobic moment; this large moment suggests that it forms an α -helix at an interface between an aqueous and an apolar phase. This suggestion was tested by examining its CD spectrum both in the absence and presence of lipid (Fig. 6). In the absence of lipid, the spectrum shows that in aqueous buffer, SI is slightly

Fig. 6. CD spectra of peptides SI and SIscr with and without liposomes in PBS, pH 7.4 (0.05 mg/mL:1.0 mg/mL peptide:lipid). **A:** Peptide SI in buffer (—) and in the presence of DPPC:DOPS liposomes (---), showing the formation of α -helical structure by the minima at 208 and 222 nm. **B:** Peptide SIscr in buffer (—) and in the presence of the liposomes (---) in which the formation of helical structure does not occur.

α -helical. Upon addition of the DPPC:DOPS liposomes, SI becomes more α -helical. Using the method of Greenfield and Fasman (1969), it is estimated that the α -helicity changes from 11% in buffer to 27% upon the addition of liposomes. In good agreement, the method of Chen and Yang (1971) gives a change from 6% to 23%. In contrast, the SIscr peptide remained in a random coil conformation in the absence and presence of liposomes. The CD

spectrum of melittin showed that the bee venom peptide was also slightly α -helical in buffer. In the presence of the DPPC:DOPS liposomes, the α -helicity was found to increase significantly. However, the increase could not be quantitated because of distortions in the spectrum caused by light scattering from particles. A rough estimate using the method of Chen and Yang (1971) indicates that the peptide is at least 40% α -helical and is probably greater than 60% at these conditions. Beschiaschvili and Seelig (1990) have recently argued that melittin bound to membranes may have 20–30% greater α -helicity than calculated by the method of Chen and Yang (1971), which suggests that melittin assumes a largely α -helical conformation. In summary, melittin has a large increase in its helicity upon mixing with negatively charged liposomes, whereas SI adopts a partial helical conformation in the presence of negatively charged liposomes.

Structural studies

Melittin and SI drastically altered the initial morphology of the liposomes (Fig. 7). Upon mixing with the negatively charged liposomes, melittin clustered the liposomes and reorganized them to form disklike structures. In some cases, the disks were stacked. These “rouleaux” have been described by others (Forte & Nordhausen, 1986; Steyrer & Kostner, 1988). Consistent with the absorbance and particle size results, the disks of lipid were smaller than the initial liposomes. The SI peptide, when mixed with the DPPC:DOPS liposomes, promoted the formation of larger vesicles, the majority of which were 200–500 nm in diameter. Apparently, amphiphilic peptides destroy small liposomes but stabilize a variety of different lipid superstructures.

Amphiphilic peptides promote membrane activity

The combined results of these experiments are summarized in Table 2. Both of the amphiphilic peptides tested, SI and melittin, were found to fuse and possibly lyse neg-

Table 2. Summary of peptide-lipid interactions, based on the results from Figures 2–7^a

Peptide	Observed membrane interactions	Structural intermediate
SI	Lyses and fuses negatively charged liposomes to form larger vesicles, but has little activity with neutral membranes	α -Helix
SIscr	Causes aggregation, but does not promote fusion or lysis of negatively charged vesicles	Random coil
Polylysine	Aggregates the negatively charged liposomes without fusing or lysing them	Random coil
Melittin	Lytic and fusogenic with both neutral and negatively charged lipid bilayers forming sheets of lipid and disklike micellar structures	α -Helix

^a Peptides are defined in Table 1 and were mixed with liposomes composed of either neutral lipids (DPPC:Chol) or a mixture of DPPC with negatively charged DOPS.

actively charged membranes. However, only melittin was observed to disrupt the neutral membranes. The scrambled sequence of SI and the positively charged polylysine apparently caused aggregation of negatively charged liposomes without disturbing the integrity of the liposomal bilayers. SI and melittin were observed to form α -helices upon mixing with the negatively charged liposomes, whereas SIscr and polylysine maintained a random coil conformation. These results suggest that amphiphilicity is important in protein-membrane interactions.

Discussion

Forces governing the membrane activity of peptides

The membrane activities of SI and melittin can be rationalized in terms of forces between peptide and lipid. As noted in Figures 2 and 3, these peptides were found to cause fusion and, apparently, lysis of liposomes. However, their membrane-associating properties were different. The SI peptide promoted fusion and diluted the encapsulated fluorexon dye only when negatively charged lipids were present in the vesicles, whereas melittin was found to fuse and probably lyse both neutral and negatively charged liposomes. We can explain this difference by the greater hydrophobicity of melittin than SI. Because of its greater $\langle H \rangle$ value (Table 1), melittin associates with membranes by hydrophobic forces as well as through interactions of the charged amino acids with the lipid head groups (Hong & Vacquier, 1986). It is strongly cationic like SI, yet largely hydrophobic through the N-terminal 20 amino acids, the majority of which are apolar. This arrangement of amino acids is pertinent to melittin's function; its design makes it very disruptive to negatively charged and to neutral lipid bilayers. In addition, this segregation of melittin's charged and hydrophobic residues to form a "hydrophobic dipole" allows melittin, in some cases, to act as a detergent to solubilize lipids into micelles (Dempsey, 1990). The reduction of the absorbance and mean particle size observed for melittin at low lipid/peptide ratios (<100) in the presence of DPPC:DOPS liposomes suggests that the formation of micelles is indeed occurring. Furthermore, the disklike structures observed by EM also appear to be micellar in nature. Peptide hydrophobicity thus plays a considerable role in the disruption of membranes by the peptide.

In contrast, SI, an amphiphilic peptide with a negative $\langle H \rangle$ value, is more hydrophilic and, hence, does not

Fig. 7. Electron micrographs of the lipid structures observed to form in the presence of the peptides. Scale bar = 500 nm. **A:** Liposomes of DPPC:DOPS untreated with peptides in PBS, illustrating their initial morphology. **B:** Bee venom melittin and DPPC:DOPS vesicles in PBS. The liposomes appear to have reorganized to form disks of lipid and rouleaux. **C:** Peptide SIb with DOPS:DPPC vesicles in PBS revealing nearly complete reorganization of the small unilamellar vesicles into larger liposomes.

depend as much on hydrophobic forces as melittin to promote its interactions with membranes. Apparently, the membrane activity of SI is more dependent than melittin on its electrostatic interactions with negatively charged lipid head groups, because the experimental evidence shows SI to be relatively inactive with the neutral liposomes. However, electrostatic interactions alone are not enough to cause lipid bilayers to fuse or reorganize themselves. The effect of polylysine on liposomes illustrates this point. The positive charges of the lysine residues provide an initial attractive force by bringing the negatively charged liposomes together (i.e., aggregation), but the absence of hydrophobic residues in polylysine fails to promote the intermixing of lipids from individual liposomes. In a similar manner, the positive charges of SIscr aggregate the negatively charged liposomes, but do not promote lysis or fusion of the lipid bilayer. If SIscr has the same amino acid composition as SI, then how does SI cause the disruption of membranes? The answer must be related to the differences in the structural arrangement of the amino acids of SI and SIscr.

Structural aspects of peptide-lipid interactions

It can be argued that SI and melittin interact with membranes as α -helices. The first argument is that both SI and melittin have large hydrophobic moments, with that of SI being the largest of any segment in the protein sequence database (Eisenberg & Wesson, 1990). Therefore, SI would be expected to coil into an α -helix at a membrane-water interface. Consistent with this hypothesis, the peptide was found to be a random coil in aqueous buffer. This is presumably because of the interactions of the charged residues with the aqueous medium. However, the addition of the negatively charged DPPC:DOPS liposomes promoted the formation of α -helical structure. About 30% α -helicity was estimated, which is lower than might be expected from the computations of Eisenberg and Wesson (1990), whose results suggest that 50% of the residues could be in a helix at a membrane interface. It is worth noting that we have observed SI to be 40–50% α -helical in the presence of hexafluoroisopropanol and trifluoroethanol (unpubl.). This observation shows that the local environment of the peptides can either promote or oppose the formation of secondary structure. Consistent with previous studies (Vogel, 1981; Beschiaschvili & Seelig, 1990), melittin displayed a significant increase in helicity upon mixing with liposomes. Even though melittin's hydrophobic moment is smaller than that for SI, its increase in α -helicity was significantly greater, again emphasizing the balanced role that electrostatic and hydrophobic forces play in the formation of secondary structure.

Electron micrographs of the SI-lipid complexes show that the peptide induces a population of unilamellar liposomes at least 10 times larger in diameter than the initial ones (Fig. 7B), suggesting that the structure stabilized by

this peptide involves a limited number of fusion events. Based upon the relative increase in surface area, it would take approximately 100 small vesicles fusing together to form one of the larger liposomes. It should be noted that the dilution of the encapsulated volume of the initial liposomes during fusion to form larger vesicles could be large enough to account for some of the increase in fluorescence observed in the lysis assay. Thus, it is possible that SI is promoting fusion of the liposomes to form larger vesicles without lysing them.

In general agreement with the electron micrographs, the mean particle size was found to increase to >300 nm as judged by laser light scattering. The high bilayer curvature of small vesicles (<50 nm) is known to destabilize liposomes because of the strain placed on the lipid packing (Stegman et al., 1989). The initial interaction of SI with the highly curved bilayer could promote fusion by facilitating insertion of the helix into the strained outer leaflet of the bilayer. As the vesicles fuse, the strain associated with the packing of the lipids is relieved (Wilschut et al., 1981). SI could catalyze this relaxation of strain by attracting pairs of small liposomes, fusing them, and then stabilizing the bilayer edges that are ordinarily unstable (Israelachvili, 1985), leading to a larger vesicle. It may be noteworthy that the size of the liposomes observed by EM is roughly the size of a mature enveloped virus. This leads to the speculative suggestion that a function of HIV gp41 C-terminus (that is, peptide SI) might be in the assembly of the virus membrane during budding from the host cell membrane. Retroviruses such as HIV are known to bud from the host cell membrane surface without cytolysis (Roizman, 1990). In contrast, other enveloped viruses, such as the paramyxoviruses, lack the C-terminal segment and often bud and break away from the host cell, leaving a hole in the cell membrane, subsequently leading to cytolysis.

A speculative molecular model for peptide-induced fusion

A hypothetical model describing the action of amphiphilic peptides, such as SIb and melittin, on negatively charged membranes is shown in Figure 8. When the cationic peptides are exposed to the negatively charged liposomes they adopt α -helical conformations in order to take energetic advantage of the interface between the polar and the apolar phases (Fig. 8A). The α -helical region of the peptide then embeds itself in the membrane surface, probably in a wedge type of interaction (Dawson et al., 1978; Terwilliger et al., 1982; Batenburg & de Kruijff, 1988). Fusion between liposomes occurs because some of the positively charged residues interact with other liposomes thus bringing them into close contact with one another (Fig. 8B). The electrical double layer potential, which would normally be repulsive (Papahadjopoulos et al., 1977; Israelachvili, 1985), is neutralized due to the electrostatic

Fig. 8. Speculative model describing membrane fusion by cationic α -helical peptides. The α -helices are shown projected down their helical axes as circles. The shaded regions are the hydrophobic cylindrical faces of the helix. Positive charges are shown on the hydrophilic faces of the helices. **A:** Initially the peptide associates with the lipid bilayer by either hydrophobic interaction or by electrostatic attraction. **B:** Formation of the helix-helix complex that juxtaposes two individual bilayers so that fusion can begin. **C:** As the helix-helix complex separates, the hydrophobic faces of the helices create an apolar bridge, allowing lipids from the previously distinct bilayers to associate. **D:** The fused bilayers reform into a new membrane. The helices remain bound to the membrane or are free to dissociate and participate in another fusion event.

screening caused by the interactions of the cationic amino acids of the peptide with the lipid (Kim et al., 1991). At this point, close apposition of the bilayers occurs, and the hydration shell (Düzgünes et al., 1981; Stegman et al., 1989) on the membrane surface is disrupted by the presence of the peptides. Once the opposing membranes are juxtaposed, the hydrophobic portions of the peptides can form a bridge that allows their lipids to associate and mix (Fig. 8C). It should be noted that the two opposing membranes need not be from separate liposomes; the intermediate of Figure 8C could also represent the constriction of a single membrane to form two separate compartments (such as in fission or budding). This model is consistent

with the observation that SI is less effective in fusing neutral (DPPC:Chol) liposomes than negatively charged liposomes.

This molecular model for cationic peptide-induced membrane fusion can also be extended to hydrophobic peptides if we assume an α -helical intermediate. Previous studies (Lear & DeGrado, 1987; Rafalski et al., 1990) indicate that hydrophobic segments representing the putative fusion regions from HIV and influenza become α -helical in the presence of membranes. Furthermore, when these hydrophobic peptides are coiled as α -helices, they show an interesting distribution of amino acids; the more hydrophobic residues tend to be projected along one side of the helix axis in a manner similar to that displayed by the amphiphilic peptides. Substitution of the hydrophobic helix into the model with the apolar residues facing the interior of the bilayer leads to the conclusion that this proposed molecular model may be generally applicable for peptide-induced membrane fusion.

Materials and methods

Hydrophobic moments

Hydrophobic moment analysis provides a convenient method for identifying potential amphiphilic α -helices in any protein sequence (Eisenberg et al., 1982a). At the same time it can locate very hydrophobic segments that are characteristic of transmembrane helices (Eisenberg et al., 1984). The hydrophobic moment, μ_H , of a protein segment is calculated from the equation

$$\mu_H = \sum_{n=1}^N \{ [H_n \sin(\delta n)]^2 + [H_n \cos(\delta n)]^2 \}^{1/2}, \quad (1)$$

where H_n is the hydrophobicity of the n th residue, and δ is the angle formed between the adjacent side chains in a periodic sequence. In α -helices, δ is taken as 100° , whereas for β structures, δ is taken as 160° . The hydrophobicity values for the individual amino acids have been calculated by various methods (Eisenberg et al., 1982b; Cornette et al., 1987) and for this study, the Eisenberg consensus scale hydrophobicities were used to calculate values of μ_H for gp41 (Eisenberg et al., 1982b) with an 11-residue window. The most amphiphilic segments identified by the analysis were then synthesized as described.

Peptides

Melittin was obtained from Sigma Chemical Co. (St. Louis, Missouri) and was purified as described by Anderson et al. (1980). The HIV peptide SI and the scrambled sequence were synthesized at the Biopolymer Synthesis Center of Caltech. The automated stepwise solid-phase syntheses were performed on an ABI 430A peptide synthesizer employing an optimized synthetic protocol of the

t-Boc chemistry (Sluka et al., 1990). PAM resins (ABI) and BHA resin (ABI) were used as a solid support. The side-chain protecting groups and the peptide-resin bonds of the completed peptides were cleaved using anhydrous liquid HF in the presence of *p*-cresol and *p*-thiocresol, as scavengers for 60 min at 0 °C (Stewart & Young, 1984). The peptides were purified by reverse-phase HPLC on a semipreparative C18 column (10 × 1 cm; Brownlee Labs). A linear gradient of 0–50% aqueous acetonitrile/0.1% TFA was run over 120 min. The purity and the primary structure of the HIV peptides were determined by analytical HPLC, amino acid analysis, and pulsed liquid-phase sequence analysis (ABI 477A-01 protein sequencer). Each peptide showed the homogeneous target sequence by all analytical methods used. Polylysine was obtained from Sigma.

Lipids

DPPC and DOPS were purchased from Avanti Polar Lipids (Birmingham, Alabama). The fluorescently labeled phospholipids NBD-PE and RH-PE were also obtained from Avanti Polar Lipids. Cholesterol was obtained from Sigma.

Chemicals

Fluorexon was purchased from Eastman Kodak and used without further purification. Sodium chloride and sodium phosphate were from Fisher Scientific. HPLC-grade acetonitrile and TFA were also from Fisher Scientific. Triton X-100 was obtained from Sigma.

Composition of solutions

Phosphate buffered saline (PBS) was made up of 100 mM NaCl, 25 mM phosphate, pH 7.4. Fluorexon solution was 100 mM fluorexon, 25 mM phosphate, pH 7.4.

Preparation of liposomes

Liposomes were prepared by sonication of lipid suspensions. The lipid compositions used were mole ratios of 3:1 DPPC:Chol, 3:1 DPPC:DOPS, or 1:1 DPPC:DOPS. The procedure involved the hydration of the dry lipid films (50 mg) with 5 mL of buffer or 100 mM fluorexon solution, pH 7.4. The suspended lipids were then sonicated with a probe sonicator (Sonics and Materials, Inc., Danbury, Connecticut) for 10–12 min under a stream of nitrogen gas, 60 °C, and a power setting of 4–5. The liposomes were allowed to cool to room temperature before use. The fluorexon-encapsulated liposomes required removal of the unencapsulated fluorexon. This was accomplished at room temperature by passing the liposomes through a Sephadex G-50–80 column equilibrated with PBS. Final lipid concentrations were determined by

HPLC analysis using a modified method of Patton et al. (1982) and were found to be 5–10 mg/mL.

Lysis assay

The lysis assay monitors the release of fluorexon, which is encapsulated at self quenching concentrations inside the liposomes. When the dye leaks out of the vesicles there is a large increase in the observed fluorescence as the fluorexon becomes diluted. The fluorescence was monitored using a Perkin-Elmer LS3 fluorimeter with excitation and emission wavelengths of 490 nm and 545 nm, respectively. Unless otherwise noted, samples were prepared by dilution of the liposomes to 0.1 mg/mL in PBS. Stock solutions of peptides in water were prepared at 10, 1, 0.1, and 0.01 mg/mL, and aliquots of the peptide solutions were added to 0.5 mL of liposome solutions. The samples were then incubated at 25 °C for 15 min and analyzed for fluorescence changes. Complete dye release was obtained by lysing the vesicles with Triton X-100 (0.1%). The % lysis could then be calculated by the equation

$$\% \text{ lysis} = \frac{I(f) - I(0)}{I(\text{tot}) - I(0)} \times 100, \quad (2)$$

where $I(f)$ = observed fluorescence, $I(0)$ = the background fluorescence of the liposomes in buffer without peptides, and $I(\text{tot})$ = the fluorescence observed after the addition of Triton X-100.

Fusion assay

Fusion of the liposomal membrane was determined by monitoring the resonance energy transfer between two fluorescent probes, NBD-PE and RH-PE (Struck et al., 1981). Liposomes labeled with 0.5% (weight ratio) of the probes were mixed in a 1:4 ratio with unlabeled liposomes and diluted to 0.1 mg/mL in PBS. Upon fusion of the liposomes, the dilution of the mixed probes diminishes the efficiency of energy transfer from the NBD-PE to the RH-PE resulting in an increase of the NBD-PE fluorescence. By using an excitation wavelength at 470 nm and by measuring the emission at 530 nm, the extent of fusion can be measured. As in the lysis assay, aliquots of stock peptide solutions were added to 0.5 mL of 0.1 mg/mL liposomes. The samples were incubated at 25 °C for 15 min, and the changes in fluorescence were observed. Liposome samples without peptides were taken to be 0% mixing, whereas a sample of liposomes labeled with 0.1% NBD-PE and RH-PE were measured as a 100% mixing standard. The % fusion can be calculated using the formula

$$\% \text{ fusion} = \frac{F(\text{obs}) - F(0)}{F(\text{tot}) - F(0)} \times 100, \quad (3)$$

where $F(obs)$ = the observed fluorescence intensity at 530 nm, $F(0)$ = the fluorescence of the 0.5% mixed probes standard without peptides, and $F(tot)$ = the fluorescence intensity of the 0.1% mixed probes standard.

Particle size and visible absorbance measurements

The changes in the size of the liposomes were assessed by laser light scattering and visible absorbance measurements. Samples were prepared by adding aliquots of peptide stock solutions to 1 mg/mL liposome preparations in PBS buffer. The samples were then incubated for 15 min at 25 °C. The absorbance at 400 nm of each sample was taken on a Hitachi U2000 UV/visible spectrometer and its particle size was measured by laser light scattering on a Leeds and Northrup Microtrac UPA.

Circular dichroism spectroscopy

CD spectra were obtained on a Jasco J-600 CD spectrometer. Samples were scanned from 250 nm to 195 nm using cells with a pathlength of 1 mm. Each spectrum was the average of four scans. Peptide concentrations were 0.05 mg/mL, and lipid concentrations were 1.0 mg/mL in PBS.

Electron microscopy

The effects of the peptides on the liposomal membrane structure were examined by negative stain EM. Samples were prepared by placing 10–15 μ L aliquots of the peptide–lipid mixtures (0.1:0.2 mg/mL) on 400-mesh copper grids previously coated with Parlodion stabilized with carbon. To improve the binding of the negatively charged liposomes to the grids, a drop of 0.2% aqueous cytochrome *c* was applied to each grid to give a uniform positive charge to the surface of the carbon film. The liposome preparations were allowed to adsorb to the film for 1 min and then rinsed with distilled water to remove any unbound material. The samples were negatively stained with 0.5% aqueous uranyl acetate for 30 s, and the excess uranyl acetate was removed by blotting with filter paper. The preparations were allowed to air dry and were examined with a Hitachi H-7000 electron microscope operated at 75 kV.

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