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Defensins promote fusion and lysis of negatively charged membranes

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Abstract

Defensins, a family of cationic peptides isolated from mammalian granulocytes and believed to permeabilize membranes, were tested for their ability to cause fusion and lysis of liposomes. Unlike α-helical peptides whose lytic effects have been extensively studied, the defensins consist primarily of β-sheet. Defensins fuse and lyse negatively charged liposomes but display reduced activity with neutral liposomes. These and other experiments suggest that fusion and lysis is mediated primarily by electrostatic forces and to a lesser extent, by hydrophobic interactions. Circular dichroism and fluorescence spectroscopy of native defensins indicate that the amphiphilic β-sheet structure is maintained throughout the fusion process. Taken together, these results support the idea that protein-mediated membrane fusion depends not only on hydrophobic and electrostatic forces but also on the spatial arrangement of the amino acid residues to form a three-dimensional amphiphilic structure, which promotes the efficient mixing of the lipids between membranes. A molecular model for membrane fusion by defensins is presented, which takes into account the contributions of electrostatic forces, hydrophobic interactions, and structural amphiphilicity.

Keywords: defensins; lipid binding; lipid-peptide interactions; membrane fusion

A defense mechanism employed by mammalian organisms against foreign microbes is the phagocytosis of these pathogens by neutrophils and macrophages (phagocytes). Phagocytes ingest the pathogens and then dispose of them through two toxic pathways. One pathway subjects the pathogen to reactive oxygen intermediates while the other exposes it to cytotoxic proteins that are stored in the cytoplasmic granules of the phagocyte. Both pathways have been implicated in the killing of bacteria, viruses, and fungi (Ganz et al., 1988).

Defensins are major components isolated from phagocyte azurophil granules (Ganz et al., 1990). They are 29-34 amino acid residues long ($M_r \approx 3{,}500$-$4{,}000$) and have been found in rats (Eisenhauer et al., 1989), rabbits (Selsted et al., 1985a), and guinea pigs (Selsted & Harwig, 1987), as well as humans (Ganz et al., 1985; Selsted et al., 1985b; Wilde et al., 1989). The amino acid sequences of the defensins have been determined and when they are aligned (Table 1) to emphasize the positions of the conserved residues, a family of cationic peptides is revealed whose net charge varies from +2 for defensin HNP-3 to +9 for defensin NP-1. Arginine accounts for all positive charges except in the rabbit defensins, where lysine is found in NP-3B at residues 7 and 21, and histidine is found at position 27 in NP-1, NP-2, NP-4, and NP-5. Another prominent feature of the defensins is the six cysteines (residues 2, 4, 10, 20, 30, 31), which form three disulfide bonds. The cationic nature of the defensins suggests that their microbicidal activity might be dependent in part upon electrostatic interactions.

The three-dimensional structure of a defensin (HNP-3) purified from human neutrophil cells has been determined by X-ray crystallography to 1.9 Å resolution (Kinemage 1; Hill et al., 1991). Also, the solution structure of a rabbit

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Abbreviations: Chol, cholesterol; DOPS, dioleylphosphatidylserine; DPPC, dipalmitoylphosphatidylcholine; HIV, human immunodeficiency virus; HNP, human neutrophil peptide; HPLC, high performance liquid chromatography; NBD-PE, 7-nitro-benzoxadiazole phosphatidylethanolamine; NP, rabbit neutrophil peptide; PB, phosphate buffer; PBS, phosphate-buffered saline; RH-PE, lissamine rhodamine phosphatidylethanolamine; SI, SI peptide; SUV, small unilamellar vesicles; TFA, trifluoroacetic acid.

Table 1. *Amino acid sequences of defensins isolated from various mammals, aligned to show residue conservation*[a]

Defensin[b]	Sequence 1 5 10 15 20 25 30	Net charge[c]	⟨H⟩
HNP-1[1]	ACYCRIPA CIAGERRYGTCIYQGRLWAFCC	+3	0.07
HNP-2[1]	CYCRIPA CIAGERRYGTCIYQGRLWAFCC	+3	0.05
HNP-3[1]	DCYCRIPA CIAGERRYGTCIYQGRLWAFCC	+2	0.02
HNP-4[2]	VCSCRLVF CRRTELRVGNCLIGGVSFTYCCTRV	+4	0.03
GPNP[3]	RRCICTTRT CRFPYRRLGTCIFQNRVYTFCC	+7	−0.27
NP-1[4]	VVCACRRAL CLPRERRAGFCRIRGRIHPLCCRR	+9	−0.37
NP-2[4]	VVCACRRAL CLPLERRAGFCRIRGRIHPLCCRR	+8	−0.27
NP-3A[4]	GICACRRRF CPNSERFSGYCRVNGARYVRCCSRR	+8	−0.43
NP-3B[4]	GRCVCRKQLLCSYRERRIGDCKIRGVRFPFCCPR	+8	−0.38
NP-4[4]	VSCTCRRFS CGFGERASGSCTVNGVRHTLCCRR	+5	−0.21
NP-5[4]	VFCTCRGFL CGSGERASGSCTINGVRHTLCCRR	+4	−0.07
RatNP-1[5]	VTCYCRRTR CGFRERLSGACGYRGRIYRLCCR	+8	−0.41
RatNP-2[5]	VTCYCRSTR CGFRERLSGACGYRGRIYRLCCR	+7	−0.33
RatNP-3[5]	CSCRTSS CRFGERLSGACRLNGRIYRLCC	+5	−0.26
RatNP-4[5]	ACYCRIGA CVSGERLTGACGLNGRIYRLCCR	+4	−0.03

[a] The mean hydrophobicity (⟨H⟩) of each defensin was calculated from the consensus scale (Eisenberg et al., 1982). Highly conserved residues are in boldface type and the disulfide linkages are shown with connecting lines.

[b] Sources: [1]human (Selsted et al., 1985b); [2]human (Wilde et al., 1989); [3]guinea pig (Selsted & Harwig, 1987); [4]rabbit (Selsted et al., 1985a); [5]rat (Eisenhauer et al., 1989).

[c] At pH 7.4, assuming the charge on histidine is neutral.

defensin (NP-5) has been determined by NMR spectroscopy (Bach et al., 1987; Pardi et al., 1988). The structures of the human and rabbit defensins agree well with each other and comparison with known defensin sequences (Table 1) suggests that all defensins have the same general conformation (Hill et al., 1991). The structural data show that defensins consist primarily of β-sheet, with no α-helices. Furthermore, the crystal structure, as well as equilibrium sedimentation studies suggests that defensins can exist as dimers or higher multimers in solution (P. Poon & V. Schumaker, pers. comm.).

The variety of organisms for which defensins are lethal suggests that a general mechanism is involved in their toxicity. In vitro, defensins have been shown to be toxic to bacteria (Greenwald & Ganz, 1987; Lehrer et al., 1989), fungi (Lehrer et al., 1985b), and enveloped viruses (Daher et al., 1986). Defensins also display cytotoxicity toward several normal and neoplastic cell lines (Lichtenstein et al., 1986). Studies with planar lipid bilayers (Kagan et al., 1990) indicate that the defensins' primary mode of action is by direct association with the lipid components in membranes. Interactions between other peptides and membranes have been studied extensively (DeGrado et al., 1982; Chung et al., 1985; Subbarao et al., 1987), but the defensins are unusual because their lipid bilayer-perturbing properties are not derived from amphiphilic, α-helical secondary structures, as is the case for most membrane-associating peptides. Instead, the β-structure, stabilized by three disulfide bonds, seems likely to be preserved during interaction with the membrane.

In order to characterize further their membrane interactions, several defensins have been isolated and their effects on SUV in vitro have been examined. The results of those experiments are reported here.

Results

Defensins selected for study

Six rabbit defensins and three human defensins were initially screened for their ability to disrupt lipid membranes. The human defensins (HNP-1, HNP-2, HNP-3) were found to behave similarly in the presence of lipid bilayers. The rabbit defensins could be divided into two groups based upon their interactions with membranes: the first group (NP-1, NP-2, NP-3A, NP-3B) was not strongly inhibited in the presence of salt, while the second group (NP-4, NP-5) displayed marked reduction in membrane activity when salt was added. Based upon these preliminary results, three defensins (HNP-1, NP-1, NP-5) in their native and acyclic (aHNP-1, aNP-1, aNP-5) conformations were selected for further study. As described in the Materials and methods, the acyclic (unfolded) derivatives of these defensins were generated by reduction of the disulfide bonds and carboxamidomethylation of the free sulfhydryls. The resulting peptides, though linearized, have the same net charge as the corresponding native molecules.

Fusion and lysis assay

Fusion of liposomal membranes was monitored by observing changes in the resonance energy transfer between fluorescent lipid probes when defensins were added to vesicle solutions. Lysis of the lipid vesicles was observed by following the increase in fluorescence of fluorexon dye caused by dilution of the dye upon release from the interior of the liposomes. The lytic and fusogenic effects of defensins on neutral and negatively charged liposomes in the presence and absence of salt were examined at different lipid-to-peptide ratios, which are summarized in Table 2. The defensins interacted strongly with negatively charged membranes, thus confirming the earlier suggestion that the membrane activity of defensins is mediated by electrostatic forces.

In general, defensins did not interact with the neutral lipid vesicles. Lysis of the DPPC:Chol liposomes by defensins was not observed at any of the conditions tested (Fig. 1). In contrast, melittin caused almost complete release of the fluorescent dye from the neutral liposomes under the same conditions. In agreement with the lysis results, fusion of DPPC:Chol vesicles by defensins was minimal (Fig. 2). Of the native defensins tested, only NP-1

Table 2. *Summary of the fusion, lysis, absorbance assays of the effects of defensins on liposomes*[a]

	3:1 DPPC:Chol						3:1 DPPC:DOPS					
	5 mM PO_4^{-3}			PBS			5 mM PO_4^{-3}			PBS		
	Fusion	Lysis	Abs.	Fusion	Lysis	Abs.	Fusion	Lysis	Abs.	Fusion	Lysis	Abs.
HNP-1	−	−	+	−	−	+	+	++	+	+	−	−
aHNP-1	+	−	++	+	−	+	+	++	+	+	+	+
NP-1	+	−	++	−	−	−	++	++	++	++	+	++
aNP-1	+	−	+	−	−	−	++	++	++	+	++	++
NP-5	−	−	−	−	−	−	++	+	−	+	−	−
aNP-5	−	−	−	−	−	−	+	++	+	−	−	−
Melittin	++	++	++	++	++	++	+	++	−	+	++	−
Poly-L-lysine	−	−	+	−	−	−	−	−	++	−	−	++

[a] Each entry represents the maximal value observed for a particular assay that each peptide listed in the left-hand column caused under the conditions given to the right. Greater than 50% fusion or lysis is denoted by ++, 20–50% by +, less than 20% by −. Increases in absorbance are assessed by comparing the absorbance changes caused by the control peptides melittin (DPPC:Chol liposomes) poly-L-lysine (DPPC:DOPS liposomes) against the changes in absorbance caused by the defensins. As in the fusion and lysis assays, a greater than 50% rise in absorbance caused by a defensin relative to melittin or poly-L-lysine is represented in the Abs. columns by ++, 20–50% by +, less 20% by −.

in PB displayed significant amounts of fusion. However, the denatured defensins, aNP-1 and aHNP-1, appeared to retain or have slightly enhanced activity relative to their native forms against the neutral membranes. Defensin NP-5 and aNP-5 did not exhibit significant amounts of fusion or lysis at any of the conditions tried with the neutral liposomes. The addition of saline tended to reduce defensins' ability to perturb the lipid bilayers. Apparently, in the absence of electrostatic interactions, the native defensins are not particularly disruptive to neutral bilayers.

When defensins were mixed with negatively charged liposomes, nearly complete fusion and lysis was observed. Both the fusogenic and lytic potential of NP-1 and aNP-1 were increased in the presence of the DPPC:DOPS liposomes when compared to the neutral DPPC:Chol liposomes. Furthermore, the inhibitory effects of salt were minimal when NP-1 was mixed with the negatively charged lipid vesicles. Both HNP-1 and aHNP-1 were also found to be more active with the negatively charged liposomes than with the neutral liposomes but unlike NP-1, their ac-

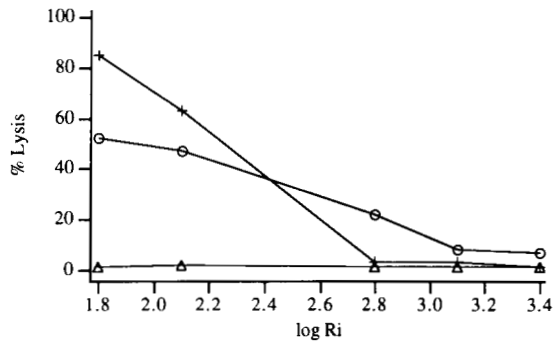

Fig. 1. Lysis of liposomes by defensins as monitored by the release of encapsulated fluorexon dye. As the dye leaks out of the liposomes, it becomes diluted and fluoresces. The % lysis observed at different lipid-to-defensin mole ratios (Ri) is shown for DPPC:DOPS and DPPC:Chol liposomes in buffer. Five 0.5-mL samples of each defensin and lipid 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. HNP-1 in PB and DPPC:DOPS liposomes, pH 7.4 (+); NP-1 in PB and DPPC:Chol liposomes, pH 7.4 (△); NP-1 in PB DPPC:DOPS liposomes, pH 7.4 (○). Measurements for each type of liposome were carried out at values of log Ri between 1.8 and 3.4.

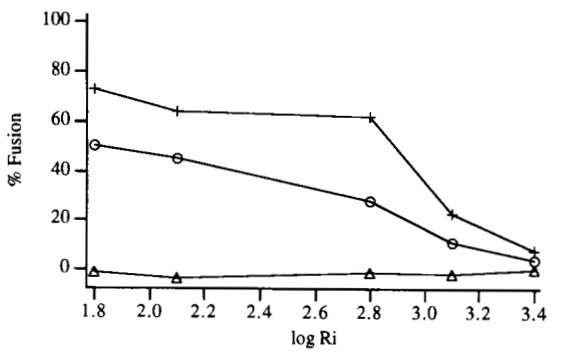

Fig. 2. Fusion of liposomes by defensins as measured by resonance energy transfer (Struck et al., 1981). The % fusion observed at different lipid-to-defensin ratios (Ri) is shown for DPPC:DOPS and DPPC:Chol liposomes in buffer. Five 0.5-mL samples of each defensin and lipid were prepared by mixing different 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. NP-5 in PBS and DPPC:Chol liposomes, pH 7.4 (△); NP-1 in PB and DPPC:DOPS liposomes, pH 7.4 (○); NP-1 in PBS and DPPC:DOPS liposomes, pH 7.4 (+). Measurements for each type of liposome were carried out at values of log Ri between 1.8 and 3.4.

tivity was reduced under isotonic saline conditions. Defensins NP-5 and aNP-5 displayed the largest salt dependence, showing complete inhibition of fusion and lysis of the negatively charged membranes in PBS. This suggests that defensins' mechanism of action may be related to their association with negatively charged membranes.

Absorbance studies

The changes in absorbance at 400 nm were used to monitor the changes in the size of the liposomes that occur when they are mixed with defensins. Table 2 compares the absorbance data with the fusion and lysis results. Overall, the absorbance changes are consistent with the fusion and lysis data. The defensins caused slight increases in absorbance in the presence of neutral liposomes, but when compared to the increase caused by melittin, the changes were minor. Not surprisingly, the absorbances increased dramatically when the defensins were mixed with the DPPC:DOPS vesicles (Fig. 3). In all cases isotonic saline was found to inhibit the increase in absorbance. Poly-L-lysine exhibited the largest increase in absorbance when mixed with DPPC:DOPS liposomes. Curiously, the absorbance of the DPPC:DOPS liposomes in the presence of melittin decreased. This effect has been observed before (Dufourc et al., 1986) and is attributed to the stabilization of micellar structures by melittin's detergent-like properties. In agreement with the fusion and lysis results, the absorbance data provide further support for a defensin–membrane model involving electrostatic interactions.

Tryptophan fluorescence spectroscopy

Changes in tryptophan fluorescence have been used (e.g., Teale, 1960) as a probe for monitoring changes in the local hydrophobic environment of a protein or peptide. Because HNP-1 contains a single tryptophan residue, the fluorescence changes of this defensin in the absence and presence of liposomes could be observed. Figure 4 displays the tryptophan emission spectra of HNP-1 and aHNP-1. No significant shifts in the wavelength maximum at 345 nm were observed for HNP-1 in buffer when either the DPPC:DOPS or DPPC:Chol liposomes were added. In contrast, aHNP-1 exhibits fluorescence changes in the presence of the negatively charged liposomes. The emission maximum of aHNP-1 in buffer at 350 nm undergoes a blue shift down to 332 nm when it is mixed with DPPC:DOPS vesicles in PB. Interestingly, the blue shift does not occur with the DPPC:Chol vesicles or with the DPPC:DOPS vesicles in PBS. The blue shift indicates that the tryptophan residue in the unfolded aHNP-1 becomes buried in the hydrophobic environment of the lipid bilayers (Mollay & Kreil, 1973). On the other hand, the local environment of tryptophan in native HNP-1 does not change in the presence of liposomal membranes, showing that the tryptophan does not come into contact

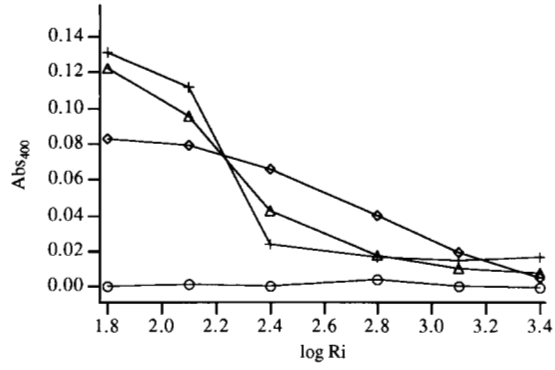

Fig. 3. Detection of aggregation and fusion by observing absorbance changes at 400 nm of liposomes mixed with defensins. The absorbance at different lipid-to-peptide ratios (Ri) is shown for the DPPC:DOPS liposomes in buffer. Six 0.5-mL samples of each defensin and lipid were prepared by adding different concentrations of peptides with 0.1 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. NP-1 in PBS, pH 7.4 (◇); NP-5 in PBS, pH 7.4 (○); poly-L-lysine in PB, pH 7.4 (+); NP-1 in PB, pH 7.4 (△). Measurements for each type of liposome were carried out at values of log Ri between 1.8 and 3.4.

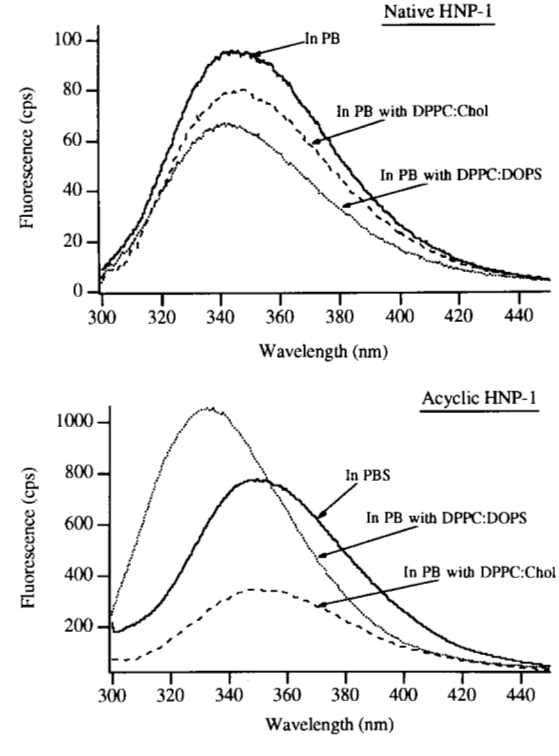

Fig. 4. Tryptophan fluorescence spectra of defensin HNP-1 in both its native and acyclic (unfolded) conformations. Samples (0.5 mL) are 0.01 mg/mL defensins in buffer and 0.100 mg/mL liposomes. Upper graph: HNP-1 in PB, pH 7.4 (——); HNP-1 in PB and DPPC:Chol liposomes, pH 7.4 (-----); HNP-1 in PB and DPPC:DOPS liposomes, pH 7.4 (·····). Lower graph: aHNP-1 in PBS, pH 7.4 (——); aHNP-1 in PB and DPPC:Chol liposomes, pH 7.4 (-----); aHNP-1 in PB and DPPC:DOPS liposomes, pH 7.4 (·····).

with the membrane and suggesting that the structure of the native peptide is maintained when it interacts with lipids.

Circular dichroism studies

Conformational changes of defensins were investigated by CD spectroscopy. Figure 5 displays several spectra that illuminate the conformational changes that native and unfolded defensins undergo in the presence of membranes. The spectra of the native defensins typically have minima at 195–200 nm for HNP-1 and 205–210 nm for NP-1 and NP-5. The single minimum observed for the native defensins is characteristic of proteins containing β-sheet and β-turn secondary structure with some random coil (Johnson, 1988). There is little change in the spectra in the presence of isotonic saline, which suggests that the native conformations are not affected by ionic strength. Similarly, the CD spectra of the native rabbit and human defensins do not exhibit any major changes in the presence of either neutral or negatively charged liposomes, indicating that the overall structure of the molecule is preserved when it interacts with lipid bilayers.

Dramatic changes in the CD spectra are found to occur when the acyclic defensins are mixed with negatively charged liposomes. In buffer, these defensins have spectra similar to that of the native defensins, suggesting that the "unfolded" defensins may actually adopt some nonrandom conformations (i.e., β-sheet and β-turn). When the acyclic defensins are added to DPPC:DOPS liposomes, large changes in the CD spectra are observed, especially in PB. The human defensin aHNP-1, shifts its minimum at 200 nm to 215 nm and gains a maximum at 195 nm indicating that an extended β-sheet structure is formed. Rabbit defensins aNP-1 and aNP-5 also undergo changes, but the single minima at 205 nm is replaced by the double minima at 208 nm and 222 nm generally associated with α-helix formation. However, the maximum α-helicity is probably less than 20% for either peptide. In the absence of the disulfide bonds that maintain the β-structure, defensins assume conformations markedly different from the stabilized native structures upon association with negatively charged lipid bilayers.

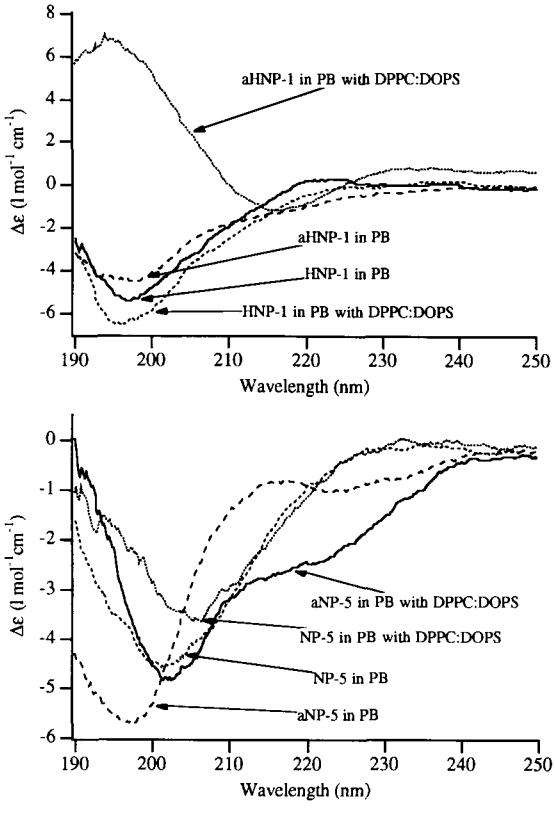

Fig. 5. Circular dichroism spectra of defensins illustrating changes in secondary structure. Upper graph: HNP-1 in PB, pH 7.4 (——); HNP-1 in PB and DPPC:DOPS liposomes, pH 7.4 (-----); aHNP-1 in PB, pH 7.4 (– – –); aHNP-1 in PB and DPPC:DOPS liposomes, pH 7.4 (·····). Lower graph: NP-5 in PB, pH 7.4 (-----); NP-5 in PB and DPPC:DOPS liposomes, pH 7.4 (·····); aNP-5 in PB, pH 7.4 (– – –); aNP-5 in PB and DPPC:DOPS liposomes, pH 7.4 (——).

Lipid binding assay

The binding of the native defensins to DPPC:DOPS liposomes was examined using a sucrose density gradient centrifugation assay. Figure 6 illustrates examples both of defensin binding to lipid (Fig. 6A) and lack of binding to lipid (Fig. 6B). The results of the lipid binding experiments are summarized in Table 3. The human defensin, HNP-1, binds lipid strongly in PB as indicated by the low lipid/defensin ratio (13 mol of lipid/mol of defensin) and the binding is slightly reduced in PBS (lipid/defensin = 20 mol/mol). NP-1 binds equally in both PB and PBS, but the total binding (lipid/defensin ≈ 90 mol/mol) is weaker than HNP-1. Binding of NP-5 is very dependent on the salt concentration as it does not bind lipid at all in PBS but does bind lipid at PB (lipid/defensin = 60 mol/mol). The interactions of defensins with lipids is influenced not only by electrostatic forces between the positively

Table 3. *Lipid/defensin ratios (mol lipid/mol defensin) calculated from sucrose density gradient centrifugation of liposomes mixed with defensins*[a]

Defensin	10 mM PO_4^{-3}	PBS[b]
HNP-1	13	20
NP-1	87	91
NP-5	60	–

[a] Low values (~10) indicate strong binding of peptide to lipid and high values (~100) indicate weak binding.
[b] PBS: 100 mM NaCl, 25 mM PO_4^{-3}, pH 7.4.

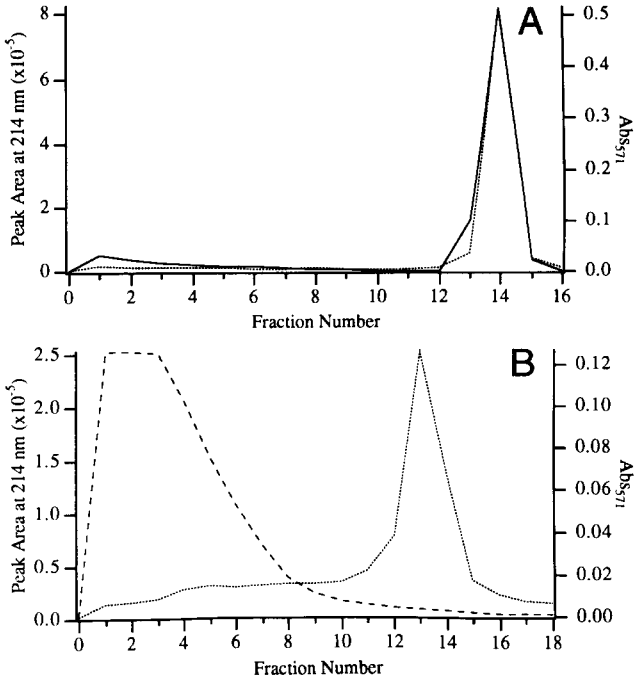

Fig. 6. Binding of defensins to liposomes as determined by fractionated sucrose density gradients. Each fraction was analyzed for peptide and lipid. Defensins HNP-1 and NP-5 are represented by solid (——) and dashed (-----) lines, respectively. Lipid fractions are represented as dotted (·····) lines. **A:** Demonstration of binding to lipid: defensin HNP-1 and 1:1 DPPC:DOPS liposomes in 10 mM PB, pH 7.4. **B:** Demonstration of lack of binding to lipid: defensin NP-5 and 1:1 DPPC: DOPS liposomes in PBS, pH 7.4.

charged residues and the negatively charged headgroups but also by the net hydrophobicity of the defensin.

Discussion

Defensins share similar properties with other membrane peptides

Previous studies on the interactions of cationic lytic peptides with membranes have focused primarily on polypeptides that are potentially α-helical (DeGrado et al., 1982; Hong & Vacquier, 1986; Suenaga et al., 1989). In contrast, defensins are formed almost entirely by β-sheet (Kinemage 1). Despite the major difference in secondary structure, there are similarities between the defensins and α-helical peptides such as melittin (Habermann & Jentsch, 1967) or SI, a highly amphiphilic segment from the C-terminus of HIV gp160 (Eisenberg & Wesson, 1990). The characteristics that defensins have in common with these α-helical peptides are: similar size (25–35 amino acids long); a net positive charge (ranging from +2 to +9); amphiphilicity; and in the cases of melittin and defensin, the potential to form multimers. These similarities pose a possibility of a general mechanism of membrane lysis and fusion that is independent of secondary structural type but involves the overall spatial arrangement of polar and hydrophobic residues.

Forces involved in defensin destabilization of lipid vesicles

It seems likely that electrostatic forces play a major role in the disruption of negatively charged liposomes by defensins. As shown in Table 2, defensins efficiently destabilize DPPC:DOPS liposomes. Decreased fusion and lysis are observed when defensins are mixed with neutral DPPC:Chol liposomes indicating that the absence of charge–charge interactions greatly reduces the ability of defensins to disrupt lipid vesicles. Alternatively, the negatively charged liposomes may be inherently less stable than the neutral liposomes because of the internal strain generated by the high bilayer curvature associated with SUV (Stegmann et al., 1989). Factors influencing the internal strain include the electrostatic repulsive forces between the charged lipids (Siegel, 1992) and the stabilizing effect of Chol on the lipid bilayer (Hoekstra, 1990). A highly strained bilayer would be more susceptible to destabilization than one that is not under significant strain. It has been observed that defensins are not particularly active in promoting fusion or lysis of DPPC SUVs (Fujii, 1991). Thus, the presence or absence of Chol is probably not the major factor in the interaction of defensins with membranes. In addition, both types of liposomes tested in this study are approximately the same diameter, so any differences in the internal strain can be attributed largely to the charge repulsion effects. This suggests that the positively charged defensins might play a role in destabilizing the negatively charged lipid vesicles by alleviating the electrostatic repulsive forces between the individual lipids and also the opposing bilayers.

Further support for the role of electrostatic interactions in defensin-mediated disruption of lipid vesicles comes from the dependence of defensin activity on salt concentration. In PBS (Table 2), defensin NP-5 experienced a dramatic reduction of fusion and lysis presumably because the charged amino acids were effectively screened away from the lipid headgroups by the ions in solution. Consistent with the fusion and lysis observations, the increases in the absorbance indicate that defensins interact more strongly with the negatively charged lipids, especially in PB. The lipid binding results (Table 3) confirm the importance of electrostatic interactions, because HNP-1 (+3) and NP-5 (+4) bind more strongly to negatively charged vesicles in PB than in PBS. In contrast, NP-1 does not show a similar salt effect, possibly because its greater number of charged residues (+9) might not be sufficiently screened in PBS to diminish the binding. Furthermore, NP-1 also requires more lipid to bind to a liposome than either HNP-1 or NP-5, presumably because the

higher net charge (Table 1) makes it more soluble in aqueous solution.

The lipid binding data also demonstrate the effects of hydrophobicity on defensins' interactions with membranes. As shown in Table 3, the lipid/defensin ratio of HNP-1 to produce binding is much smaller than NP-1 and NP-5 (13 vs. 90 and 60). HNP-1 has the smallest number of positively charged residues, so binding apparently results from an effective combination of hydrophobic and electrostatic forces. The binding of melittin to lipid bilayers has been studied extensively (Vogel, 1981; Bradrick & Georghiou, 1987; Beschiaschvili & Seelig, 1990). The present work suggests that the human defensins have membrane-associative properties similar to melittin. Both melittin (Dufourcq & Faucon, 1977) and human defensins interact strongly with negatively charged lipid bilayers. Lipid binding of melittin has also been reported to be enhanced (Batenburg et al., 1987) in the presence of the negatively charged lipids. The lipid-to-melittin ratios required to bind melittin completely (<10 for negatively charged lipids at 100 mM saline, pH 7.3; Batenburg et al., 1987) is comparable to the value calculated by us for binding of human defensin HNP-1 (lipid/defensin = 20 mol/mol at 100 mM saline, pH 7.4) to the DOPS:DPPC liposomes. On the other hand, the cationic, α-helical SI peptide behaves much like the rabbit defensins. It lyses and fuses negatively charged membranes but does not bind to lipid significantly in isotonic saline at neutral pH (Fujii, 1991). This implies that the defensins are similar to α-helical fusogenic peptides with respect to their effects on membranes even though they are composed of β-structure. Instead of a requirement for α-helices in membrane lysis and fusion, it is apparent that the mechanism of peptide-induced membrane fusion is governed by a balance of hydrophobic and electrostatic forces with structural amphiphilicity.

Structural aspects of defensin interaction with membranes

The three-dimensional structure of the dimeric form of human defensin HNP-3 has been determined (Kinemage 1; Hill et al., 1991) by X-ray crystallographic methods. Each defensin monomer consists of three strands of antiparallel β-sheet incorporating 60% of the residues. Two β-turns and three disulfide bonds add further restrictions to the conformational freedom of the monomer, whose folding pattern resembles that of a paperclip. The dimer is formed by joining identical β-strands of the two monomers together to create a symmetrical six-stranded β-sheet. This extended β-sheet twists and curls to form a basket-shaped structure that has a small solvent-accessible channel passing through it. The base of the basket is hydrophobic while the top, which contains the N- and C-termini of the two defensin monomers, is polar. In the crystal structure, two dimers associate with one another by hydrophobic contact between the bases of each dimer basket. This dimer-of-dimers may be an essential feature of defensins' interaction with membranes.

The mechanism by which native defensins destabilize and fuse membranes does not appear to depend on any major structural or conformational changes of the monomer. The CD spectra of defensins (Fig. 5) in aqueous buffer and with the addition of liposomes are very similar, indicating that the structure is stable even in the presence of lipid. In addition, the tryptophan fluorescence maximum at 345 nm (Fig. 4) does not shift and no major changes in intensity are observed, indicating that the indole ring of the tryptophan does not experience local environmental changes. Because the tryptophans are located near the solvent minichannel formed by the two monomers and consequently, away from the exterior surface of the dimer, the absence of changes in fluorescence suggests that the defensin structure probably does not undergo a large conformational rearrangement. The surface area of the two tryptophan residues accessible to the aqueous solvent has been calculated in the HNP-3 dimer to be approximately 20% of the total tryptophan surface area. If this solvent-accessible surface area was transferred to a more hydrophobic environment because of a structural rearrangement, it seems likely that a change in the fluorescence spectra would then have been observed. The combined CD and fluorescence evidence (Figs. 4, 5) suggests that any mechanism proposed describing native defensin-induced membrane association requires that the lipids do not interact with the interior of a defensin dimer; rather lipid interactions depend mainly on contact with the amino acid residues on the outer surface of the dimer. Furthermore, the three intrachain disulfide bonds (Table 1) lend structural rigidity to each individual defensin molecule, making it difficult for conformational changes to occur within the monomer and perhaps, within the dimer. This is quite different from membrane-active α-helical peptides such as melittin (Dempsey, 1990) or the designed β-structure peptides of Ono et al. (1990), which undergo changes in secondary structure upon contact with lipid bilayers. Similarly, proteins such as colicin (Parker et al., 1989) and diphtheria toxin (Choe et al., 1992) have also been proposed to undergo structural rearrangements during interaction with a membrane.

The acyclic defensins promoted the fusion and lysis of negatively charged liposomes, in contrast to our prediction that unfolding them would destroy membrane activity. However, it is interesting to note that effective membrane activity of the acyclic defensins appears to be correlated with conformational changes. The acyclic defensins apparently undergo major structural changes upon contact with lipid bilayers as judged by CD and fluorescence spectroscopy. The tryptophan of aHNP-1 is observed to undergo a blue shift in fluorescence of nearly 20 nm with the negatively charged liposomes, suggesting that the indole ring of the unfolded peptide comes into

close contact with the hydrophobic core of the bilayer. In addition, the changes in the CD spectra of the acyclic defensins suggests that conformational changes occur when the defensins interact with the negatively charged lipid bilayers. Thus, it is possible that the unfolded defensins promote membrane destabilization by undergoing structural transitions to form alternative amphiphilic conformations in a manner similar to those observed for other peptides such as melittin or SI.

A speculative model for defensin-induced membrane fusion

There have been numerous models proposed for protein-mediated membrane fusion. Some of these models are based upon the interactions of melittin with lipid membranes (Batenburg & de Kruiff, 1989; Dempsey, 1990), whereas others have been formulated from studies on hydrophobic segments found in the envelope coat proteins of many viruses (Hoekstra, 1990; White, 1990). We propose a speculative model (Fig. 7) describing the action of defensin on lipid membranes. In Figure 7A, two defensin dimers are shown associated with one another through their hydrophobic patches at the base of the basket as the dimers are associated in the crystal structure (Hill et al., 1991). The defensin dimer-of-dimers is attracted to the negatively charged liposomes by electrostatic interactions with the arginines on one side of the dimer–dimer complex (Kim et al., 1991). Those arginine residues on the opposite side of the dimer-of-dimers, not interacting directly with the membrane, are free to attract another liposome, thus forming an intermediate complex (Fig. 7B). The electrostatic repulsive layer that normally inhibits close interaction of opposing negatively charged bilayers (Israelachvili, 1985) is neutralized by the cationic defensin dimers, and the hydration shell (Stegmann et al., 1989)

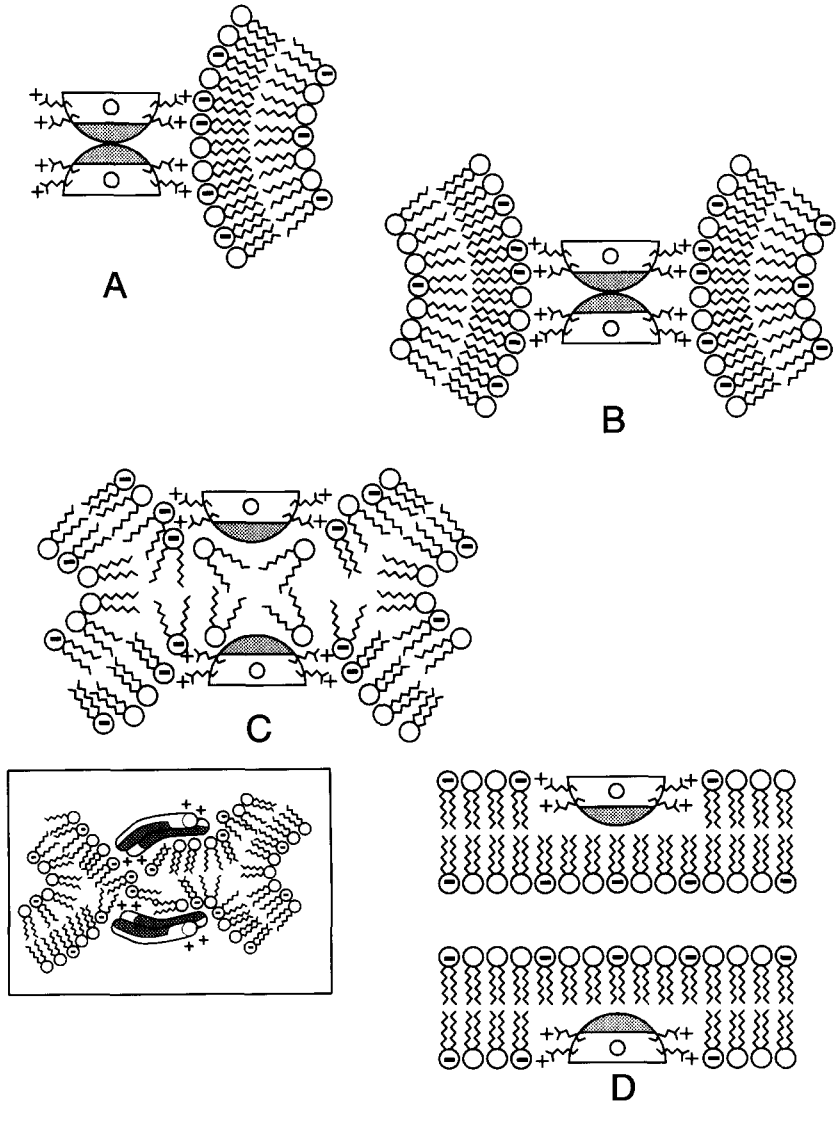

Fig. 7. Speculative model for the interactions of a defensin dimer-of-dimers complex with a charged lipid bilayer. Two defensin dimers associate with each other by hydrophobic contact at their bases (shaded regions) as in the crystal structure of Hill et al. (1991). Four of the six arginine residues are shown as positively charged arms extending from the defensins. **A:** Initial interaction of defensins with the lipid bilayer is an electrostatic attraction between the positively charged arginines and the negatively charged headgroups. **B:** A second bilayer becomes attracted to the other side of the defensin complex thereby bringing the two bilayers close together. **C:** The hydration shell surrounding the vesicle is disrupted, which destabilizes the membrane. The hydrophobic chains of the lipids from both bilayers are free to exchange with each other thus initiating fusion. **D:** The fused bilayers reform into a larger structure. **Inset:** A comparative model of melittin interacting with a lipid bilayer. The hydrophobic portions (shaded regions) of a dimer from a tetramer, which can function in the same manner as the hydrophobic faces of the defensin dimer complex. The positively charged amino acids are situated at the ends of the melittin dimer-of-dimers and initiate lipid bilayer fusion by electrostatic attraction to the lipid headgroups. In short, melittin and defensin can fuse bilayers by the same fundamental mechanism.

is disrupted, allowing the bilayer interior to be accessible to the protein. During a thermal fluctuation that separates the two defensin dimers at their base, several hydrocarbon chains of the lipid bilayer can associate with the hydrophobic patches of the separated defensins. This requires the lipid chains to be in a fluid state so that they may rotate freely out of the bilayer plane into the gap created by the separating dimers. These rotated hydrocarbon chains form a hydrophobic bridge that spans the liposomes and facilitates the mixing of the lipids (Fig. 7C). Because the force binding the two dimers is removed once the hydrophobic base is covered by lipid chains, the defensin complex can dissociate, thereby allowing complete mixing of the lipids (Fig. 7D). At this stage, the defensin dimer may remain bound to the lipid bilayer or it might possibly dissociate from the membrane, where it might reform another dimer–dimer complex and participate in further fusion events.

This model is reminiscent of the wedge models proposed for α-helical peptides, such as melittin (Dawson et al., 1978; Terwilliger et al., 1982; Batenburg & de Kruiff, 1988) or SI (Fujii et al., 1992). If the defensin dimer is imagined to be an amphiphilic wedge, then Figure 7C illustrates the similarity of the defensins to the wedges formed by an α-helix. The tip of the wedge is hydrophobic and orients itself into the membrane while the charged arginine residues at the top of the defensin wedge are free to interact with lipid headgroups. In a similar manner, melittin, which is known to form tetramers (Habermann, 1972; Terwilliger et al., 1982), could have a fusion intermediate analogous to the one proposed here for defensins. Figure 7 illustrates the comparison of tetrameric melittin (inset) and the tetrameric defensin intermediates (Fig. 7C). The defensin dimer-of-dimers has been replaced by a melittin dimer-of-dimers. Initially, electrostatic interactions bring the positively charged amino acids on the edges of the melittin tetramer into close association with the negatively charged lipids of opposing vesicles. The melittin tetramer can dissociate into a dimer-of-dimers, thus creating a hydrophobic bridge that allows the lipids to mix. Results of studies on the aggregation state of melittin in membranes have been reported that are both compatible (Vogel & Jähnig, 1986; Talbot et al., 1987) and incompatible (Hermetter & Lakowicz, 1986; Altenbach & Hubbell, 1988) with this model.

In summary, defensins cause extensive fusion and lysis of negatively charged lipid vesicles. Contributions from electrostatic forces, hydrophobic interactions, and from structural amphiphilicity appear to be important factors in the ability of defensins to disrupt membranes and bind lipid. The fact that native defensins are composed primarily of β-structure, rather than α-helix, does not significantly reduce their ability to interact with membranes relative to other lytic peptides. In fact, these findings are comparable to the results observed for the cationic α-helical peptides such as melittin. However, unlike melittin, native defensins do not undergo large conformational rearrangements in the presence of lipid bilayers because the three disulfide bonds stabilize the structure of each defensin monomer. In the past, protein-induced membrane fusion and lysis were thought to be mediated primarily by an α-helical intermediate. Several studies (Ono et al., 1990; Epand et al., 1992) have recently implicated the formation of β-type structure in protein–membrane interactions, suggesting that other factors may play a significant role in protein–membrane interactions. Taken together, our findings support the idea that a general mechanism for protein-mediated membrane fusion can be proposed based upon the formation of structures that depend on the spatial arrangement of the amino acids rather than the secondary structural type.

The discovery that unfolding the defensins does not appreciably destroy their ability to fuse and lyse negatively charged membranes raises the question of why defensins in nature exist in a tight, conformationally restrained structure. Studies have shown (Lehrer et al., 1985a) that reduction and alkylation of the defensin disulfide bonds does abolish their antimicrobial activities. Thus, the fusogenic activity of acyclic defensins suggests that the tendency of the linear peptide to interact with a phospholipid bilayer is not sufficient to confer a microbicidal effect. There are several reasons that may explain why acyclic defensins fuse and lyse model membranes but do not kill pathogenic organisms. First, it is conceivable that additional postbinding events between a folded defensin and its target may be required for microbicidal killing, and that the acyclic molecule is inert in this regard. For example, channel formation could be necessary for killing and it may be that the acyclic peptides do not form structures that can assemble to form channels. Second, it is possible that the linear peptide is not microbicidal because it is degraded by cell proteases. Native defensin, on the other hand, is quite resistant to proteolytic degradation. Although the results of this study support the idea that defensins' mechanism of killing involves membrane interactions, further investigation will be needed to completely understand the nature of these interactions.

Materials and methods

Peptides

Native defensins were isolated from rabbit (Selsted et al., 1984; Lichtenstein et al., 1986) and human neutrophils (Ganz et al., 1985), as described, and quantitated by amino acid analysis. Linearized defensins were produced by reduction with dithiothreitol and *S*-carboxamidomethylation with iodoacetamide as described previously (Lehrer et al., 1985a) and were purified by reverse-phase HPLC. Melittin was purchased from Sigma Chemical Co. (St. Louis, Missouri) and purified according to the method of

Anderson et al. (1982). Poly-L-lysine (MW = 3,000) was also obtained from Sigma.

Lipids

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

Chemicals

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

Composition of solutions

PBS: 100 mM NaCl, 25 mM phosphate, pH 7.4. PB: 5–10 mM phosphate, pH 7.4. Fluorexon: 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 either DPPC:Chol or DPPC:DOPS in 3:1 mole ratios. The procedure involved the hydration of the dry lipid films (50 mg) with 5 mL of buffer or fluorexon solution. The suspended lipids were then sonicated with a probe sonicator (Sonics and Materials Inc., Danbury, Connecticut) for 2–3 min under a stream of nitrogen gas, 45 °C, and a power setting of 4–5. The liposomes were allowed to cool to room temperature before filtration through a 0.22-μm filter (Millipore GS). Unencapsulated fluorexon dye was removed from the liposomes by passing the room temperature liposomes through a Sephadex G-50–80 column equilibrated with buffer before the filtration step. The vesicles were sized on a Leeds and Northrup Microtrac UPA and the mean diameters were approximately 30 nm for both types of liposomes. Final lipid concentrations were determined by HPLC analysis (Patton et al., 1982).

Lysis assay

The lysis assay monitors the release of fluorexon, which is encapsulated at self-quenching concentrations inside the liposomes. The fluorexon is diluted as it leaks out of the vesicles, which results in an increase in fluorescence. The fluorescence was monitored using a Perkin-Elmer fluorimeter with excitation and emission wavelengths of 470 nm and 535 nm, respectively. Unless otherwise noted, samples were prepared by dilution of the liposomes to 0.1 mg/mL in either PBS or 10 mM phosphate buffer. Stock solutions of peptides in water were prepared at different concentrations, and aliquots were added to 0.5 mL of liposome solutions to give different lipid/peptide ratios. The samples were incubated at 25 °C for 15 min and then analyzed for fluorescence changes. Complete dye release was obtained by lysing the vesicles with Triton X-100 (0.5%). The % lysis could then be calculated by the equation

$$\% \text{ lysis} = \frac{I(\text{f}) - I(0)}{I(\text{tot}) - I(0)} \times 100, \quad (1)$$

where $I(\text{f})$ = 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 measuring the resonance energy transfer between two fluorescent probes NBD-PE and RH-PE (Struck et al., 1981). Liposomes labeled with 0.5% (w/w) of the probes were mixed in a 1:4 ratio with unlabeled liposomes and diluted to 0.1 mg/mL in either PBS or 10 mM phosphate buffer. Upon fusion of the liposomes, dilution of the mixed probes diminishes the efficiency of energy transfer from NBD-PE to RH-PE, resulting in an increase of the NBD-PE fluorescence. The extent of fusion is measured by exciting at a wavelength of 470 nm and monitoring the emission at 535 nm. As in the lysis assay, aliquots of the peptide stock solutions were added to 0.5 mL of 0.1 mg/mL liposomes. Samples were incubated at 25 °C for 15 min and changes in fluorescence observed. The liposome samples labeled with 0.5% probes without peptides were taken to be a 0% fusion standard while a sample of liposomes labeled with 0.10% NBD-PE and RH-PE was taken as a 100% fusion standard. The % fusion can be calculated using the formula

$$\% \text{ fusion} = \frac{F(\text{obs}) - F(0)}{F(\text{tot}) - F(0)} \times 100, \quad (2)$$

where $F(\text{obs})$ = the observed fluorescence intensity at 535 nm, $F(0)$ = the fluorescence of the 0.5% mixed probes standard without peptides, and $F(\text{tot})$ = the fluorescence intensity of the 0.10% mixed probes standard.

Absorbance measurements

The changes in the size of the liposomes by aggregation or fusion were assessed by monitoring the absorbance at 400 nm. Samples (0.5 mL) were prepared by adding aliquots of peptide stock solutions to 0.1 mg/mL liposome

preparations in buffer. The samples were then incubated for 15 min at 25 °C. The absorbance at 400 nm of each sample was measured on a Hitachi U2000 UV/visible spectrometer. The extent of aggregation or fusion was estimated by comparing the increase in absorbance caused by the defensins with melittin (DPPC:Chol) and poly-L-lysine (DPPC:DOPS).

Tryptophan fluorescence spectroscopy

The local environment of the tryptophan residue in HNP-1 and aHNP-1 was monitored by fluorescence spectroscopy. Samples (0.5 mL) of peptides (0.05 mg/mL) in buffer (PBS or 5 mM phosphate) and samples of peptides with lipids (0.05 mg/mL:1.0 mg/mL) were prepared and the fluorescence measured on a Hitachi F4500 fluorimeter by using an excitation wavelength of 280 nm and an emission scan range of 300–450 nm.

Circular dichroism spectroscopy

Circular dichroism spectra were obtained on a Jasco J-600 spectrometer. Samples were scanned from 250 nm to 190 nm (PB) or to 195 nm (PBS) using cells with a pathlength of 1.0 mm. The sample chamber was purged with nitrogen gas to minimize interferences from atmospheric oxygen. Each spectrum was the average of four scans. Peptide concentrations were 0.05 mg/mL, while lipid concentrations were 1.0 mg/mL.

Lipid binding assay

The binding of peptide to lipid was assayed by the difference in sedimentation on a sucrose gradient between free defensin and defensin bound to lipid. Liposomes containing 0.5% RH-PE (5 mg/mL) were added in 100-μL aliquots to 25 μL of peptide stock solutions and diluted to 250 μL total volume with buffer. The samples were incubated for 15 min at 25 °C. Sucrose density gradients from 0% to 40% in buffer were made on a Biocomp Gradient Master 105 (Fredericton, New Brunswick, Canada) and chilled to 4 °C. The samples were then layered on the top of the gradient and centrifuged in a Beckman LS-5 ultracentrifuge equipped with an SW-41 swinging bucket rotor for 2 h at 35,000 rpm, 4 °C. The gradients were fractionated on an ISCO model 185 fractionator and the fractions were collected with a Retriever II fraction collector. Each fraction was analyzed for the presence of lipid by measuring the absorbance at 570 nm. Peptide analysis was performed on each fraction using a Waters HPLC system equipped with a Waters C18 μbondapak column (3.5 × 25 mm) and a Model 480 UV/visible detector. The peptides were eluted with a gradient of 0.05% TFA in water and acetonitrile and detected at a wavelength of 214 nm. Lipid-to-defensin ratios were calculated on a mole/mole basis.

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