A Novel Linear Amphipathic β-Sheet Cationic Antimicrobial Peptide with Enhanced Selectivity for Bacterial Lipids*

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All known naturally occurring linear cationic peptides adopt an amphipathic α-helical conformation upon binding to lipids as an initial step in the induction of cell leakage. We designed an 18-residue peptide, (KIGAKI)3-NH2, that has no amphipathic character as an α-helix but can form a highly amphipathic β-sheet. When bound to lipids, (KIGAKI)3-NH2 did indeed form a β-sheet structure as evidenced by Fourier transform infrared and circular dichroism spectroscopy. The antimicrobial activity of this peptide was compared with that of (KIAGKIA)3-NH2, and it was better than that of GMASKAGAIAGKIAYKAL-NH2, all of which form amphipathic α-helices when bound to membranes. (KIGAKI)3-NH2 was much less effective at inducing leakage in lipid vesicles composed of mixtures of the acidic lipid, phosphatidylglycerol, and the neutral lipid, phosphatidylcholine, as compared with the other peptides. However, when phosphatidylethanolamine replaced phosphatidylcholine, the lytic potency of PGLa and the β-helical model peptides was reduced, whereas that of (KIGAKI)3-NH2 was improved. Fluorescence experiments using analogs containing a single tryptophan residue showed significant differences between (KIGAKI)3-NH2 and the α-helical peptides in their interactions with lipid vesicles. Because the data suggest enhanced selectivity between bacterial and mammalian lipids, linear amphipathic β-sheet peptides such as (KIGAKI)3-NH2 warrant further investigation as potential antimicrobial agents.

A diverse collection of host defense peptides discovered in a wide range of species shares the common characteristics of a net positive charge and the ability to form amphipathic structures. Many of these peptides appear to exert their protective effect by permeabilizing the membranes of target organisms. The efficacy of these peptides results from their ability to disrupt prokaryotic membranes at concentrations that are not harmful to host membranes. (1)

Frog skin is a particularly rich source of defense peptides, including magainins (2) and PGLa(3) (4). These linear cationic peptides containing 21–23 amino acid residues demonstrate broad antimicrobial activity; however, relatively high concentrations are necessary to kill most target organisms. It is possible to enhance antimicrobial activity through simple modifications of the native peptides. For instance, substituting Ala for Glu-19 in magainin 2-amide substantially increases antimicrobial activity (5, 6).

Conformational studies of magainins revealed a significant increase in α-helical content in nonpolar solvents or upon binding to lipid bilayers (7). Subsequently, a large body of experimental evidence has accumulated to support the notion that these peptides are mostly α-helical when bound to membranes (8–13).

PGLa (see Table I) possesses greater Gram-positive antimicrobial activity than magainin 2 (1). PGLa is largely α-helical when bound to lipid bilayers (14) and appears to form pores in membranes (15). A more potent derivative of PGLa in which glycines at positions 1 and 8 are replaced by lysines contains three heptamer repeats of sequence KXXXKXXX, where X represents a nonpolar residue as shown in Table I. A 21-residue-amidated peptide containing three heptamer repeats of KIAGKIA possesses high antimicrobial and relatively low hemolytic activity (1). When KIAGKIA adopts an α-helical conformation, the peptide is highly amphipathic with all six lysines clustered on the helical face (Fig. 1). Using a consensus hydrophobicity scale, the hydrophobic moment, a quantitative measure of amphipathicity (16) for this peptide, is much greater as an α-helix (0.40) as compared with a β-sheet (0.16).

To determine whether a highly amphipathic α-helix is a prerequisite for potent antimicrobial activity, we synthesized a peptide KLAGLAK with a similar amino acid content but with a heptamer repeat that separates the six lysines into two groups of three on the helical face, resulting in a large decrease in hydrophobic moment to 0.25 (see Table I and Fig. 1). Like KIAGKIA, KLAGLAK cannot form a highly amphipathic β-sheet structure. Because all known naturally occurring linear antimicrobial peptides have the capability to form at least a reasonably amphipathic α-helical structure (17, 18), we de-
signed a new peptide that can form a highly amphipathic β-sheet rather than an α-helix. This 18-residue peptide contains the hexameric repeat KIGAKI (Table I). The values of hydrophobic moment as an α-helix and a β-sheet are 0 and 0.63, respectively, as shown in Fig. 1. The three model peptides, KIAGKIA, KLAGLAK, and KIGAKI, possess equal charge (+7) and nearly equal mean hydrophobicity values. We compared the antimicrobial and hemolytic activity of these peptides, used CD and FTIR spectroscopy to determine the conformation of the peptides in solution and when bound to lipid bilayers, and measured the ability of the peptides to induce leakage in and bind to LUV of varying lipid composition. Our results show that KIGAKI does indeed adopt a β-sheet conformation when bound to lipids and is comparable in antimicrobial activity to KIAGKIA and KLAGLAK. KIGAKI appears to possess greater selectivity for bacterial versus mammalian lipids as compared with the α-helical peptides tested.

EXPERIMENTAL PROCEDURES

Materials—All peptides were synthesized using Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry on an advanced chem tech model 90 peptide synthesizer. The crude peptides were purified by reverse phase high pressure liquid chromatography. Purity was checked by reverse phase high pressure liquid chromatography, capillary electrophoresis, and electrospray mass spectrometry. POPC, POPE, POPG, and *Escherichia coli* polar lipid extract were used as supplied from Avanti Polar Lipids, Inc. *E. coli* DPG, calcine, TFE, and buffer materials were from Sigma. Phosphorus content in lipid stock solutions was determined by a spectrophotometric analysis (19).

**Antimicrobial and Hemolytic Assays—**Antimicrobial susceptibility testing against *Staphylococcus aureus* (ATCC 29213), *E. coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853) was performed.
of peptide was added to achieve the desired lipid-to-peptide ratio (256–
were added to 1.5 ml of buffer in a stirred cuvette at 25 °C. An aliquot
measuring the time-dependent increase in fluorescence of calcein (ex-
tored using a Perkin-Elmer LS-5B luminescence spectrometer by
adex G-50 column and calcein-free buffer. Calcein leakage was moni-
ted using a modification of the National Committee for Clinical Laboratory
 Standards microdilution broth assay (6). Mueller-Hinton broth (BBL)
was used for diluting the peptide stock solution and the bacterial
inoculum. The inoculum was prepared from mid-logarithmic phase
cultures. Microtiter plate wells received aliquots of 100 μl each of
the inoculum and peptide dilution. The final concentration of the peptide
solution ranged from 0.25 to 256 μg/ml in 2-fold dilutions. The final
concentration of bacteria in the wells was 5 × 10^7 colony-forming
units/ml. Peptides were tested in duplicate. In addition to the test
peptide, three standard peptides and a nontreated growth control were
included to validate the assay. The microtiter plates were incubated
overnight at 37 °C, and the absorbance was measured at 600 nm. MIC
is defined as the lowest concentration of peptide that completely inhib-
its the growth of the organism. Hemolysis at peptide concentrations of
500 μg/ml was determined using a 5% suspension of freshly drawn
human erythrocytes, which had been washed twice in phosphate-buff-
ered saline. After incubation at 37 °C for 30 min, the suspension was
centrifuged at 10,000 × g for 10 min, and the absorbance at 400 nm was
measured. Complete hemolysis was determined by adding 0.2% Triton
X-100 in the place of the peptide.

CD Spectroscopy—CD spectra were measured using a Jasco J-715
spectropolarimeter. Spectra were recorded from 250 to 190 nm at a
sensitivity of 100 millidegrees, resolution of 0.1 nm, response of 8 s,
bandwidth of 1.0 nm, and a scan speed of 20 nm/min with a single
accumulation. The buffer contained 10 mM potassium phosphate, 150
mM KCl, 1 mM EDTA, pH 7.0. The peptide concentration in buffer and
TFE/buffer mixtures was 20 μM. LUV were prepared from aqueous
dispersions of POPG at a concentration of ~1 mg/ml in phosphate
buffer. After 5 freeze/thaw cycles, the mixture was extruded 10 times
through a 0.1-m pore polycarbonate membrane in an Avanti mini-
extruder apparatus, resulting in ~100-nm diameter LUV. The lipid and
peptide concentrations in the cuvette were 100 and 5 μM, respectively.

FTIR Spectroscopy—Mixtures of POPG (4 μmol) and peptide (0.2
μmol) were co-dissolved in 2:1 CHCl3/CH3OH. The solvent was removed
by evaporation followed by evaporation under high vacuum. The mix-
tures were suspended in D2O buffer (20 mM PIPES, 1 mM EGTA, pH
7.0), isolated by centrifugation, and placed between CaF2 windows
using a 25-μm Teflon spacer. FTIR spectra were collected using a
Mattson Polaris FTIR spectrometer with a HgCdTe detector. A total of
250 interferograms were co-added, and Fourier was transformed with
triangular apodization to generate absorbance spectra with 2 cm
resolution and data points encoded every 1 cm
 with a signal-to-noise
ratio of better than 500 (6).

Peptide-induced Leakage from Calcein-loaded LUV—The ability of
the peptides to release calcein (Mw = 623) from LUV of varying lipid
composition was compared. LUV were prepared as above with the
exception that the buffer consisted of 50 mM HEPES, 100 mM NaCl, 0.3
mM EDTA, 80 mM calcein, pH 7.4. Calcein-loaded vesicles were separ-
ated from free calcein by size exclusion chromatography using a Seph-
adex G-50 column and calcein-free buffer. Calcein leakage was moni-
tored using a Perkin-Elmer LS-5B luminescence spectrometer by
measuring the time-dependent increase in fluorescence of calcein (ex-
citation = 490 nm, emission = 520 nm). LUV containing 8 nmol of
peptide were added to 1.5 ml of buffer in a stirred cuvette at 25 °C. An aliquot
of peptide was added to achieve the desired lipid-to-peptide ratio (256–
8). Complete leakage was determined by the addition of 20 μl of 10%
Triton X-100. Each value represents at least nine different measure-
ments of at least three different LUV preparations.

Fluorescence Measurement of Peptide Binding to LUV—Peptide
binding to LUV was assessed by measuring the shift in the emission
maximum of the tryptophan residue in W-KIAGKIA, W-KLAGLAK,
and W-KIGAKI. LUV were prepared as described previously for the
leakage experiments with the exception that calcein was omitted.
Fluorescence spectra were collected from 290 to 400 nm using an
excitation wavelength of 280 nm. The peptide concentration was kept
constant at 3 μM.

Differential Scanning Calorimetry—Lipid films were made by solv-
ent evaporation under nitrogen of solutions of DiPoPE in chloroform/
methanol (2:1, v/v). Last traces of solvent were removed in vacuum for
2 h. The films were then hydrated by vortexing at room temperature
with 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN3, pH
7.4. The final lipid concentration was 3.7 mg/ml. The lipid suspension
was degassed under vacuum before being loaded into a NanoCal high
sensitivity scanning calorimeter (CSC, Spanish Forks, UT). A heating
scan rate of 0.75 °C/min was generally employed. Transition tempera-
ture (Tm) was fitted using parameters to describe equilibrium with a
single van’t Hoff enthalpy and the transition temperature reported as
that for the fitted curve. Data were analyzed with the program Origin,
version 5.0.

RESULTS

Comparison of Antimicrobial and Hemolytic Activities of the
Peptides—The antimicrobial and hemolytic activities of the
three model peptides are compared with those of magainin
2-amide and PGLa in Table II. KIAGKIA, KLAGLAK, and
KIGAKI are significantly more potent against all three microor-
ganisms than either magainin 2-amide or PGLa. At 500 μg/ml,
all of the peptides tested showed little hemolytic activity.

Secondary Structure of the Peptides—The conformation of the
peptides was assessed by CD and FTIR spectroscopy. As
shown in Fig. 2, the CD spectra of the three model peptides are
characteristic of a random structure (with a minimum below
200 nm) in buffer (panel A). TFE is often used as a membrane mimetic
to lower the polarity of the solvent. In 50% TFE (panel
B), the spectra show the minima near 208 and 222 nm, which
indicates α-helical content. The amount of helical structure in
KIAGKIA and KLAGLAK is approximately the same, whereas
the helical content of KIGAKI is slightly lower under these
conditions. Finally, the spectra of the peptides in the presence
of POPG LUV (lipid-to-peptide ratio of 20:1) reveal that both
KIAGKIA and KLAGLAK are mainly α-helical with the helical con-
tent of KIGAKI slightly greater than that of KLAGLAK.

The conformation of KIGAKI is distinctly different in the presence
of POPG vesicles as compared with TFE. This spectrum with a single minimum just below 220 nm suggests β-sheet
structure (20).

FIG. 2. CD spectra of KIAGKIA (dashed line), KLAGLAK (dash-dotted line), and
KIGAKI (solid line) in (A) aqueous buffer,
(b) 50% TFE/buffer, and (C) POPG LUV
(lipid-to-peptide ratio = 20).
The amide I’ vibrational bands of the three peptides bound to POPG at a 20:1 molar ratio of lipid to peptide are shown in Fig. 3. The amide I’ band for KIAGKIA is centered close to 1650 cm⁻¹, which primarily indicates α-helical conformation. In contrast, the band for KLGLAK is shifted slightly to lower frequency and is broader, suggesting less α-helical content in agreement with the CD data. The amide band for KIAGKI is markedly different with a maximum below 1620 cm⁻¹ and a small peak near 1680 cm⁻¹, consistent with β-sheet conformation (21).

Peptide-induced Leakage from Calcein-loaded LUV—A comparison of PGLa and the three model peptides in their ability to release calcein from LUV with varying lipid composition is shown in Fig. 4. All peptides were able to induce leakage from anionic LUV composed of POPG (panel A). The order of potency is PGLa > KIAGKIA > KLGLAK > KIAGKI. Thus, PGLa, the peptide with the weakest antimicrobial activity, is the most potent in inducing calcein release. In POPC LUV (panel B), the level of calcein release was much lower than in POPG LUV; however, it is clear that the three α-helical peptides (PGLa, KIAGKI, and KLGLAK) were more active than KIAGKI in permeabilizing POPC vesicles.

To better relate the leakage experiments to bacterial membranes, calcein-loaded LUV composed of E. coli polar lipids were tested. These results shown in panel C are strikingly different. The order of potency here is KIAGKI = KIAGKIA > KLGLAK > PGLa. The correlation between the antimicrobial activity and leakage in E. coli LUV is much better than in POPG or POPC LUV. The composition of E. coli polar lipids is 67% PE, 23% phosphatidylglycerol, and 10% DPG. Therefore, we decided to compare leakage rates in LUV containing POPG as the anionic component and either POPC or POPE as the neutral component. The ability of the peptides to increase the permeability of LUV with neutral-to-acidic lipid ratios of 1:1, 2:1, 3:1, and 4:1 is shown in Fig. 5. The general trend is a reduction in leakage as the neutral-to-acidic lipid ratio increases. In POPC/POPG LUV (panels A–D), PGLa is the most potent peptide at all ratios followed closely by KIAGKIA. The lytic activity of KLGLAK falls off sharply at higher neutral lipid content. KIAGKI is the least potent peptide at all ratios. The results change markedly, however, when POPC is replaced by POPE (panels E–H). At 2:1 POPE/POPG, leakage rates for the three α-helical peptides decrease significantly, whereas the activity of KIAGKI is slightly enhanced in comparison to 2:1 POPC/POPG. By 4:1 POPE/POPG, the activity of PGLa is quite low even at high peptide levels. KIAGKI and KIGAKI are the most potent with leakage rates only slightly below those observed in E. coli LUV.

Because E. coli plasma membrane lipids contain DPG as a third major lipid component, LUV were prepared with a ternary mixture of POPG, DPG, and either POPE or POPC (Fig. 6). LUV containing POPG as the neutral lipid (panel A) are highly susceptible to the lytic activity of the α-helical peptides but not the β-sheet peptide. In contrast, the properties of LUV formed by combining POPE, POPG, and DPG (panel B) are quite similar to those of LUV made from the E. coli lipid extract (Fig. 4C). Only the activity of KIAGKI is enhanced by replacing POPC with POPE in the ternary mixture.

Fluorescence Emission in Tryptophan-containing Analogs—W-KIAGKIA, W-KLGLAK, and W-KIAGKI (see Table I) were synthesized to assess the interaction of the peptides with LUV of varying lipid composition. Because each peptide contains a single tryptophan residue, fluorescence emission can be used to monitor binding and local polarity. The antimicrobial activity and secondary structure of these analogs were similar to those of the parent peptides (data not shown). In aqueous solution, the emission maxima for the three peptides were nearly identical (354–356 nm). The change in emission maximum to lower the wavelength (blue shift) under different conditions is shown in Fig. 7. In 50% TFE, the emission maximum decreased by 4–5 nm.
nm for each peptide. Emission spectra in the presence of LUV were measured at a lipid-to-peptide ratio of 20. The shift observed in the presence of POPC LUV (3 nm) was smaller than in 50% TFE for all peptides. The largest blue shifts were observed in the presence of POPG LUV. For W-KIAGKIA (Fig. 7A), the shifts in the presence of POPC/POPG were slightly larger than POPE/POPG at ratios above 1:1. This enhancement is much greater for W-KLAGLAK (Fig. 7B), where the blue shifts with POPC/POPG LUV are approximately twice as large as the corresponding POPE/POPG LUV at neutral-to-acidic ratios ≥2. The presence of either POPC or POPE decreased the magnitude of the blue shifts observed for W-KIAGKIA (Fig. 7C) to a much greater degree as compared with the other peptides; however, the shifts observed with POPE/POPG LUV were greater than those with POPC/POPG LUV at all ratios below 4.

The blue shifts observed for W-KIAGKIA in the presence of LUV containing E. coli lipids or ternary mixtures of POPC/POPG/DPG or POPE/POPG/DPG were nearly equal (Fig. 7A). For W-KLAGLAK, however, the shift observed in the presence of POPC/POPG/DPG was greater than that with either E. coli lipids or POPE/POPG/DPG (Fig. 7B). Only W-KIAGKIA showed larger blue shifts in the presence of E. coli lipids or POPE/POPG/DPG as compared with POPC/POPG/DPG (Fig. 7C).

Differential Scanning Calorimetry—An indication of the effects of membrane additives on the curvature properties of membranes may be assessed by their effect on the bilayer-to-hexagonal phase \( T_{H} \). Only KLAGLAK caused an appreciable increase in \( T_{H} \). The change in \( T_{H} \) of DiPoPE as a function of peptide concentration is shown in Table III.

**DISCUSSION**

PGLa and the three model peptides, KIAGKIA, KLAGLAK, and KIGAKI, possess no defined secondary structure in solution but adopt a conformation that appears to maximize amphipathic character upon interacting with lipid bilayers. Like PGLa, KIAGKIA and KLAGLAK can form an amphipathic \( \alpha \)-helix at the bilayer surface. KIGAKI was designed to mimic KIAGKIA and KLAGLAK in terms of net charge and hydrophobicity but to form an amphipathic \( \beta \)-sheet instead of an \( \alpha \)-helix. In 50% TFE, KIGAKI is mainly helical, but when bound to LUV, the drive to form an amphipathic structure dominates, and the resulting conformation is \( \beta \)-sheet as shown by CD and FTIR spectroscopy (Figs. 2 and 3). A comparison of antimicrobial activity (Table II) shows that KIAGKIA and KIGAKI are significantly more active than PGLa with KLAGLAK only slightly active. Notably, KIGAKI is the least hemolytic of the three model peptides and approximately the same as magainin 2-amide and PGLa.

Several other linear amphipathic \( \beta \)-sheet peptides have been examined previously. An 18-residue Lys-Leu repeat was re-
ported to have no appreciable antimicrobial or hemolytic activity (22). Peptides containing 6–12 residues with repeats of either SVKV or Lys-Val were shown to adopt a β-sheet structure in the presence of lipid (23). Although some of these peptides could induce leakage in lipid vesicles, none were antimicrobial below a concentration of 100 μg/ml. The peptide FKVKFKVKVK was able to inhibit the growth of E. coli, S. aureus, and P. aeruginosa at concentrations comparable to the peptides in this study, although the hemolytic activity of this peptide was not tested (24). FKVKFKVKVK was shown by CD to adopt a β-sheet structure in the presence of either 50% TFE or 25 mM sodium dodecyl sulfate.

Recently, a series of (KL)nK-NH₂ peptides containing 9–15 residues (all dansylated at the NH₂ terminus) was studied by Castano et al. (25). The amide I vibrational band in the infrared spectra (either dry, at the air/water interface, or inserted into a lipid monolayer) of these peptides was very similar to that of KIGAKI (Fig. 3), centered near 1620 cm⁻¹. All of the peptides induced both leakage in lipid vesicles and hemolysis with activity increasing as a function of length. The antimicrobial activity of these peptides was not examined.

Shai and co-workers (26–28) studied diastereomeric antimicrobial peptides based on 12-mers containing Lys and Leu or derivatives of pardaxin (28). These peptides were derived from all l-amino acid parent compounds that can form amphipathic α-helices. Based on changes in the amide I infrared band, the conformation of the diastereomeric peptides was interpreted to be mainly β-sheet; however, the appearance of the amide I infrared band is significantly different than that of the peptides in this study or those examined by Castano et al. (25). Instead of a relatively narrow band near 1620 cm⁻¹, the diastereomeric peptides gave rise to broad bands centered between 1640 and 1650 cm⁻¹. Clearly, the conformation of these peptides is significantly different than that of KIGAKI when bound to lipid bilayers.

PGLa, KIAGKIA, KLAGLAK, and particularly KIGAKI were not very effective at inducing leakage in phosphatidylcholine LUV, but all were significantly more active with phosphatidylglycerol LUV. The binding of PGLa to membranes was shown recently to be dominated by electrostatic and not hydrophobic effects (15). Thus, the increased binding probably accounts for the greater leakage rates observed in POGP versus POPC LUV. However, at lower peptide levels, PGLa is more effective than the other peptides at inducing leakage in LUV containing POPG alone or POPC/POPG mixtures (Fig. 5). This contrasts with the antimicrobial activities (Table II) that show PGLa as the least potent peptide. However, in LUV composed of E. coli polar lipids, the activity of PGLa is markedly reduced, whereas that of KIGAKI is enhanced compared with the other peptides (Fig. 4).

Because PE is the major uncharged polar lipid in E. coli plasma membranes, we examined the effect of replacing POPC by POPE. In LUV containing equimolar amounts of POPG and neutral lipid, only slight differences were observed. As the proportion of POPE in the LUV increased, however, the leakage rates resembled those in E. coli LUV more closely (Fig. 5). In a comparison of ternary mixtures of either POPE/POPG or POPC/POPG/POPE, the presence of phosphatidylcholine greatly enhances the activity of PGLa and the other α-helical peptides while reducing the activity of KIGAKI (Fig. 6).

One may legitimately question whether the relatively high level of peptide necessary to induce leakage in LUV composed of E. coli polar lipid extract and other lipid mixtures with a high proportion of PE is relevant to the inhibitory effect upon bacterial growth. We can estimate the number of peptide molecules/bacterium in the MIC assay. The assay mixture contains 10⁵ bacteria in a volume of 0.2 ml. The lowest MIC value reported here is 8 μg/ml. With a molecular weight of ~2000, the amount of peptide in the assay (1.6 μg) translates to ~4 × 10¹⁴ molecules. Thus, there are ~4 × 10¹⁴ peptide molecules/bacterium even at the lowest MIC value. How does this relate to the number of lipid molecules in the plasma membrane? For a large bacterium of size of 2 × 4 μm (i.e. even larger than the bacteria tested here), the surface area is ~3 × 10²⁷ nm². If the average surface area of a lipid molecule is estimated to be ~0.7 nm², then the number of lipid molecules on the outer surface of the plasma membrane is ~4 × 10⁷. Therefore, conservatively, there are ~100 peptide molecules for each lipid molecule on the exterior of the bacterial plasma membrane. For smaller bacteria or for higher MIC values, the number of peptides/lipid is proportionally higher.

This does not mean, however, that all of the peptides are bound to the plasma membrane. Many peptide molecules may be bound to lipopolysaccharide, peptidoglycan, teichoic acid, or other components of the cell envelope beyond the plasma membrane, whereas other peptides may remain free in solution. The estimate does point out, however, that a real potential exists for a very large number of peptides to interact with the plasma membrane surface at antimicrobial concentrations. Further experiments will be necessary to determine the binding affinity and location of the peptides on intact bacteria.

What is the explanation for the observed differences between phosphatidylcholine and PE in the leakage experiments? One obvious possibility is that the peptides bind differently to LUV containing phosphatidylcholine or PE as the neutral lipid. We used tryptophan-containing analogs of the three model peptides to study their interactions with LUV. A blue shift in the maximum of the tryptophan emission band results from the decrease in polarity surrounding the indole side chain as the peptide binds to and penetrates the bilayer surface. Minimal and maximal blue shifts were observed in the presence of POPC and POPG LUV, respectively (Fig. 7). A comparison of LUV with either POPC/POPG or POPE/POPG reveals that W-KIAGKIA and W-KLAGLAK showed a larger blue shift with
POPC/POPG. In contrast, W-KIGAKI showed a larger blue shift with POPE/POPG. An increased blue shift could result from more peptide molecules binding to LUV, a more hydrophobic environment of the tryptophan side chains in bound peptides (i.e. deeper insertion into the bilayers) or some combination of the two. Thus, only a qualitative assessment of peptide-lipid interaction can be inferred from the data. In the case of POPG, although all three peptides demonstrated a large shift (15–23 nm), suggesting that a significant fraction of the peptide molecules is bound to LUV, the precise fraction cannot be determined. Moreover, a small blue shift does not necessarily mean that little or no peptide is bound. If peptide binding to the surface of LUV is not associated with a change in polarity surrounding the indole ring, no change in the fluorescence emission spectrum would be expected. The large differences in blue shifts observed between the α-helical and β-sheet peptides suggest that the manner by which these peptides interact with LUV containing a mixture of neutral and acidic lipids is dissimilar. We plan to carry out surface plasmon resonance spectroscopy experiments to measure binding to LUV directly. Once binding is determined independently, a comparison of changes in the fluorescence emission spectrum will be more informative.

The curvature-modulating property (29) of KLAGLAK differs from KIAGKIA and KIGAKI in that KLAGLAK promotes a more positive membrane curvature (Table III). This property appears to have consequences for the lipid dependence of the lytic activity of magainin 2, which was shown to induce positive curvature to a slightly greater extent than KLAGLAK (30). The curvature effects of these peptides can be rationalized in terms of their structure. In comparing the two α-helical model peptides, the six lysine residues are clustered together in KIAGKIA, whereas in KLAGLAK they are separated by three glycine residues in a helical wheel projection (Fig. 1). Lysines have a special role in the binding of peptides to bilayers because of the amphiphilic nature of their side chain (i.e. four

![FIG. 7. Shifts in the emission maximum of tryptophan fluorescence of (A) W-KIAGKIA, (B) W-KLAGLAK, and (C) W-KIGAKI. The emission peak positions in aqueous solution were 356 nm (W-KIAGKIA), 354 nm (W-KLAGLAK), and 355 nm (W-KIGAKI). The peptide concentration was 3 μM. The lipid-to-peptide ratio was 20 for measurements in the presence of LUV. The LUV abbreviations are PC, POPC; PC/PG, POPC/POPG; PE/PG, POPE/POPG; PG, POPG; E, coli, E. coli; polar lipids; PE/PG/DPG, POPE/POPG/DPG (6.7:2.3:1); and PC/PG/DPG, POPC/POPG/DPG (6.7:2.3:1). Standard errors are <± 2 nm for all measurements.]

<table>
<thead>
<tr>
<th>Table II</th>
<th>Antimicrobial and hemolytic activities</th>
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<tbody>
<tr>
<td>Peptide</td>
<td>Minimum inhibitory concentrationb</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
</tr>
<tr>
<td>Magainin 2-NH₂</td>
<td>64</td>
</tr>
<tr>
<td>PGLa</td>
<td>32</td>
</tr>
<tr>
<td>KIAGKIA</td>
<td>8</td>
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<tr>
<td>KLAGLAK</td>
<td>8</td>
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<tr>
<td>KIGAKI</td>
<td>8</td>
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a See Table I for peptide sequences.
b For minimum inhibitory concentrations, the differences of greater than a factor of two are considered significant.

<table>
<thead>
<tr>
<th>Table III</th>
<th>Effect of peptides on bilayer-to-hexagonal phase transition temperature (T_H) of DiPoPE</th>
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<tr>
<td>Peptide</td>
<td>∆T_H/∆ mole fractiona</td>
</tr>
<tr>
<td>KIAGKIA</td>
<td>132 ± 65</td>
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<tr>
<td>KLAGLAK</td>
<td>1077 ± 52</td>
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<tr>
<td>KIGAKI</td>
<td>157 ± 142</td>
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</table>

a Slope of T_H versus mole fraction of peptide determined by regression analysis.
hydrophobic methylene groups between the α-carbon atom and the side chain amino group) (31). In the case of KIAGKIA, the clustered lysine residues will allow the peptide to insert more deeply in the bilayer and thereby promote less positive curvature (32). In KIAGKIA, the two groups of lysine residues are at the interface between the hydrophobic and hydrophilic sides of the amphipathic helix as they are in class A peptides (33), resulting in an increased positive curvature. This difference in insertion might not be reflected in the fluorescent properties of the tryptophan-substituted analogs, because the Trp residues should mimic the interaction with DiPoPE. If the interactions with DiPoPE can be generalized to other lipids, the membrane disruption caused by KIAGKIA and KIGAKI may well be different from magainin-like peptides.

We have demonstrated that KIAGKIA, designed to adopt a highly amphipathic β-sheet, possesses a combination of equivalent antimicrobial activity and superior selectivity compared with the α-helical peptides in this study. Since KIAGKIA appears to bind preferentially to PE-containing bilayers and induces leakage in LUV-rich in PE to a greater extent than the α-helical peptides while maintaining low hemolytic activity, a more detailed examination of the mechanism of this peptide and an exploration of other peptides with amphipathic β-sheet potential is warranted.

REFERENCES

A Novel Linear Amphipathic β-Sheet Cationic Antimicrobial Peptide with Enhanced Selectivity for Bacterial Lipids

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