Molecular Basis for Prokaryotic Specificity of Magainin-Induced Lysis†

Ewan M. Tytler,* G. M. Anantharamaiah, Donald E. Walker, Vinod K. Mishra, M. N. Palgunachari, and Jere P. Segrest

Atherosclerosis Research Unit, Departments of Medicine and Biochemistry, University of Alabama at Birmingham Medical Center, Birmingham, Alabama 35294

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ABSTRACT: Magainins and mastoparans are examples of peptide antibiotics and peptide venoms, respectively. They have been grouped together as class L amphipathic helixes [Segrest, J. P., et al. (1990) *Proteins 8*, 103−117] because of similarities in the distribution of Lys residues along the polar face of the helix. Class L venoms lyse both eukaryotic and prokaryotic cells whereas class L antibiotics specifically lyse bacteria. The structural basis for the specificity of class L antibiotics is not well understood. Sequence analysis showed that class L antibiotics have a Glu residue on the nonpolar face of the amphipathic helix; this is absent from class L venoms. We synthesized three model class L peptides with or without Glu on the nonpolar face: $18L_{MG}$ (LGSIWKFIKAFVGGIKKF), $[E^{14}]18L_{MG}$ and $[G^5,E^{14}]18L_{MG}$. Hemolysis, bacteriolysis, and bacteriostasis studies using these peptides showed that the specificity of lysis is due to both the presence of a Glu residue on the nonpolar face of the helix and the bulk of the nonpolar face. Studies using large unilamellar phospholipid vesicles showed that the inclusion of cholesterol greatly inhibited leakage by the two Glu-containing peptides. These results cannot be attributed to changes in the phase behavior of the lipids caused by the inclusion of cholesterol or to differences in the secondary structure of the peptides. These results suggest that eukaryotic cells are resistant to lysis by magainins because of peptide−cholesterol interactions in their membranes that inhibit the formation of peptide structures capable of lysis, perhaps by hydrogen bonding between Glu and cholesterol. Bacterial membranes, lacking cholesterol, are susceptible to lysis by magainins.

Since the recent discovery of the magainins (Zasloff, 1987; Giovannini et al., 1987), there has been considerable interest in the structure−function relationships of these peptides (Table 1). Little is known about the mechanism and specificity of the antibacterial activity of magainins. Many of these studies are aimed at producing peptides with increased activity and potential clinical applications (Cruciani et al., 1991). Increased antibacterial activity with minimal cytotoxicity is needed to improve the therapeutic index of these compounds. To achieve this goal, the structural basis for the prokaryotic specificity of the magainins needs to be elucidated.

Magainins have been classified as class L (lytic) amphipathic α-helixes (Segrest et al., 1990) because of their secondary structural homology to class L peptides from bee and wasp venoms, such as mastoparans and bombolitins. Class L amphipathic helixes are cationic. They possess a high hydrophobic moment per residue, a broad nonpolar face, and a narrow polar face, with bimodal clustering of Lys residues (Segrest et al., 1990, 1994). The magainins are specifically toxic to bacteria, fungi, and protozoa and are not hemolytic (Zasloff, 1987), while the mastoparans are both antibacterial and hemolytic (Argiolas & Pisano, 1983, 1984; Katsu et al., 1990). Some empirical studies have shown that the antibacterial activity of magainin 2 (MG-2)[1] from *Xenopus laevis* (Zasloff, 1987) can be increased by amino acid substitutions that increase the helical propensity (Chen et al., 1988). While most of the peptide analogs designed are based on the manipulation of primary structure, a systematic approach to understanding the specificity of the antibacterial activity of magainins has not been described.

We have previously shown that the hemolytic activity of model class L peptides can be explained by the reciprocal wedge model (Tytler et al., 1993). In this model, class L peptides fold into an amphipathic α-helical structure, upon association with phospholipid, such that the polar face of the peptide is associated with the polar head group of the phospholipid. The lipid-associated class L amphipathic helix, when viewed in cross section, is an inverted wedge shape in which the hydrophobic face of the helix forms the base of the inverted wedge while the polar face forms the apex. This structure is expected to force the alkyl chains of phospholipid apart, inducing a negative curvature on the membrane surface, and thus will cause localized disruption of the lipid bilayer structure. This hypothesis is supported by the results of differential scanning calorimetry studies that indicate that class L peptides promote the formation of inverted lipid phases in phosphatidylethanolamine. We have

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* Author to whom correspondence should be addressed.

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[1] Abbreviations: C, cholesterol; CD, circular dichroism; CF, carboxyfluorescein; CFU, colony-forming units; C_l, lethal concentration; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; LCAT, lecithin:cholesterol acyltransferase; LPS, lipopolysaccharide; LUV(s), large unilamellar vesicle(s); MG-2, magainin 2; NAPB, 10 mM sodium phosphate buffer, pH 7.4; ONPG, *o*-nitrophenyl β-D-galactoside; PBS, phosphate-buffered saline, pH 7.4; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PPC, monopalmitoylphosphatidylcholine; TFE, trifluoroethanol; TSB, tryptic soy broth; $18L_{MG}$, peptide LGSIWKFIKAFVGGIKKF.

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Table 1: Biological Activities of Magainins, Mastoparans, and Their Peptide Analogs

magainins	mastoparans
bactericidal (Zasloff, 1987)	bactericidal (Katsu et al., 1990)[a]
fungicidal (Zasloff, 1987)	hemolytic (Argiolas & Pisano, 1983, 1984)
antiprotozoan (Zasloff, 1987)	stimulate phospholipase A_2 (Argiolas & Pisano, 1983, 1984)
antimalarial (Gwadz et al., 1989)	inhibit protein kinase C and Na,K-ATPase (Raynor et al., 1991)
trypanocidal (Huang, et al., 1990)	activate GTP-binding proteins (Higashijima et al., 1988)
antitumor (Cruciani et al., 1991)	bind calmodulin (Cachia et al., 1986)
decrease sperm motility (deWaal et al., 1991)	activate mast cells (Argiolas & Pisano, 1983, 1984), pancreatic b cells
activate mast cells (Hook et al., 1990)	(Yokokawa, et al., 1989), platelets (Ozaki et al., 1990), PMN (Perianin & Snyderman, 1989), and pulmonary alveolar cells (Joyce-Brady et al., 1991)

[a] Mastoparan has a lethal concentration of 0.1 μM against *E. coli* D31 (E. M. Tytler, unpublished results).

shown that the hemolytic activity of a series of rationally designed class L peptide analogs can be related to the hypothetical angle subtended by the apex (apical angle) of the inverted wedge shape.

It has been suggested that the prokaryotic specificity of the magainins is due to differences in the lipid composition of eukaryotic and prokaryotic cell membranes (Matsuzaki et al., 1989). Bacterial membranes are rich in negatively charged lipids such as phosphatidylglycerol (PG) and lipopolysaccharide (LPS), while the outer leaflet of eukaryotic cell membranes is predominantly zwitterionic lipids such as phosphatidylcholine (PC) and sphingomyelin (Op den Kamp, 1978). Eukaryotic cell membranes contain cholesterol while bacterial cell membranes do not. On the basis of these differences, Matsuzaki et al. (1989) concluded that the higher affinity of these cationic peptides for anionic phospholipids than for zwitterionic phospholipids is responsible for the prokaryotic specificity of the magainins. This hypothesis cannot completely explain the specificity of the magainins since the class L venom mastoparan, which lyses both prokaryotic and eukaryotic cells (Argiolas & Pisano, 1983; Katsu et al., 1990), also has a higher affinity for anionic phospholipids than for zwitterionic phospholipids (Katsu et al., 1990).

Since the secondary structures of the class L antibiotics and venoms are very similar (Segrest et al., 1990), we considered the possibility that the prokaryotic specificity of the magainins is due to a subtle difference in the secondary structure of these two groups of class L peptides. In this report, we have compared the amino acid sequences of the majority of known class L venoms and antibiotics to identify these differences. We have also considered that interactions between cholesterol and a specific amino acid in the sequence of class L antibiotics may be involved in the specificity of the lytic activity of these peptides. We have investigated the effect of specific amino acid substitutions on the bactericidal and hemolytic activity of rationally designed model peptide analogs of the magainins. This has allowed us to determine a structural basis for the prokaryotic specificity of the class L antibiotic peptides.

EXPERIMENTAL PROCEDURES

Bacteria. Escherichia coli ML-35 (Martinez & Carroll, 1980), a stain that has cytoplasmic β-galactosidase activity and is lactose permease-deficient ($i-$, $y-$, $z+$), was a gift from Dr. Robert I. Lehrer (Department of Medicine, University of California, Los Angeles). This strain was grown to stationary phase in tryptic soy broth (TSB), washed three times with 10 mM sodium phosphate buffer (pH 7.4) (NAPB), diluted to a density of $\sim 10^8$ CFU mL^{-1} (A_{620} = 0.35), and stored on ice before use (Lehrer et al., 1988, 1989). *E. coli* D31 (Monner et al., 1971), a mutant of *E. coli* K12 that expresses a truncated LPS, was obtained from the *E. coli* genetic stock center (Yale University) and from Dr. Michael Zasloff. This strain was grown to mid-log phase in TSB, diluted to a density of $\sim 10^7$ CFU mL^{-1} (A_{600} = 0.01), and mopped onto LB agarose plates with a sterile wipe before the application of peptides.

Peptides. The model peptide analogs were synthesized by the solid-phase method using *t*-BOC chemistry. Peptides were cleaved from the resin using anhydrous HF and purified by reversed-phase HPLC (Anantharamaiah, 1986). The peptides were judged to be greater than 99% pure by analytical reversed-phase HPLC. MG-2 was obtained from Bachem Inc. (Torrance, CA). Peptides were dissolved in phosphate-buffered saline (pH 7.4) (PBS). Peptide concentration was determined by quantitative amino acid analysis using the PICO-TAG method (Pierce, Rockford, IL).

Hemolysis of Erythrocytes. Erythrocytes were collected from EDTA-treated human blood by centrifugation and washed three times with PBS to remove plasma and buffy coat (Tosteson et al., 1985). The hematocrit was determined and the cells were diluted to 10% hematocrit. Erythrocytes (1%) in PBS with or without peptide were incubated for 10 min at 37 °C. Hemolysis was measured as hemoglobin content of the supernatant after centrifugation at 16000*g* for 3 min. The absorbance of the supernatants was measured at 540 nm against a buffer blank. Baseline hemolysis was hemoglobin release in the presence of PBS, and 100% hemolysis was hemoglobin release in the presence of 0.1% Triton X-100.

Membrane Permeabilization of E. coli ML-35. Permeabilization of the inner membrane of *E. coli* ML-35 was measured as the hydrolysis of *o*-nitrophenyl β-D-galactoside (ONPG) by cytoplasmic β-galactosidase (Lehrer et al., 1988, 1989). Briefly, *E. coli* was incubated at a density of $\sim 1.5 \times 10^7$ CFU mL^{-1} in 1% TSB and 1.67 mM ONPG in NAPB. ONPG hydrolysis at 37 °C was measured as the absorbance at 420 nm with a Gilford Response spectrophotometer against a reference containing bacteria in 1% TSB in NAPB without ONPG. Light scattering at 660 nm, a measurement of bacterial density, was measured concurrently. Absorbance values from five sample cells were measured sequentially at 1 min intervals.

Leakage of Aqueous Contents from Dye-Entrapped Large Unilamellar Vesicles. Large unilamellar vesicles (LUVs) were made from dried films of dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylcholine (DOPC), and

cholesterol (C) in a 2:1:1 or 2:1:0 molar ratio, rehydrated in 100 mM recrystallized carboxyfluorescein (CF) (Weinstein et al., 1986) in 150 mM NaCl, 1 mM Na_2 EDTA, 0.002% NaN_3, and 20 mM PIPES (pH 7.4) by vortex mixing. After five freeze/thaw cycles, the LUVs were made by extrusion through a 100 nm filter (Hope et al., 1985). Vesicles with encapsulated contents were separated by size exclusion chromatography on Sephadex G-75. The phospholipid concentration was 42.5−59.8 μM. Fluorescence emission intensity was measured at 520 nm using an excitation wavelength of 470 nm with a SLM 8000 spectrofluorometer. CF-dependent fluorescence was measured after 5 min of incubation with peptide at 37 °C. Increases in CF-dependent fluorescence were due to decreased self-quenching, reflecting leakage of CF from the vesicles (Weinstein et al., 1986). Fluorescence emission at 5 min ($F(1)$) was measured relative to initial fluorescence, ($F(0)$), before the addition of peptide, and 100% leakage ($F(100)$) was taken as the fluorescence obtained in the presence of 0.1% Triton X-100. Percentage leakage was calculated with the equation, %leakage = $[F(1) - F(0)]/[F(100) - F(0)]100$.

RESULTS

Computer Analysis of Class L Amphipathic Helixes. COMBO and COMNET analyses (Jones et al., 1992) of naturally occurring class L peptides were carried out to determine the radial and axial distributions of positively and negatively charged amino acid residues, respectively. COMBO analysis of class L as a whole has been described elsewhere (Tytler et al., 1993). Class L peptides are cationic. The positively charged amino acids are almost exclusively Lys in both groups (Tytler et al., 1993); only crabrolin has an Arg residue (Argiolas & Pisano, 1984). In class L antibiotics as well as in venoms, the positively charged amino acid residues are clustered at the center of the polar face, subtending an angle of 140° (Figure 1). The clustering is bimodal in the venoms, while this clustering pattern is less pronounced in the antibiotics. The vast majority (94% in venoms, 89% in antibiotics) of the positively charged amino acid residues are contained within an arc of 100° (Figure 1). The figure also shows that eight out of the eleven antibiotics analyzed possess a negatively charged amino acid residue on the nonpolar face of the amphipathic helix (Figure 1A), close to the carboxy terminal (Figure 1C) of the peptide. The negatively charged amino acid residue is almost exclusively Glu, with the exception of dermaseptin, which has an Asp residue (Mor et al., 1991). This is unlike the peptide venoms (Figure 1B,D) in which four of the peptides analyzed (*Polites* mastoparan and bombolitins II, III, and IV) (Argiolas & Pisano, 1983, 1985) possess an Asp residue on the polar face of the amphipathic helix (Figure 1B), close to the amino terminal of the peptide (Figure 1D).

Design of Model Peptide Analogs of Magainins. On the basis of the preceding sequence analysis, the following analogs of the model lytic peptide 18L (GIKKFLGSIWKFIKAFVG) (Tytler et al., 1993) were designed and synthesized: $18L_{MG}$ (LGSIWKFIKAFVGGIKKF), $[E^{14}]18L_{MG}$, and $[G^5,E^{14}]18L_{MG}$ (Figure 2) in which (i) the radial distribution of amino acids in 18L was retained (Figure 2A), while the axial distribution was changed so that the residue adjacent to the hydrophobic amino acids is close to the carboxy terminal of the peptide (Figure 2B) as in MG-2, (ii) a Glu residue was substituted for a Gly residue adjacent to the hydrophobic amino acids, and (iii) a Gly residue was substituted for a Trp residue to reduce the bulk of the polar−nonpolar interface on the opposite side of the helix. The biological activities of these peptides were compared with that of MG-2, which was included as a standard peptide antibiotic.

Secondary Structure of Model Peptides. The circular dichroism (CD) spectra of the model peptides (Figure 3) were recorded to calculate their ability to form α-helixes. CD spectra of these peptides could not be measured in a bilayer of dimyristoylphosphatidylcholine due to substantial light scattering (data not shown). To measure the secondary structure of these peptides in an environment that mimics a lipid bilayer, their CD spectra were measured in the presence of monopalmitoylphosphatidylcholine (PPC), a lysophospholipid that forms micelles in aqueous solution, and in the presence of 50% TFE, a solvent with a low dielectric constant, similar to that of a lipid membrane. All of the peptides possessed predominantly random coil structure in PBS (Figure 3, Table 2). However, they all adopted α-helical structure in 50% TFE and PPC (Figure 3, Table 2). The α-helicity of the analogs with the $Gly^{14} \rightarrow Glu^{14}$ substitution was higher than that of $18L_{MG}$ in 50% TFE and in the presence of PPC (Table 2). The α-helicity of MG-2 was similar to that of the model peptides in 50% TFE (Table 2), but was much higher than that of the model peptides in the presence of PPC (Table 2).

Hemolytic Activities of Model Peptides. To compare the ability of these peptides to lyse eukaryotic cells, the hemolysis of human erythrocytes was measured (Figure 4). The rank order of hemolytic activity was $18L_{MG} > [E^{14}]18L_{MG} > [G^5,E^{14}]18L_{MG}$ = MG-2 (Figure 4).

Bacteriolytic and Bacteriostatic Activities of MG-2 and the Model Peptides. In order to measure the bactericidal activity of the peptides, three different assay systems were used. Inner membrane permeabilization was measured as cytoplasmic β-galactosidase activity. While MG-2 and the three model peptides permeabilized the inner membrane of *E. coli* ML-35, the kinetics of lysis was different for the four peptides (Figure 5A). Permeabilization by $18L_{MG}$ was very rapid with no time lag. Permeabilization by MG-2 and $[G^5,E^{14}]18L_{MG}$ was preceded by lag periods of 12 and 25 min, respectively. Each peptide had fully permeabilized the bacterial membranes within 90 min, with the exception of $[E^{14}]18L_{MG}$ (Figure 5A). Each peptide also stopped the division of *E. coli* ML-35, as measured by light scattering at 660 nm (Figure 5B). The density of bacteria decreased following incubation with each peptide, with time lags similar to those observed in the membrane permeabilization measurements. To quantitate the bactericidal activity of these peptides, inhibition of *E. coli* D31 growth was measured on agarose plates (Table 3). The rank order of bactericidal activity was $18L_{MG} > [E^{14}]18L_{MG} > [G^5,E^{14}]18L_{MG} >$ MG-2 (Table 3). The $[G^5,E^{14}]18L_{MG}$ analog had virtually no hemolytic activity (Figure 4); furthermore, this peptide is twice as active as MG-2 against *E. coli* D31.

Effect of Cholesterol on the Membrane Activity of Model Peptides. To determine whether membrane cholesterol was involved in the cellular specificity of these peptides, large unilamellar vesicles (LUVs) with and without cholesterol were used. The results show that lysis by the analogs with the $Gly^{14} \rightarrow Glu^{14}$ substitution was significantly lower in the cholesterol-containing LUVs than in the cholesterol-free

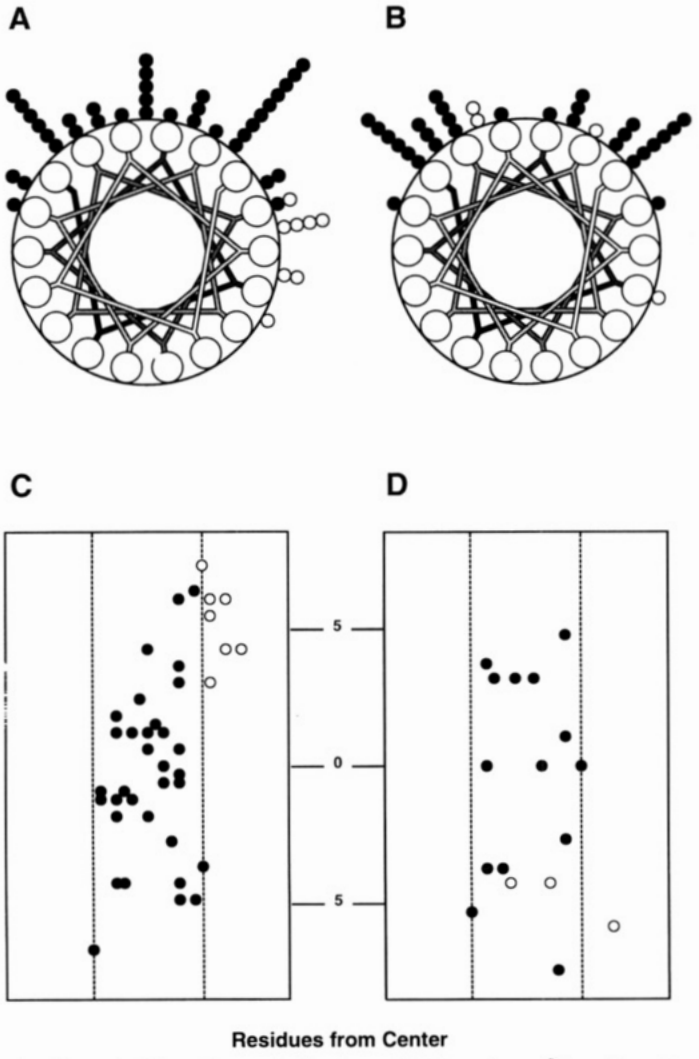

FIGURE 1: Distribution of positively charged (●) and negatively charged (○) amino acid residues in class L amphipathic helixes: (A) radial distribution in antibiotics; (B) radial distribution in venoms; (C) axial distribution in antibiotics: (D) axial distribution in venoms. The COMBO program (Jones et al., 1992) determines the radial distribution of amino acids in a set of idealized amphipathic α-helixes. This program superimposes and averages a set of helical wheel (Shiffer & Edmundson, 1967) representations. Before the wheels are superimposed, each wheel is rotated so that the positively charged residues are in the Snorkel orientation (Segrest et al., 1990). The count of positively and negatively charged residues is displayed at every 10° position around a helical wheel diagram, in which the polar face points toward the top of the page and the long axis of the α-helix is oriented perpendicular to the plane of the page and is viewed from the amino-terminal end (Figure 1A,B). The COMNET program (Jones et al., 1992) determines the axial distribution of amino acids in a set of idealized amphipathic α-helixes. This program superimposes and averages a set of helical net (Lim, 1978) representations. The α-helix is displayed as a cylinder cut along the center of the nonpolar face and flattened. The nets are superimposed so that the midpoint of each helix coincides and the positively charged residues are in the Snorkel orientation. The count of positively and negatively charged residues is displayed at every 10° position on a helical net diagram, with the polar face at the center of the diagram and the carboxy terminal at the top of the diagram (Figure 1C,D). The dashed lines represent the boundaries of the polar face. In both classes this represents an angle of 140°. The database used comprises known class L sequences. Antibiotics: bombinin [6−24] (Gibson et al., 1991), bombinin-like peptides 1−4 (Gibson et al., 1991), dermaseptin [1−29] (Mor et al., 1991), magainins 1 and 2 (Zasloff, 1987), PGQ (Moore et al., 1991), caerulein precursor fragment (1+5)[1−24], and xenopsin precursor fragment (1+3)[1−23] (Gibson et al., 1986). Venoms: bombolitins I−V, crabrolin, mastoparan, mastoparans C, M, and X, and *Polites* mastoparan (Argiolas & Pisano, 1983, 1984, 1985). The termination of the amphipathic helical domains of these peptides was predicted by the rules of Segrest et al. (1992).

LUVs (Figure 6). The rank order of lytic activity in both cholesterol-free and cholesterol-containing LUVs was identical (Figure 6): $18L_{MG}$ > $[E^{14}]18L_{MG}$ > $[G^5,E^{14}]18L_{MG}$.

DISCUSSION

The structural basis for the prokaryotic specificity of class L antibiotics is not well understood. Since these peptides form amphipathic α-helixes, and since the majority of the differences in function of amphipathic helix classes are due to the distribution of charged amino acids (Segrest et al., 1990), we compared the distribution of charged amino acids in naturally occurring class L antibiotics and venoms (Figure 1). This analysis showed that the major difference between the two groups of peptides is the presence of a conserved Glu residue on the nonpolar faces of the antibiotic peptides that is not present in the peptide venoms (Figure 1).

In previous studies on the activation of the enzyme lecithin:cholesterol acyltransferase (LCAT) by peptide analogs of apolipoprotein A-I, we had observed that analogs that had a Glu at a similar position on the nonpolar face were the most effective in stimulating LCAT activity (Anantharamaiah et al., 1990). We hypothesized that this was due to interactions between cholesterol and this Glu residue (Anantharamaiah et al., 1990). These two observa-

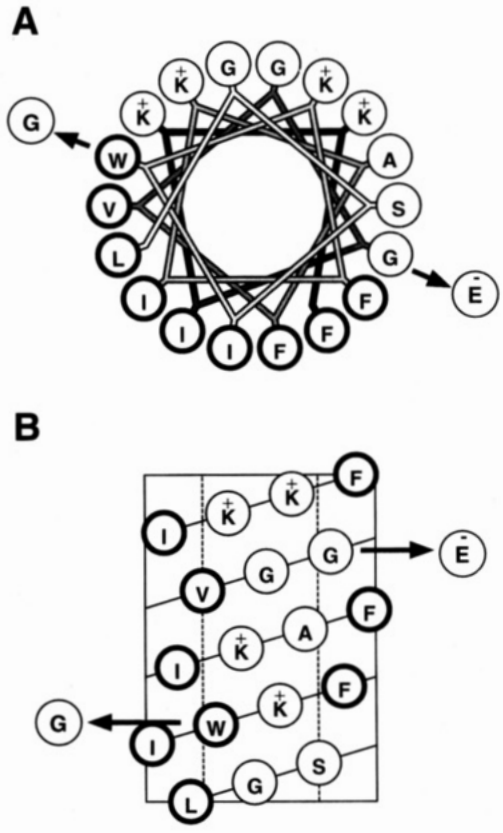

FIGURE 2: (A) Helical wheel and (B) helical net diagrams of $18L_{MG}$. The peptide $18L_{MG}$ (LGSIWKFIKAFVGGIKKF) is shown in (A), a helical wheel diagram (Shiffer & Edmundson, 1967) generated using the WHEEL program (Jones et al., 1992) (the long axis of the helix is oriented perpendicular to the plane of the page and is viewed from the amino-terminal end), and in (B), a helical net diagram (Lim, 1978) generated using the HELNET program (Jones et al., 1992). The α-helix is seen as a cylinder cut through the nonpolar face and flattened. The center of the polar face lies in the center of the figure and is oriented to rise out of the page. The carboxy terminal is at the top of the diagram. Hydrophobic residues are highlighted. The dashed lines represent the boundaries of the polar face; this represents an angle of 100°. The arrows indicate the substitutions of Gly for Trp and Glu for Gly at residues 5 and 14, respectively.

tions led us to hypothesize that the Glu residue on the nonpolar faces of the class L antibiotics could also interact with the cholesterol found in eukaryotic membranes.

We have previously shown that the hemolytic activity of model class L peptides can be explained by the reciprocal wedge model (Tytler et al., 1993). In this model, the shape of a lipid-bound class L peptide, when viewed in cross section, is an inverted wedge. This inverted wedge shape disrupts lipid bilayer structure and promotes inverted lipid phases. The experimental evidence suggests that the hypothetical apical angle of the inverted wedge shape determines the hemolytic activity of class L peptides (Tytler et al., 1993). It follows that a small apical angle is desirable to minimize the hemolytic activity of these peptides.

To test the hypothesis that the Glu residue on the nonpolar faces of class L antibiotics controls the specificity of these peptides by interacting with cholesterol, we synthesized three model class L peptides: $18L_{MG}$, $[E^{14}]18L_{MG}$, and $[G^5,E^{14}]$-$18L_{MG}$, the latter two peptides having a $Gly^{14} \rightarrow Glu^{14}$ substitution. The analog with an additional $Trp^5 \rightarrow Gly^5$ substitution would be expected to have a small apical angle according to the reciprocal wedge model (Tytler et al., 1993). We tested these peptides for their ability to lyse bacteria, human red blood cells, and LUVs, with or without cholesterol.

MG-2 and all three model peptides arrested growth and lysed *E. coli*. (Figure 5A,B, Table 3). Both Gram-positive and Gram-negative bacteria are sensitive to magainins (Zasloff, 1987). Gram-positive bacteria have a single cell membrane, while Gram-negative bacteria have two membranes surrounding the cell. The outer membrane of Gram-negative bacteria serves as a barrier to hydrophobic molecules, while the inner membrane contains all of the components required for electron transport and oxidative phosphorylation. The outer leaflet of the outer membrane contains LPS, a complex anionic phosphorylated glycolipid, while the inner leaflet of the outer membrane is enriched in the phospholipid PE. The inner membrane lipids are PE, and the anionic lipids PG and cardiolipin. The bactericidal mechanism of magainins has been studied more extensively in Gram-negative bacteria.

It has been shown that MG-2 interacts with LPS from *Salmonella typhimurium* and disorders the structure of outer membrane lipids of this bacterium (Rana et al., 1991). Westerhoff et al. (1989) have shown that the magainins PGLa and MG-2 both dissipated the membrane potential of *E. coli* D31, an LPS mutant with a truncated carbohydrate moiety (Monner et al., 1971). These authors also showed that MG-2 dissipated the membrane potential of rat liver mitochondria and uncoupled oxidative phosphorylation. Our present studies show that MG-2 and the three model class L peptides permeabilize both the outer and inner membranes of *E. coli* ML-35 (Figure 5A), a strain with a wild-type LPS (Martinez & Carroll, 1980). Figure 5A shows that bacteriolysis is a part of the mechanism of the bactericidal activity of naturally occurring and model class L peptides. It appears that the kinetics of bacteriolysis, measured as cytoplasmic β-galactosidase activity (Figure 5A), is not correlated with the bacteriostatic activity, measured as growth inhibition (Table 3). This suggests that some other event or events, in addition to lysis, are involved in the bactericidal activity of these peptides. However, it is possible that slow-acting peptides may be just as effective as fast-acting peptides in lysing bacteria over long incubation periods.

The model peptide $18L_{MG}$ was hemolytic to human erythrocytes (Figure 4), while the two analogs containing the $Gly^{14} \rightarrow Glu^{14}$ substitution had significantly lower hemolysis than the parent analog (Figure 4). The analog with the additional $Trp^5 \rightarrow Gly^5$ substitution had the lowest hemolysis, comparable to that of MG-2 (Figure 4).

If there are interactions between the Glu of class L antibiotics and cholesterol, they could inhibit the lysis of eukaryotic cells. This was studied using dye-entrapped LUVs and an artificial membrane system, with and without cholesterol. The LUV experiments show that, although the presence of cholesterol in a model membrane had only a small effect on dye leakage by $18L_{MG}$, its presence significantly reduced dye leakage by analogs containing the $Gly^{14} \rightarrow Glu^{14}$ substitution (Figure 6A,B). Leakage from cholesterol-containing LUVs is qualitatively similar to the hemolysis data (Figure 4). These results are consistent with an interaction between the Glu residue and membrane-associated cholesterol. This interaction may involve hydrogen bond formation between the acidic side chain of Glu and the

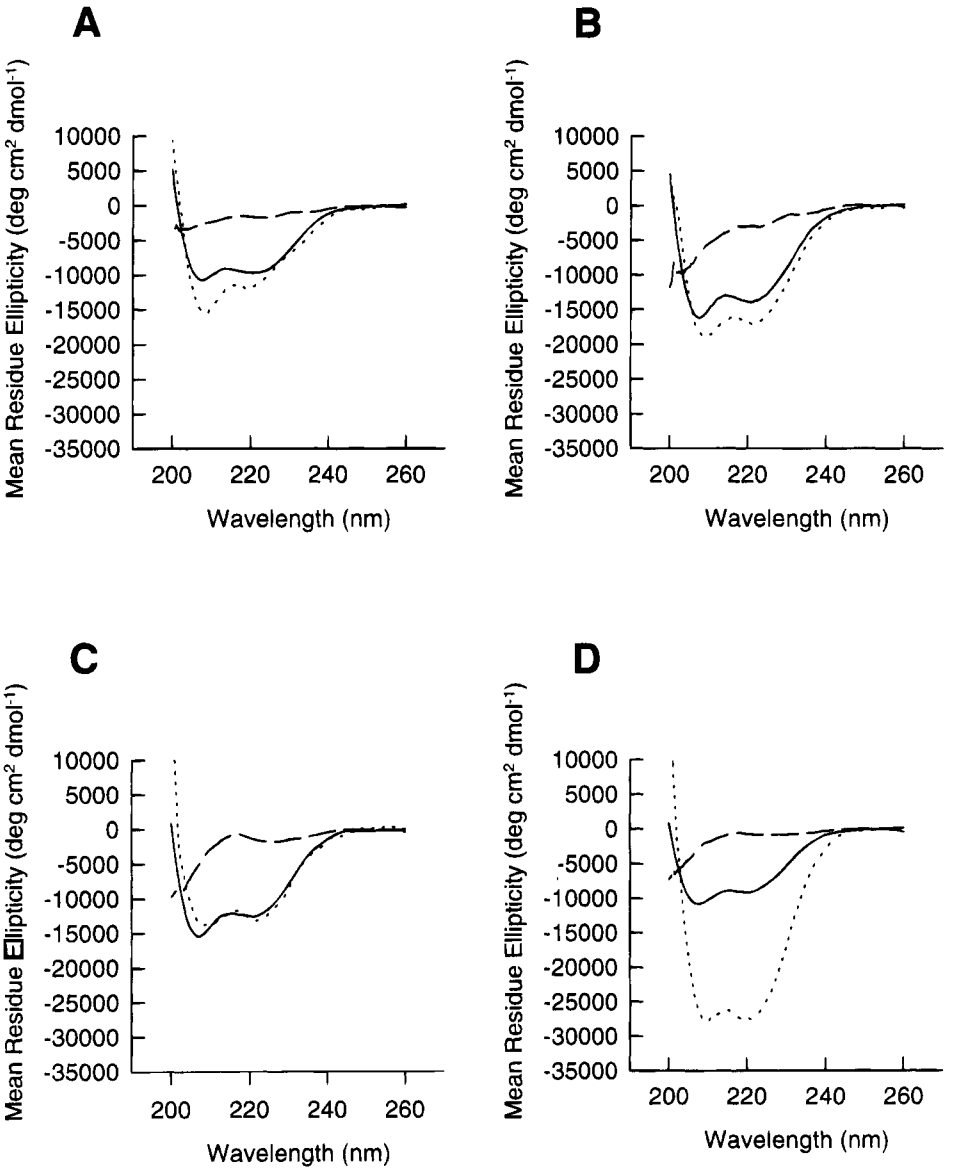

FIGURE 3: Circular dichroism (CD) spectra of (A) $18L_{MG}$; (B) $[E^{14}]18L_{MG}$; (C) $[G^5,E^{14}]18L_{MG}$; and (D) MG-2. CD spectra were measured at 25 °C in either PBS (pH 7.4) (dashed lines), 50% trifluoroethanol (TFE) in PBS (solid lines), or 10 mM monopalmitoylphosphatidylcholine (PPC) in PBS (dotted lines). Peptide concentration was 10 μM in the presence of PPC and 20 μM in the presence of PBS and 50% TFE for all peptides except $[E^{14}]18L_{MG}$, in which the concentration was 12 μM.

Table 2: Percentage α-Helicity of MG-2, $18L_{MG}$, and Related Analogs Deduced from CD Spectra[a]

peptide	α-helicity in PBS (%)	α-helicity in 50% TFE (%)	α-helicity in 10 mM PPC (%)
$18L_{MG}$	12.1	32.3	36.8
$[E^{14}]18L_{MG}$	15.2	42.7	51.0
$[G^5,E^{14}]18L_{MG}$	11.7	39.6	41.3
MG-2	9.8	30.6	77.1

[a] Circular dichroism (CD) spectra were obtained using an AVIV 62 DS spectropolarimeter. The CD was measured at 25 °C in a 2.0 mm path length cell. Four scans were averaged, and the reported spectra were corrected for the appropriate baselines. The corrected data were used to calculate the mean residue ellipticities. Percent α-helicity was calculated with the equation, % α-helix = $([\theta]_{222} + 3000)100/39000$, where $[\theta]_{222}$ is the mean residue ellipticity at 222 nm (deg cm² dmol⁻¹).

hydroxyl group of cholesterol, as previously suggested for LCAT activation by apo-A-I (Anantharamaiah et al., 1990).

The inclusion of cholesterol in multilamellar vesicles of DOPE/DOPC, at the molar ratios used in this study, destabilized the bilayer phase and promoted isotropic motion and the formation of an inverted hexagonal phase (Tilcock et al., 1982). This effect cannot explain the reduction in peptide-induced leakage from cholesterol-containing LUVs (Figure 6B). The effect of cholesterol on membrane stability is ambiguous, as the inclusion of cholesterol in multilamellar vesicles of DOPE/DPPC stabilizes the bilayer structure (Cullis et al., 1978).

The reciprocal wedge model (Tytler et al., 1993) can explain the differences between the activities of $[G^5,E^{14}]18L_{MG}$ and $[E^{14}]18L_{MG}$ in the hemolysis (Figure 4) and liposomal leakage experiments (Figure 5). The substitution of Trp^5 for Gly, a less bulky amino acid, gives this peptide a smaller apical angle, making it less potent in perturbing bilayer structure and thus decreasing its hemolytic activity (Tytler et al., 1993).

The putative interaction between Glu and cholesterol could inhibit peptide-induced lysis of eukaryotic cells in a number of ways. Several authors have shown that MG-2 forms ion-selective channels in liposomes (Duclohier et al., 1989;

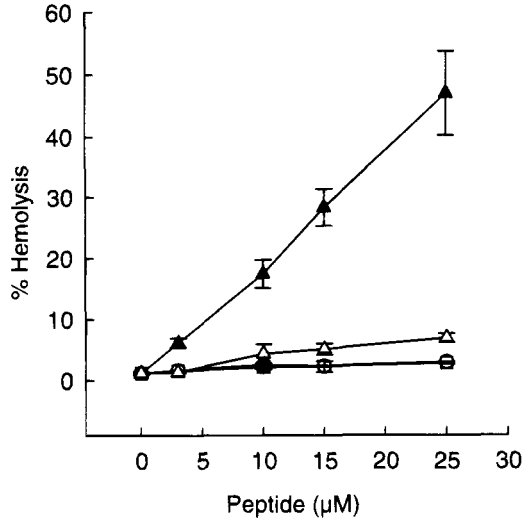

FIGURE 4: Hemolysis of human erythrocytes by MG-2, $18L_{MG}$, and related analogs. Erythrocytes were mixed with either 1, 3, 10, or 25 μM $18L_{MG}$ (▲), $[E^{14}]18L_{MG}$ (△), $[G^5,E^{14}]18L_{MG}$ (□), or MG-2 (○). Error bars represent the range of duplicate samples.

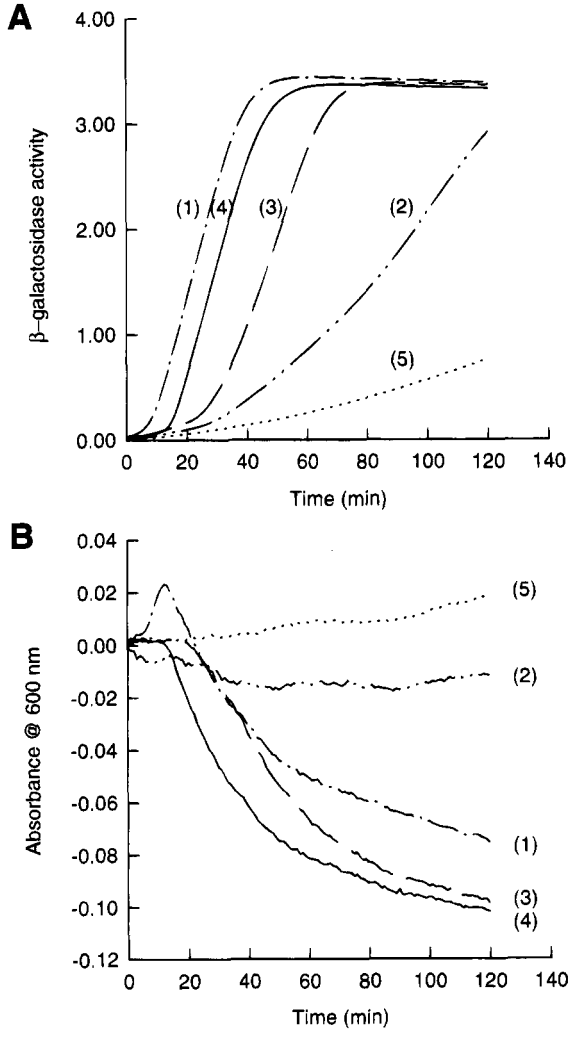

FIGURE 5: Kinetics of membrane permeabilization of *E. coli* by MG-2, $18L_{MG}$, and related analogs: (A) β-galactosidase activity; (B) light scattering. *E. coli* ML-35 was incubated with 5 μM $18L_{MG}$ (1), $[E^{14}]18L_{MG}$ (2), $[G^5,E^{14}]18L_{MG}$ (3), or MG-2 (4) or without peptide (5).

Cruciani et al., 1992) and have concluded that MG-2 is in a perpendicular (transmembrane) orientation. In contrast, Bechler et al. (1992, 1993) have provided evidence from NMR studies that MG-2 is oriented parallel to the membrane surface. The results of our study cannot distinguish between the perpendicular or parallel orientation of these peptides. If the peptides cause lysis when oriented parallel to the membrane surface, the reduction in leakage from LUVs observed with the inclusion of cholesterol may be due to either (a) weakening of interactions between the peptide and the membrane (Bechler et al., 1992), (b) effects on the

Table 3: Antibacterial Activity of Model Peptides[a]

peptide	lethal concentration (μM)
$18L_{MG}$	0.008
$[E^{14}]18L_{MG}$	0.5
$[G^5,E^{14}]18L_{MG}$	7.5
MG-2	15.8

[a] Aliquots (10 μL) of peptide solutions in PBS were spotted at different locations on LB agarose plates, which had been mopped with *E. coli* D31, in a serial dilution. The plates were incubated overnight at 37 °C, and the diameter of the zones of growth inhibition was measured. There is a linear relationship between the natural log of the amount of peptide in nanomoles (n) and the square of the diameter in centimeters (d) of the zones of inhibition. The lethal concentration C_l (Hultmark et al., 1983) for each peptide was calculated from this relationship, using the following equation: $C_l = 2.93/[ak(10)^{l/k}]$, where a is the thickness of the agarose plate in centimeters, k is the slope of the line relating ln n to d^2, and l is the intercept of the line relating ln n to d^2.

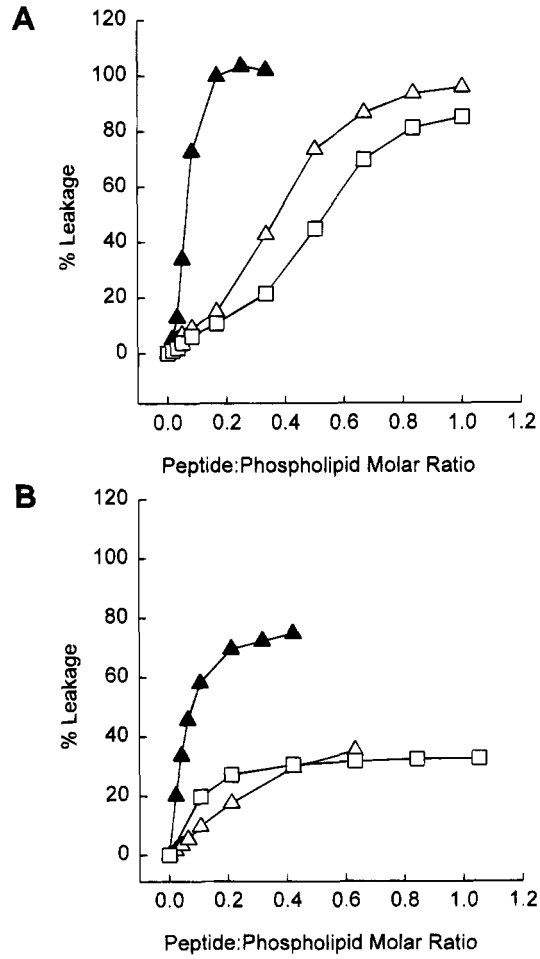

FIGURE 6: Leakage of aqueous contents from dye-entrapped large unilamellar vesicles (LUVs): (A) DOPE/DOPC vesicles; (B) DOPE/DOPC/C vesicles. LUVs were incubated with increasing concentrations (1−60 μM) of $18L_{MG}$ (▲), $[E^{14}]18L_{MG}$ (△), or $[G^5,E^{14}]18L_{MG}$ (□).

secondary structure of the peptide (Jackson et al., 1992), (c) effects on the cross-sectional shape of the peptide (Tytler et al., 1993), or (d) reduction in the penetration of the peptide into cholesterol-containing membranes. If the peptides have to be in a perpendicular orientation before lysis occurs, the effects of cholesterol on LUV leakage can be explained by all of the preceding reasons or by cholesterol specifically interfering with the reorientation of the peptide from a parallel to a perpendicular orientation.

The biological activities of these peptides are dependent on their secondary structure. The CD results of this study are consistent with previous studies on MG-2 that showed that it has a random coil structure in aqueous buffer and an α-helical structure when bound to anionic lipids (Matsuzaki et al., 1991; Jackson et al., 1992). The percentage α-helicity of MG-2 in PPC (Table 2) was similar to that in the presence of PG (Matsuzaki et al., 1991). It has also been shown that the α-helicity of MG-2 was not affected by the inclusion of cholesterol in DMPG liposomes (Jackson et al., 1992). The α-helicity of the model peptides with the $Gly^{14} \rightarrow Glu^{14}$ substitution is higher than that of $18L_{MG}$ in the presence of 50% TFE or 10 mM PPC (Table 2). Glu^{14} has the potential to form a salt bridge with Lys^{17}, the $i + 3$ amino acid, thus stabilizing the α-helix (Marqusee & Baldwin, 1987). This is consistent with the observation that the class L venom bombolitin III has higher α-helicity than bombolitin I in aqueous solution due to salt bridge formation between an Asp and an $i + 4$ Lys residue (Bairaktari et al., 1990a). This effect was not observed in the presence of micelles of the anionic detergent sodium dodecyl sulfate (Bairaktari et al., 1990b). The α-helicities of the model peptides were about half that of MG-2 in PPC, while their biological activities were much higher. The differences in the biological activities of these peptides cannot be attributed to differences in secondary structure.

In this study, using synthetic model peptides of the class L amphipathic helixes, designed with computer assistance, we have achieved a 2-fold increase in bactericidal activity over that of MG-2 (Table 3) while maintaining the hemolysis below 1%. Thus, replacement of a neutral amino acid on the nonpolar face by Glu and reduction in the bulk of its nonpolar face converted a peptide that possessed hemolytic and antibacterial activity to a nonhemolytic peptide with antibacterial activity. We have shown that the presence of a Glu residue on the nonpolar faces of these peptides determines the specificity of their lytic activity. Results from interaction of peptides with cholesterol-containing vesicles are consistent with an interaction between Glu and cholesterol in eukaryotic cell membranes being involved. These studies for the first time provide an explanation for the specificity of antibiotic, amphipathic helical peptides.

ADDED IN PROOF

It has been shown recently that cholesterol depletion of human erythrocytes made these cells more susceptible to hemolysis by MG-2 without affecting their susceptibility to melittin (Matsuzaki et al., 1995). These authors also found that inclusion of cholesterol in LUV of phosphatidylserine made these vesicles less susceptible to lysis by $[W^{12}]$MG-2 without affecting their susceptibility to melittin.

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