Structural location determines functional roles of the basic amino acids of KR-12, the smallest antimicrobial peptide from human cathelicidin LL-37

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Abstract

Cationic antimicrobial peptides are recognized templates for developing a new generation of antimicrobials to combat superbugs. Human cathelicidin LL-37 is an essential host defense molecule in human innate immunity. Previously, we identified KR-12 as the smallest antibacterial peptide of LL-37. KR-12 has a narrow activity spectrum since it is active against Gram-negative *Escherichia coli* but not Gram-positive *Staphylococcus aureus*. The functional roles of the basic amino acids of KR-12, however, have not yet been elucidated. An alanine scan of cationic amino acids of KR-12 provided evidence for their distinct roles in the activities of the peptides. Bacterial killing and membrane permeation experiments indicate that the R23A and K25A mutants, as well as the lysine-to-arginine mutant, were more potent than KR-12. Another three cationic residues (K18, R19, and R29) of KR-12, which are located in the hydrophilic face of the amphipathic helix, appeared to be more important in clustering anionic lipids or hemolysis than R23 and K25 in the interfacial region. While the loss of interfacial R23 or K25 reduced peptide helicity, underscoring its important role in membrane binding, the overall increase in peptide activity of KR-12 could be ascribed to the increased peptide hydrophobicity that outweighed the role of basic charge in this case. In contrast, the mutations of interfacial R23 or K25 reduced peptide bactericidal activity of GF-17, an overlapping, more hydrophobic and potent peptide also derived from LL-37. Thus, the hydrophobic context of the peptide determines whether an alanine substitution of an interfacial basic residue increases or decreases membrane permeation and peptide activity.

Introduction

The lasting potency of natural antimicrobial peptides (AMPs) justifies current research efforts in elucidating their functions and eventually developing them into novel antimicrobial agents against antibiotic-resistant superbugs.¹⁻³ The participation of and contributions from many laboratories around the world led to a substantial increase in the number of such peptides. As of May 2013, there were 2224 AMPs in the antimicrobial peptide database (APD), including 192 bacteriocins, 293 plant AMPs, and 1685 animal

defense peptides (<http://aps.unmc.edu/AP/main.php>).^{4, 5} The majority of the peptides in the APD database contain less than 50 amino acids.

The net charge of these peptides can be negative, zero, but usually positive with an overall average of +3. The hydrophobic content of the AMPs in the APD covers a broad range with a peak at 40-50%.⁶ The amino acid use in natural AMPs appears to be biased. Some residues such as alanines, arginines, cysteines, glycines, isoleucines, lysines, leucines, and serines are more abundant (5.5-11.6% according to the statistical interface of the APD) than other residues. Although natural AMPs can fold into various 3D structures (α -helices, β -sheets, a hybrid of α and β structures, and non- α structures), they are frequently cationic and amphipathic, thereby highly suitable for targeting a variety of anionic bacterial components such as membranes and nucleic acids.⁶

In humans, multiple AMPs have been identified. The well-known peptide families are cathelicidins, defensins, and histatins.⁷⁻⁹ Some other molecules such as chemokines and α -amyloid peptides also possess antimicrobial activity,^{10, 11} further expanding the repository of natural defense molecules and linking the innate and adaptive immune systems. While there are more than two dozens of defensins, only one cathelicidin gene is identified in the human genome. The precursor protein of human cathelicidin, however, can be cleaved into LL-37 in epithelial cells or ALL-38 in reproductive systems. Although there is one additional alanine at the N-terminus of ALL-38, LL-37 and ALL-38 were shown to have similar antimicrobial activity.^{14, 15} To understand the relationship of 3D structure with antimicrobial activity, we determined a high-quality structure for full-length LL-37 by triple-resonance NMR spectroscopy.¹⁶ Importantly, this structure determined in the SDS micelles is retained in a new membrane model dioctanoyl phosphatidylglycerol (D8PG), an anionic lipid that has exact the same head group as the major anionic phosphatidylglycerols (PGs) in bacterial membranes. It is proposed that human LL-37 binds to the bacterial lipopolysaccharides (LPS) or inner membranes through a long amphipathic helix spanning residues 2-31 (Fig. 1A), while the C-terminal tail remains disordered.

Based on NMR studies, we also identified the major antimicrobial region of human LL-37 corresponding to residues 17-32 (Fig. 1B). A corresponding peptide GF-17 is active against both *S. aureus* and *E. coli*.^{12, 17} The broad spectrum activity of GF-17 makes it a useful template for engineering novel compounds against these important pathogens. Resistant Staphylococcal strains have been identified in both clinical and community settings. *E. coli* also poses risks to the public. For example, some *E. coli* strains can cause food poisoning or water contamination. Our subsequent studies revealed that the major antimicrobial region could be further shortened.^{17, 18} Using a series of synthetic peptides, KR-12, corresponding to residues 18-29 of human LL-37 (Fig. 1C), was identified as the smallest antibacterial peptide.¹⁶ Unlike GF-17, this short peptide is active against *E. coli* but not *S. aureus*. The selective antimicrobial activity of KR-12 is interesting. Because of low cytotoxicity of this peptide, it constitutes a useful template for designing bacteria-specific antimicrobial agents. Furthermore, unlike GF-17, KR-12 does not cause media precipitation even at high concentrations we used.¹² This makes KR-12 an excellent model peptide for us to learn the minimal requirements for antimicrobial activity, especially against Gram-negative bacteria. This study focuses on functional roles of basic amino acids of KR-12. In particular, a specially engineered *E. coli* strain is included here to provide insight into membrane permeation. Here we report the differential effects of these cationic side chains in terms of antimicrobial activity, membrane permeation, lipid clustering, and hemolysis. An attempt is made to correlate these properties with the 3D structure of KR-12. Our results suggest that the properties of cationic residues of KR-12 to a large extent are determined by their locations in the 3D structure. This observation is important for understanding the functional roles of cationic residues of natural AMPs as well as for designing or predicting AMPs.

Materials and methods

Peptide design

Two series of peptides were designed based on the sequence of KR-12 (Fig. 1). The first series consists of single alanine mutants and the second series compares the effects of lysines with arginines. In addition, a double mutant was generated by changing K18 to A18 and R19 to Q19. We assumed that polar Q19 is a better mimic of charged R19 than A19. In addition, we intended to avoid the appearance of a string of hydrophobic residues in the sequence as a consequence of double alanine substitutions. The amino acid sequences of these designed peptides are given in Table 1. All the peptides were chemically synthesized and purified to >95% (Genemed Synthesis, TX). Peptide quality was verified by mass spectrometry as well as reverse-phase HPLC.

Peptide hydrophobicity

One of the methods to estimate peptide hydrophobicity is to measure the retention times of peptides on a reverse-phase HPLC column.¹⁹ In brief, the retention time of the peptide was measured on a Waters HPLC system equipped with an analytical reverse-phase Waters symmetry C8 column (150 × 3.9 mm). The peptide detected at 254 nm and 280 nm was eluted with a gradient of acetonitrile (containing 1% TFA) from 5% to 95% at a flow rate of 1 ml/min.

Antimicrobial activity assays

The assays were conducted as described.²⁰ In brief, a 2 ml culture was grown overnight. A fresh culture (2 ml) was inoculated with 10 µl of the overnight culture and incubated at 37°C (240 rpm) until the bacterial growth reached the logarithmic stage ($OD_{600} \sim 0.5$). The culture was then diluted and 90 µL aliquots placed into a 96-well microplate with $\sim 10^6$ colony forming unit (CFU) per well. The bacterial wells were then treated with a series of peptide solutions (10 µL) with two-fold dilution. A broad range of peptide concentrations ranging from 10 to 320 µM was utilized. The assays were performed in duplicates for each peptide and repeated four times at different dates. The plate was incubated at 37°C overnight (~ 16 h) and read with a ChroMate 4300 Microplate Reader at 630 nm (GMI, Ramsey, MN). The lowest peptide concentration that fully inhibits the bacterial growth was taken as the minimal inhibitory concentration (MIC). The average and standard deviation of MIC values for each peptide were calculated.

Growth inhibition and killing experiments

These experiments were performed by modifying the activity assays above. In particular, the starting OD_{600} was set at ~ 0.1 with the shaker rotation at 100 rpm. For each peptide, the bacteria were treated at various concentrations at 0, 1, 2, 4, and 8-fold of the MIC of KR-12 (40 µM).¹⁶ The plates were read every 30 minutes for a total duration of 3 h. The experiments were duplicated for each peptide and the averaged values were presented. For comparison, we define a growth inhibition and killing (IK) index for each peptide by taking the ratio between the maximal OD drop due to a peptide under investigation and a reference peptide that brought OD to zero (also see the text).

Electron microscopy

For transmission electron microscopy (TEM), the *E. coli* K12 strain was grown in LB medium at 37°C (240 rpm) to attain mid logarithmic phase. Five mL of the culture was then treated with the peptide KR-12 at a concentration of $8 \times \text{MIC}$ for 1 h. The cells were then pelleted on a bench top centrifuge 5415D (Eppendorf) at 15,000 rpm for 5 min. They were then washed consecutively 3 times with fresh PBS, pH 7.4 and fixed with a solution of 2%

paraformaldehyde, 2% glutaraldehyde in 0.1 M PBS. Control sample (i.e. no peptide treatment) were processed in the same manner. Ten μL of the bacterial suspension was then adsorbed onto 200 mesh copper grids coated with formvar and silicon monoxide for 2 min, dried with the help of a blotting paper and then negatively stained by two drops of Nanovan® (Nanoprobes). Samples were observed in an FEI Technai G2 TEM operated at 80 kV accelerating voltage at the University of Nebraska Medical Center (UNMC).

Membrane permeation

The experiments were performed using a mutant *E. coli* strain developed and provided by Lehrer et al.²¹ The *E. coli* strain ML-35p was engineered specifically to monitor permeation of both inner and outer membranes (OM) of the same bacterial strain. *E. coli* ML-35p is constitutive for cytoplasmic β -galactosidase, lacks lac permease, and expresses a plasmid-encoded periplasmic β -lactamase. Two chromogenic reporter molecules are used to monitor permeabilization of outer and inner membranes in a single assay. Nitrocefin, a chromogenic cephalosporin, cannot cross the OM and is excluded from the periplasmic space. However, permeabilization of OM allows nitrocefin to enter the periplasm where it is cleaved by a β -lactamase and produces a color change that can be monitored spectrophotometrically at 486 nm. Since there is no lactose permease in this strain of *E. coli*, o-nitrophenylgalactose (ONPG) cannot traverse its inner membrane (IM) to be cleaved by cytoplasmic β -galactosidase to o-nitrophenol, unless permeabilization of the inner membrane occurs. ONPG cleavage produces a color change that can be measured spectrophotometrically at 420 nm.

Bacteria were grown in tryptic soy broth (TSB) from a single colony, overnight, at 37°C. After 3 washings in phosphate buffer pH 7.4 (10 mM phosphate buffer, 100 mM NaCl), it was diluted to $\sim 10^6$ CFU/ml in incubation buffer (phosphate buffer pH 7.4 containing 0.3 mg/ml TSB) and added to all wells in a non culture-treated polystyrene microplate, together with increasing concentrations of peptide solutions, in phosphate buffer. Each well also contained 30 μM nitrocefin or 2.5 mM ONPG in phosphate buffer. Absorbance was followed simultaneously at 490 nm and at 450 nm (these were the closest to the maximum value which the fixed wavelength microplate allowed us to use) for 36 min, taking readings every two minutes at 37°C with a few seconds of shaking prior to each reading. OM permeabilization was followed for only 20 min to avoid artifacts due to spontaneous hydrolysis at longer times. A SpectraMax Pro microplate reader equipped with MaxSoft Plus software (Molecular Devices, Sunnyvale, CA) was used.

Hemolysis

Hemolytic analysis of selected peptides was performed using an established protocol as described.²² Briefly, blood cells (UNMC Blood Bank) were washed three times with PBS and diluted to a 5% solution. After peptide treatment, incubation at 37°C for one hour, and centrifugation at 13,000 rpm, aliquots of the supernatant were transferred to a fresh 96-well microplate. To assess peptide cytotoxicity, the amount of hemoglobin released was measured at 545 nm. The percent lysis was calculated by assuming 100% hemoglobin release when human blood cells were treated with 2% Triton X-100, and 0% release when incubated with PBS buffer.

Preparation of multilamellar liposome vesicles (MLVs) and large unilamellar vesicles (LUVs)

Lipids were dissolved in chloroform/methanol, 2/1 (v/v). The solvent was then evaporated with nitrogen to deposit a film on the wall of a glass test tube. Final traces of solvent were removed for 2-3 hours in an evacuated chamber. The films were suspended in the indicated buffer by vortexing at room temperature to form MLVs. Peptides were mixed with the lipid

by dissolving them in the buffer that was used to hydrate the lipid films. When LUVs were prepared, the lipid films were made devoid of peptides. They were hydrated with buffer and were further processed with 5 cycles of freezing and thawing, followed by 10 passes through two stacked 0.1 μm polycarbonate filters (Nucleopore Filtration Products, Pleasanton, CA) in a barrel extruder (Lipex Biomembranes, Vancouver, BC) at room temperature. LUVs were stored on ice and used within a short time after preparation.²³

Differential scanning calorimetry (DSC)

Measurements were made in a Nano II Differential Scanning Calorimeter (Calorimeter Sciences Corporation, Lindon, UT) as described.²³ Lipid films (POPE:TOCL = 75:25) were hydrated at room temperature with peptide solutions in PIPES buffer (20 mM PIPES, 140 mM NaCl, 1 mM EDTA, pH 7.4) or with PIPES buffer alone, vortexed vigorously to make MLVs and then degassed before loading into the sample cell of the calorimeter. Controls using peptide solutions in the sample cell in the absence of lipid showed no transition. Degassed PIPES buffer was placed in the reference cell. The concentration of phospholipids in the samples was maintained at 2.5 mg/mL and the lipid to peptide molar ratio at 10. The cell volume was 0.34 mL. Samples were equilibrated in the calorimeter at 0°C and successive heating and cooling scans were carried out between 0 and 35°C at a scan rate of 1.0°C/min with a delay of 5 minutes between sequential scans in a series to allow for thermal equilibration. The resulting curves were analyzed by using the fitting program provided by Microcal Inc. (Northampton, MA) and plotted with Origin version 7.0.

Circular dichroism (CD)

CD spectra were recorded from 260 to 195 nm on an AVIV model 215 spectropolarimeter. A quartz cell with a 0.1 cm path length was placed in a thermally controlled cell holder. The machine was equipped with a Peltier junction thermal device and a Thermo Neslab M25 circulating bath. The sample temperature was maintained at 25°C in all wavelength scans. Spectra were obtained with a wavelength step of 1 nm and an averaging time of 3 s for each data point, with a 30 s equilibration time between points. The KR-12 peptide and its mutants were measured at a concentration of 0.2-0.3 mg/mL in sodium phosphate buffer pH 7.4, without or with the addition of D8PG, at a lipid to peptide molar ratio of 20. Secondary structure analysis was carried out with the program CDPro.

Results

Antimicrobial activity of KR-12 and its mutants

Our previous study revealed that KR-12 is active against Gram-negative *Escherichia coli* K12, but not the Gram-positive community-associated strain *S. aureus* USA300 or clinical strain UAMS-1.^{16, 23} To elucidate the effects of the basic residue mutation on antimicrobial activity, we measured MIC values for KR-12 and its mutants against *E. coli* K12 (Table 1). In the table, the mutation is indicated by appending the name of the substituted residue after KR-12. For example, KR-12A18 represents a mutation of K18 to A18 (i.e. K18A). KR-12 displayed on average an MIC of 64 μM , similar to the previous value (Fig. 1). While the activity of the K18A mutant is comparable to KR-12, substitution of residues R19, K25, or R29 with an alanine caused a slight drop in anti-*E. coli* activity (MIC 120-140 μM). To validate the roles of K18 and R19, we also designed a double mutant by changing K18 to alanine and R19 to glutamine. This double mutant KR-12A18Q19 became even less active (MIC 240 μM), supporting that both K18 and R19 contributed to the antimicrobial activity of KR-12. However, the R23A mutation increased peptide activity (MIC 40 μM). We also compared the effect of lysines vs. arginines. When the three arginines (R19, R23, and R29) of KR-12 were mutated to lysines, the resulting peptide KR-12K (MIC 106 μM) was less active than its parent peptide. Nevertheless, KR-12R, an arginine analog obtained

by changing K18 and K25 of KR-12 to arginines, became more potent (MIC 30 μ M) than KR-12. Thus, substitutions of lysines with arginines made KR-12 more effective against *E. coli*.

In order to better understand the activity differences of these peptides, we also obtained growth inhibition and killing curves for KR-12 and its mutants (Fig. 2). In the absence of peptides, the OD of *E. coli* K12 increased exponentially until the static phase. The bacterial growth was hindered to varying degrees depending on the type and concentration of the peptide. In the case of KR-12, the OD dropped by ~ 0.05 when the culture was treated at 320 μ M (Fig. 2A). Most of the peptide mutants behaved more or less similarly (Fig. 2, B-I). Peptide KR-12A23, however, was more potent and was able to reduce the OD to zero at 180 min (Fig. 2D), indicating near complete bacterial lysis.¹² To quantitate the inhibitory ability of these peptides based on their inhibition and killing (IK) curves (Fig. 2), we defined an IK index by taking the ratio between the maximal OD drop at 180 min by a KR-12 peptide and that by a control peptide GF-17A18 known to lyse bacteria (Fig. 2J).¹² The IK indexes are listed in Table 1. It is clear that KR-12A23 was most effective (IK index 1) followed by KR-12A25 and KR-12R (IK index ~ 0.5). Mutant peptides KR-12A18, KR-12A19, and KR-12A29 (IK index 0.2-29) were similar to KR-12 (IK index 0.33). The double mutant and the lysine analog KR-12K appeared to be poorest (IK index 0.12-0.13) (Table 1).

To view the bacterial killing effect, we also conducted a TEM study of the parent peptide KR-12. The *E. coli* images showed a marked difference in the presence or absence of the peptide. The untreated bacteria (Fig. 3A) showed a uniform staining of the dye and the surface appeared to be homogeneously opaque, while the peptide-treated cells became transparent (Fig. 3B). It is likely that the cytoplasm of these ghost cells leaked out due to the action of the peptide that compromised membrane integrity.

Membrane penetration ability of the KR-12 peptides

The time-dependent response curves of the reporter molecules for *E. coli* ML-35p after treatment with KR-12 or its mutants are presented in the left (nitrocefin) and right (ONPG) columns of Fig. 4. Peptide permeability increased with time and concentration in all the cases except for the double mutant and KR-12K that were not able to penetrate the inner membranes. To compare the membrane permeation ability of these peptides, we derived the peptide concentrations required to achieve an OD value of 0.2 (Table 2). We noticed that the double mutant was able to pass the OM barrier at ~ 170 μ M but failed to penetrate the IM even at the highest peptide concentrations tested. Because our activity assays indicate that KR-12A18Q19 was the poorest among these KR-12 peptides, the failure in penetrating the IM should be the critical step that determined the poor activity of this peptide. As a consequence, we turned our attention to peptide concentrations for penetrating the IM. Peptides KR-12A23, KR-12A25, and KR-12R appeared to be most potent since they reached an OD of 0.2 around 100 μ M (Table 2). These experiments identified KR-12A23, KR-12A25, and KR-12R as the most potent peptides. As a comparison, we also measured the membrane penetration ability of GF-17, which is known to be much more potent against *E. coli* K12 (MIC = 7.5 μ M) (Fig. 1). This peptide was able to achieve the same level of penetration (i.e. OD 0.2) at 5 μ M (Fig. 4J). Therefore, KR-12 and its mutants are poor in penetrating bacterial membranes compared to GF-17.

Lipid clustering by the KR-12 mutants

Lipid clustering also contributes to bacterial killing.²⁴ KR-12 is established by different techniques to be able to cluster lipids in a membrane system that mimic Gram-negative bacteria.²⁵ Therefore, these KR-12 mutants (Table 1) provide us a good opportunity to elucidate the effects of various cationic side chains of KR-12 on lipid clustering. For this

purpose, we used the same membrane model system established previously.²³ This system was proved to be effective for testing lipid-clustering ability of various antimicrobial agents. The DSC results are shown in Fig. 5. To facilitate the comparison, we define D_{pvl} (a deviation ratio of peptide vs. lipids) based on the changes in transition temperatures T_m with and without peptide relative to the wild type KR-12. The equation and calculated results are given in Table 3 and its footnotes. Because there are two transition temperatures in the majority cases, we assumed the merging of the two transition temperatures when one

T_m was obtained from DSC deconvolution. In this scale, the wild type peptide KR-12 has a D_{pvl} value of 1.

A mutant peptide has a D_{pvl} greater than 1 if it has a stronger lipid clustering ability than KR-12. Based on these calculated values, KR-12R, the arginine analog, had the strongest lipid clustering ability in Table 3. Of the single alanine mutants, lipid-clustering ability reduced in the absence of K18 or R29, while the R19A mutant (KR-12A19) showed a slightly better clustering ability than KR-12. There were no changes when either R23 or K25 was mutated. It appears that K18 and R29 of KR-12 were more important in lipid clustering than R19, R23, or K25. In addition, the changes of arginines to lysines did not matter due to a similar lipid-clustering ability of KR-12K and KR-12. However, substitutions of K18 and K25 with arginines greatly increased peptide lipid clustering ability. These results underscore the importance of arginines in lipid clustering in the case of KR-12.

Hemolysis

Natural AMPs can be broadly classified into two families: cytotoxic and non-toxic. Cytotoxic peptides are not cell selective since they cause lysis of both bacteria and human cells. Such peptides are less interesting in terms of antibiotic development. Many full-length natural AMPs are cytotoxic to some extent. However, their truncated fragments can be more selective. Such peptides are better templates for developing the next generation of antibiotics. Our current knowledge suggests that high hydrophobicity of AMPs is an important factor in determining peptide cytotoxicity.⁶ Hemolysis is a convenient experiment as a first measure of peptide cytotoxicity. This is because the release of hemoglobin from erythrocytes is easy to follow by a colorimetric method. Since KR-12 contains the minimal requirements for anti-*E. coli* activity, it is predicted that this peptide will have low cytotoxicity. Indeed, KR-12 only caused ~10% lysis of erythrocytes at 100 μ M. It is also important to elucidate which cationic residues contribute more to cytotoxicity. Such knowledge is useful for improving peptide selectivity. At the same peptide concentration, the mutants of KR-12 showed some variations in hemolysis (Table 3). While KR-12A23 and KR-12A25 showed similar lytic capability to the wild type KR-12, little or no lysis was observed for KR-12A18, KR-12A19, KR-12A29, or the K18/R19 double mutant. Thus, K18, R19, and R29 of KR-12 appeared to be slightly more important for hemolysis than R23 and K25. Regarding the lysine or arginine substitutions, a small percentage of lysis was observed for KR-12K, the lysine analog, whereas more lysis (58%) was observed for KR-12R, the arginine analog. Thus, increase in arginines may also increase the unwanted hemolytic ability of the peptide. Under the same conditions, a more hydrophobic peptide GF-17 caused nearly complete lysis, confirming the important role of peptide hydrophobicity in hemolysis.

Conformations of KR-12 and its mutants

The 3D structure of KR-12 was previously determined by NMR spectroscopy in a novel membrane-mimetic model we established (Fig. 6). In particular, D8PG has an identical head group as the major anionic lipids in bacterial membranes. Therefore, the KR-12 structure determined in complex with this lipid should allow us to better correlate the structure with antibacterial activity. In the NMR structure, residues 20-28 of KR-12 are in the helical

region.¹⁶ This is consistent with the CD spectrum of the peptide in the presence of D8PG (Fig. 7). The helical structure of KR-12 did not form in PBS buffer (pH 7.4). Likewise, the peptide mutants of KR-12 were also in the random-coiled conformation in PBS buffer, but became helical in the presence of D8PG. It is thus established that the formation of the helical structure of KR-12 and its mutants all resulted from their interactions with anionic D8PG. The secondary structures of these peptides were calculated and the results are summarized in Table 4. For each peptide, no or low percentage of β -sheet structure was found, further supporting the dominance of helical conformation in lipid micelles. Of note, KR-12A23 and KR-12A25 are less helical in D8PG than KR-12 or its other single alanine mutants. Because R23 and K25 are located in the interface of the amphipathic helical structure of KR-12 (Fig. 6), it seems that interfacial basic residues play an important role in binding to D8PG. These results suggest that the D8PG model is unique in being able to reveal the subtle difference of the KR-12 mutants in interactions with bacterial membranes.

Discussion

Although over 2000 AMPs have been registered into the APD database,^{4, 5} there is great interest in host defense peptides from humans. It appears that each human AMP plays a unique role in protecting us from infection.

For example, HBD-1 needs to be reduced to be effective against bacteria presumably in an oxygen-limited environment,²⁶ while HD-6 does not kill bacteria directly but can accumulate and oligomerize on the surface of bacteria to keep them away from human cells.²⁷ HNP-1 and HBD-3 inhibit the growth of Gram-positive bacteria by binding to the cell wall precursor lipid II.^{28, 29} Thus, human defensins can help the host at different stages of bacterial invasion, under different conditions, and by using different mechanisms. Besides defensins, human cathelicidin is also deployed for host defense. Human LL-37 is now recognized to have multiple functional roles, ranging from antimicrobial to immune modulation.³⁰ We have been focusing on the structure and activity relationship of human LL-37. This peptide uses a long and continuous amphipathic helix to interact with the outer and inner membranes of *E. coli* (Fig. 1A).¹⁶

To improve our understanding, it is also useful to identify the functional domains of LL-37. Recently, we showed that, LL-23, a natural fragment corresponding to the N-terminal 23 residues of human LL-37, is primarily involved in other functions such as modulation of chemokine release since the peptide was active only against susceptible bacterial strains of *E. coli* and *S. aureus*.¹³

The poor antibacterial activity of LL-23 can be attributed to the segregation of the hydrophobic surfaces into two regions by a hydrophilic serine since a mutation of S9 to an alanine or valine did increase peptide activity. This unique structure of LL-23 is retained in three membrane-mimetic micelles: SDS, D8PG, and dodecylphosphocholine (DPC). Using NMR spectroscopy, we previously mapped the major antimicrobial region to GF-17, a central fragment corresponding to residues 17-32 of LL-37 (Fig. 1A).¹⁷ Indeed, GF-17 showed an MIC against *E. coli* comparable to that of LL-37 (Fig. 1B), indicating that bacterial killing does not require the presence of the entire LL-37 peptide sequence.

However, we witnessed activity drop when additional residues were removed from GF-17. In particular, KR-12 (Fig. 1C) is only active against *E. coli*, so its antimicrobial activity spectrum is narrower than that of GF-17. Such an activity spectrum difference between KR-12 and GF-17 may be explained by peptide properties and their modes of physical interactions with bacterial membranes. GF-17 is sufficiently hydrophobic, allowing the peptide to effectively eliminate both *S. aureus* and *E. coli*. KR-12 is less hydrophobic as a

consequence of removing several hydrophobic residues from GF-17, including F17 from the N-terminus and, L31 and V32 from the C-terminus (Fig. 1). This reduced hydrophobicity of KR-12 is consistent with our observation that GF-17 is able to lyse lipid vesicles, whereas KR-12 is unable to.²³ Correspondingly, GF-17 is able to lyse *E. coli*,²³ but not KR-12 (Fig. 2). In terms of the modes of interaction, GF-17 is able to both disrupt membranes and cluster lipids. The weak inhibitory effect of KR-12 on *E. coli* may be due to its ability to cluster anionic lipids, but not vesicle or bacterial lysis. This lipid clustering ability of KR-12 has been proved by a variety of techniques.^{23, 25} By generating various single-residue mutants of cationic amino acids, our current study sheds further light on the antimicrobial activity difference of these two peptides. In the lipid clustering process, K18, R19, or R29 of KR-12 appeared to be more important than R23 or K25. Interestingly, K18 and R19 are also important lipid clustering residues in the case of GF-17. What is different is that R23 was also important in the case of GF-17, but not in the case of KR-12. Such a difference in lipid clustering also explains the high potency of GF-17 compared to KR-12. Because K18, R19, and R29 are located on the hydrophilic surfaces in the 3D structures of both GF-17 and KR-12,^{12, 16} it appears that non-interfacial basic residues are more important than interfacial residues in lipid clustering. In addition, K18 and R29 are located at the terminal region of KR-12 and thereby tend to be more mobile. This can be seen in Fig. 6 where K18 clearly possesses two conformations even at the backbone level. We propose that flexible cationic side chains play an important role in clustering anionic lipids in membrane-mimetic vesicles. This agrees with our current knowledge that those flexible peptide mimics are most powerful lipid clustering agents investigated to date.^{24, 31}

Interfacial residues, however, play an essential role in bacterial killing because mutation of R23 or K25 of GF-17 substantially reduced anti-*E. coli* activity.¹² Interestingly, mutation of the same two basic residues of KR-12, especially R23, led to an increase in antibacterial activity against *E. coli*. Such a difference was unanticipated. We propose that these results are not conflicting with each other, but reflect the elegant balance between peptide hydrophobicity and basic charge. In both cases, it seems that membrane penetration ability corresponds well with antimicrobial activity of GF-17 and KR-12. A poor membrane penetration of the R23A mutant of GF-17 explains its reduced activity compared to the parent peptide. In the case of KR-12, increased membrane penetration of the R23A mutant (Table 3) corresponds well to its inhibitory and killing index (Table 1). In particular, the R23A mutant of KR-12 is able to lyse *E. coli*, similar to a GF-17 mutant (Fig. 2), albeit at a high concentration. The importance of interfacial R23 in membrane binding is firmly established because we detected direct interactions between the R23 side chain and D8PG by NMR.³²

Under the sufficiently hydrophobic context of GF-17, the loss of this critical R23 hindered membrane binding and was also reflected in the decrease in peptide activity. In the case of the less hydrophobic peptide KR-12, a loss of basic R23 or K25 also reduced membrane binding of the peptide. This is reflected in the less helical conformation of these two peptides in complex with anionic D8PG (Fig. 7). In terms of antibacterial activity, however, additional factors came into play. When R23 of KR-12 is mutated to an alanine, it appears that the gain in hydrophobicity outweighs the loss in a basic charge, enabling the peptide mutant to lyse *E. coli* at an elevated peptide level. This line of reasoning agrees with our HPLC measurements, which indicate that the R23A mutation makes the peptide more hydrophobic (Table 1). Therefore, interfacial basic amino acids, especially R23, are critical in modulating peptide properties of both KR-12 and GF-17 in terms of membrane binding. In terms of antibacterial activity, however, the hydrophobic context of the peptide sequence appears to determine whether antibacterial activity is tuned up or down. Considering the general difficulty in correlating peptide activity with a single parameter such as helicity, net charge, or hydrophobicity,²⁰ our observation underscores the importance of a combined

consideration of charge and hydrophobicity in interpreting antimicrobial activity of cationic AMPs.

We also examined the differences between lysines and arginines in KR-12. In all the cases investigated here, KR-12 became more potent against *E. coli* when lysines were mutated to arginines. This includes higher antimicrobial activity, hemolysis, anionic lipid clustering, and membrane penetration. Previously, we also found that the arginine analog of GE-18, a peptide obtained by inserting E16 in front of F17 of GF-17, is more potent against methicillin-resistant *S. aureus* (MRSA) USA300 than the lysine analog.¹² It appears that the arginine analogs of AMPs could be more active against both Gram-positive and Gram-negative bacteria. Likewise, arginine analogs of AMPs could be more potent against HIV-1.³³ The beneficial effects of arginines might not be limited to antimicrobial activity. For example, human defense peptides tend to have a higher arginine content than natural AMPs from other life domains (e.g. bacteria, plants, insects, fish, and reptiles).^{4, 5} It is proposed that the incorporation of more arginines in these peptides confer other biological functions such as immune modulation.³⁴ However, just as the properties of the cationic residues in KR-12 are largely determined by their position in the sequence, the consequences of the substitution of arginines for lysines is also modulated by the nature of the peptide into which the substitution is made. For example, it has recently been shown that in several, but not all, of a number of cell penetrating peptides (CPPs) and AMPs, there is little change in some properties when the substitution of all Lys for all Arg is made in the sequence.³⁵ Therefore, the lysine-to-arginine mutational effects could depend on both residue position and peptide sequence, as well as a specific physical effect measured by a particular method.

Conclusions

Human cathelicidin LL-37 is a multiple functional defense peptide whose antimicrobial activity does not require the entire peptide sequence (Fig. 1). KR-12 contains the minimal antimicrobial peptide sequence against Gram-negative bacteria such as *E. coli*. It showed little cytotoxicity to human red blood cells and is inactive against Gram-positive *S. aureus*.²³ Based on their locations in the 3D structure, the basic amino acids in KR-12 may be classified into two groups. The first group consists of R23 and K25, which are two interfacial basic residues in the amphipathic helix of KR-12 (Fig. 6). These two interfacial residues occupy an important position that controls peptide ability to bind to membranes (Fig. 4) since a loss of either R23 or K25 hinders its binding to D8PG micelles, leading to reduced helix formation (Fig. 7). The second group includes K18, R19, and R29, which are located on the hydrophilic face of the amphipathic helix structure (Fig. 6). These cationic residues appear to be important in clustering anionic lipids (Fig. 5), thereby disturbing bacterial physiology and inhibiting their growth.¹⁶ However, the interfacial cationic side chains are essential in determining peptide activity as well as their interaction with bacterial membranes. It is novel to note that the hydrophobic context of the LL-37 derived peptide determines whether a mutation of an interfacial basic residue increases or decreases membrane permeation and antimicrobial activity. In the case of a sufficiently hydrophobic GF-17, such a mutation compromised peptide activity in every aspect we measured. In the less hydrophobic KR-12 case, the increased hydrophobicity as a result of alanine substitution outweighs the effect of the basic charge. Since basic amino acids on the hydrophilic surface tend to contribute more to hemolysis, they may be mutated to improve peptide selectivity. Our findings documented herein improved our understanding of the structure and activity relationship of human LL-37 and its fragments in particular (Fig. 1) and perhaps other cationic helical AMPs in general.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Amino acid sequences, three-dimensional structures and activities of human LL-37 (A), GF-17 (B), and KR-12 (C)

The 3D structure for each is depicted in a ribbon diagram. The MIC (μ M) for each peptide against *E. coli* K12 is provided on the right side of the peptide sequence and structure.^{12, 13} In the LL-37 sequence, the disordered C-terminal region is underlined and the positions of the five cationic residues investigated in this study using KR-12 as a model are indicated with Arabic numbers.

Fig. 2. Growth curves of KR-12 and its mutants

E. coli K12 was treated with each peptide at 0 μ M (open triangles), 40 μ M (open squares), 80 μ M (open circles), 160 μ M (filled squares), and 320 μ M (filled circles) corresponding to 0, 1, 2, 4, and 8 $\times$ MIC of KR-12 (MIC = 40 μ M). Each panel (A to J) recorded the changes in the *E. coli* growth due to the treatment of a particular peptide as indicated. A known potent peptide GF-17A18 is used as a control and treated at 30 μ M (filled squares). GF-17A18 is as active as GF-17.¹²

Fig. 3. Electron microscopy of KR-12. (A) Untreated. (B) KR-12 treated at $8 \times \text{MIC}$. In this view, KR-12 treatment caused the death of two bacterial cells.

Fig. 4.

The outer and inner membrane permeation assays with the *E. coli* ML-35p strain in the presence of KR-12 and its mutants: (A) KR-12; (B) KR-12A18; (C) KR-12A19; (D) KR-12A23; (E) KR-12A25; (F) KR-12A29; (G) KR-12A18Q19; (H) KR-12K; (I) KR-12R. As a control, GF-17 is provided as panel (J). There are two panels for each peptide. The OM assay (left panel) was carried out for 20 minutes while the IM one (right panel) was followed for 26 minutes, taking readings every 2 minutes at 37°C.

Fig. 5. Differential scanning calorimetric analysis as an indication of lipid clustering ability of KR-12 and its mutants

Positive values are heatings (odd numbers) while negative values (even numbers) are coolings. All graphs are presented in the same scale. The first cycle of heating and cooling between 0 to 35°C corresponds to the lipid alone, followed by two cycles of the lipid containing peptide at a lipid to peptide ratio of 10.

Fig. 6. Three-dimensional structure of KR-12 bound to D8PG

(A) An ensemble of backbone structures indicates two conformers for residue K18; (B) A representative of one conformer; and (C) A representative of another conformer. The structure of KR-12 in complex with D8PG (molar ratio 1:10) was determined by NMR spectroscopy at 25°C and pH 5.4.¹⁶ Residues 20-28 of KR-12 are helical.

Fig. 7. CD spectra of KR-12 and its mutants in PBS or D8PG at room temperature and pH 7.4. The molar ratio of peptide/D8PG was 1:20. The left panel corresponds to peptide in buffer and the right panel to peptide in D8PG, at a lipid to peptide ratio of 20. All graphs are presented in the same scale. The wavelength was scanned from 260 down to 190 nm, every nm.

Table 1Amino acid sequence, antibacterial activity, net charge, and hydrophobicity of KR-12 and its mutants^a

Peptide	Sequence	<i>E. coli</i> K12 MIC (μ M)	IK index	Net charge	HPLC (min)
KR-12	KRIVQRIKDFLR-NH ₂	64 $\pm$ 22	0.33	+5	9.283
KR-12A18	<u>A</u> RIVQRIKDFLR-NH ₂	63 $\pm$ 21	0.29	+4	9.885
KR-12A19	K <u>A</u> IVQRIKDFLR-NH ₂	120 $\pm$ 43	0.20	+4	9.724
KR-12A23	KRIVQ <u>A</u> IKDFLR-NH ₂	40 $\pm$ 20	1.0	+4	9.959
KR-12A25	KRIVQRI <u>A</u> DFLR-NH ₂	112 $\pm$ 44	0.53	+4	9.798
KR-12A29	KRIVQRIKDFL <u>A</u> -NH ₂	140 $\pm$ 100	0.20	+4	9.535
KR-12A18Q19	<u>A</u> <u>Q</u> IVQRIKDFLR-NH ₂	240 $\pm$ 86	0.13	+3	10.342
KR-12K	K <u>K</u> IVQ <u>K</u> IKDFL <u>K</u> -NH ₂	106 $\pm$ 55	0.12	+5	9.017
KR-12R	<u>R</u> RIVQRI <u>R</u> DFLR-NH ₂	30 $\pm$ 11	0.47	+5	9.332

^aMutated residues are underlined and in bold.

Table 2Concentration-dependent membrane permeation of KR-12 and its mutants^a

Peptide	Peptide concentration (μ M) for reaching OD = 0.2	
	Nitrocefin (OD ₄₉₀)	ONPG (OD ₄₅₀)
KR-12	154	308
KR-12A18	>480	480
KR-12A19	>324	324
KR-12A23	120	120
KR-12A25	180	90
KR-12A29	96	192
KR-12A18Q19	168	>>672
KR-12K	>420	>>420
KR-12R	44	88
GF-17 ^b	5	5

^aObtained from Fig. 4.^bGF-17 is used as a control.

Table 3

Quantification of lipid cluster ability and hemolysis of KR-12 peptides

Peptide	Tm1 ^a	Tm2	Tm1+ Tm2	D _{pvl} ^b	Hemolysis% ^c
KR-12	1.2	4.6	5.8	1.0	10.6
KR-12A18	2.3	2.3	4.6	0.79	0
KR-12A19	2.1	4.5	.6	1.14	0
KR-12A23	1.5	4.2	5.7	0.98	8.5
KR-12A25	1.7	4	5.7	0.98	9.4
KR-12A29	2.3	2.3	4.6	0.79	0.2
KR-12A18Q19	0.4	0.4	0.8	0.14	0
KR-12K	2.1	3.7	5.8	1.0	5
KR-12R	3.8	5	8.8	1.52	58

^aThe changes in temperature of the transitions with respect to the lipid without peptide in °C.

^bD_{pvl} is defined as the ratio of (Tm1+ Tm2)/5.8. The sum of transition temperatures is used as a measurement of the total deviation from the pure lipid due to the effect of peptide. The sum of 5.8 for KR-12 is used as a reference value. In this scale, a D_{pvl} value > 1 indicates a stronger lipid clustering ability, while a lower value suggests a poorer ability.

^cRelative to 2% triton X-100, 100 µM GF-17 caused 100% hemolysis under the same assay conditions.

Table 4

Secondary structure analysis of KR-12 and its mutants based on the CD spectra in PBS buffer or D8PG (bold)

Peptide	Environment	-Helix%	-Sheet%	Turns%	Coils%
KR-12	Buffer	8	40	24	28
	D8PG	57	0	21	22
KR-12A18	Buffer	8	32	24	36
	D8PG	60	0	14	26
KR-12A19	Buffer	18	39	19	24
	D8PG	65	0	14	21
KR-12A23	Buffer	5	43	21	31
	D8PG	35	6	21	39
KR-12A25	Buffer	5	41	22	32
	D8PG	39	9	21	31
KR-12A29	Buffer	8	34	20	38
	D8PG	62	0	13	25
KR-12A18Q19	Buffer	7	38	22	33
	D8PG	61	5	13	21
KR-12K	Buffer	5	44	21	30
	D8PG	47	8	17	28
KR-12R	Buffer	9	33	23	35
	D8PG	67	0	11	22