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Review

High-quality 3D structures shine light on antibacterial, anti-biofilm and antiviral activities of human cathelicidin LL-37 and its fragments☆

Guangshun Wang [a,*], Biswajit Mishra [a], Raquel F. Epand [b], Richard M. Epand [b]

[a] Department of Pathology and Microbiology, College of Medicine, University of Nebraska Medical Center, 986495 Nebraska Medical Center, Omaha, NE 68198-6495, USA
[b] Biochemistry and Biomedical Sciences, McMaster University Health Sciences Centre, Hamilton, Ontario L8S 4K1, Canada

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ABSTRACT

Host defense antimicrobial peptides are key components of human innate immunity that plays an indispensible role in human health. While there are multiple copies of cathelicidin genes in horses, cattle, pigs, and sheep, only one cathelicidin gene is found in humans. Interestingly, this single cathelicidin gene can be processed into different forms of antimicrobial peptides. LL-37, the most commonly studied form, is not only antimicrobial but also possesses other functional roles such as chemotaxis, apoptosis, wound healing, immune modulation, and cancer metastasis. This article reviews recent advances made in structural and biophysical studies of human LL-37 and its fragments, which serve as a basis to understand their antibacterial, anti-biofilm and antiviral activities. High-quality structures were made possible by using improved 2D NMR methods for peptide fragments and 3D NMR spectroscopy for intact LL-37. The two hydrophobic domains in the long amphipathic helix (residues 2–31) of LL-37 separated by a hydrophilic residue serine 9 explain its cooperative binding to bacterial lipopolysaccharides (LPS). Both aromatic rings (F5, F6, F17, and F27) and interfacial basic amino acids of LL-37 directly interact with anionic phosphatidylglycerols (PG). Although the peptide sequences reported in the literature vary slightly, there is a consensus that the central helix of LL-37 is essential for disrupting superbugs (*e.g.*, MRSA), bacterial biofilms, and viruses such as human immunodeficiency virus 1 (HIV-1) and respiratory syncytial virus (RSV). In the central helix, the central arginine R23 is of particular importance in binding to bacterial membranes or DNA. Mapping the functional roles of the cationic amino acids of the major antimicrobial region of LL-37 provides a basis for designing antimicrobial peptides with desired properties. This article is part of a Special Issue entitled: Interfacially Active Peptides and Proteins. Guest Editors: William C. Wimley and Kalina Hristova.

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* Corresponding author. Tel.: +1 402 559 4176; fax: +1 402 559 4077.
E-mail address: gwang@unmc.edu (G. Wang).

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1. Introduction

Antimicrobial peptides are host defense molecules of the innate immunity of all life forms and have remained potent for millions of years [1–4]. Interest in antimicrobial peptides has been on the rise since the discoveries of cecropins from silk moths by Hans Boman, magainins from African frogs by Michael Zasloff, and α-defensins from human blood by Robert Lehrer in the 1980s [5–7]. According to the antimicrobial peptide database (APD), over 2000 such peptides have been characterized from a variety of biological sources, ranging from bacteria, protozoa, fungi, and plants, to animals [8,9]. Antimicrobial peptides collected in the APD (http://aps.unmc.edu/AP) are usually small (on average 32 amino acids), cationic (average net charge +3.2) and contain on average 40% hydrophobic amino acids. Such sequences are ideal for recognizing and neutralizing anionic microbes. Therefore, there is a strong desire to develop antimicrobial peptides into a new generation of therapies to meet the growing antibiotic resistance problem.

One of the best studied antimicrobial peptides is human cathelicidin LL-37, a 37-residue cationic peptide starting with a pair of leucines [10–15]. Although the first cathelicidin was identified in 1988 [16], a cathelicidin-related therapeutic practice could be traced to over 100 years ago. Niels Finsen won Nobel Prize in 1903 for his light therapy to treat tuberculosis. This therapy is now linked to LL-37. Light can induce the synthesis of dihydroxylated form of vitamin D, which binds to the receptor to induce the expression of human cathelicidin gene [17,18]. The processed antimicrobial peptide LL-37 might have been responsible for the pathogen killing. Modern scientific results further underscore the significance of antimicrobial peptides to human health. The lack of human LL-37 can cause the disease of morbus Kostmann [19]. In acute and chronic lesions from patients with atopic dermatitis, the expression of LL-37 as well as human beta defensin 2 (hBD-2) is suppressed [20]. *In vitro* antimicrobial assays demonstrated antimicrobial activity of LL-37 against various pathogenic microbes, including superbugs, HIV-1, influenza virus A, fungi, and parasites [21–27]. LL-37 may be an important cancer marker and its fragments may be developed into new anticancer agents [28]. These facts explain the increased literature related to human cathelicidin LL-37 [29].

Human hCAP-18/LL-37 was first discovered in 1995 by three laboratories [30–32]. In this article, hCAP-18 represents the precursor protein (*i.e.*, human cationic protein of 18 kDa), while human cathelicidin is reserved for the mature peptide (*e.g.* LL-37) that exerts the antimicrobial action. The mature cathelicidin antimicrobial peptides from different biological sources vary in amino acid, activity, and 3D structure. However, the precursor proteins share a highly conserved "cathelin" domain. Cathelicidins have been found not only in mammals but also in birds, amphibians, and fish. While many animals contain multiple genes encoding different cathelicidins, humans, rats, and mice have only one cathelicidin gene [10]. The single human cathelicidin gene, however, can generate different forms of cathelicidin peptides (Fig. 1A). In neutrophils, the precursor protein hCAP-18 is cleaved by proteinase to release LL-37 [33]. In the human reproductive system, hCAP-18 in the sperm is processed into ALL-38 within the vagina by gastricsin [34]. Compared to LL-37, ALL-38 contains one additional alanine at the N-terminus. These two forms of peptides have comparable antimicrobial activities. In human skin, other proteases can process human cathelicidin into smaller fragments, which may or may not have antimicrobial activity [35]. The various fragments of LL-37 further expand the human defense arsenal. In addition to antimicrobial activity, human LL-37 possesses many other functions such as chemotaxis, immune modulation, and wound healing (Fig. 1B) [10–15].

This review discusses the advances in structural determination of LL-37 and its important fragments. The high-quality structures of LL-37 and its fragments set the stage for us to explain why certain LL-37 fragments have stronger antimicrobial activities than others. In particular, the central portion of LL-37 is now recognized as the key antibacterial, antibiofilm and antiviral region. Remarkably, this portion corresponds to the central helix in the 3D structure of LL-37 determined by 3D NMR spectroscopy. Our systematic studies of the impact of alanine scanning of cationic amino acids of the major antimicrobial region of LL-37 on membrane permeation, disruption, and lipid clustering provide insight into its mechanism of action.

2. NMR studies of human LL-37 yielded atomic pictures for understanding its interactions with bacterial membranes

2.1. Bacterial membranes and membrane models for structural studies by NMR

Biological membranes are complex and may contain multiple layers with various molecules such as membrane proteins, carbohydrates, and lipids [36–38]. The membranes of different bacteria also differ. The membrane compositions for a select set of bacteria are listed in Table 1. A cartoon view of the surface of Gram-positive and Gram-negative bacteria is given in Fig. 2. While Gram-positive bacteria possess a cell wall outside the membrane, Gram-negative bacteria contain an outer membrane. Furthermore, even Gram-negative bacteria can have different membrane compositions (Table 1). In addition, some resistant bacterial strains have developed mechanisms to modify their membrane surface properties in order to compromise the effect of cationic antimicrobial peptides [39,40]. However, there are also common features. For example, bacterial membranes are characterized by a large negative potential due to the dominance of anionic lipids such

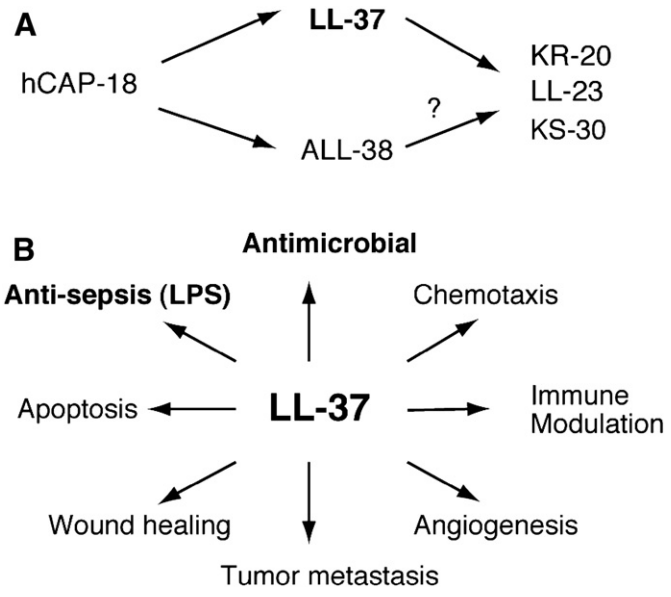

Fig. 1. The processing pathways (*A*) and multiple functions (*B*) of human cathelicidin peptides. In human sweat, LL-37 can be processed into shorter peptides such as KR-20, LL-23, and KS-30 [35]. It is unclear whether ALL-38 can be processed in a similar manner in the human reproductive system [34].

Table 1
Lipid compositions of selected bacteria [101].

Bacterial species	CL (% total lipid)	PG (% total lipid)	PE (% total lipid)
Gram-negative bacteria			
Escherichia coli	5	15	80
Escherichia cloacae	3	21	74
Yersinia kristensenii	20	20	60
Proteus mirabilis	5	10	80
Klebsiella pneumoniae	6	5	82
Pseudomonas aeruginosa	11	21	60
Gram-positive bacteria			
Staphylococcus aureus	42	58	0
Streptococcus pneumonia	50	50	0
Bacillus cereus	17	40	43
Bacillus polymyxa	8	3	60

as phosphatidylglycerols (PG) and cardiolipin (CL) [41,42]. This potential constitutes the major driving force for cationic peptides to recognize the anionic bacterial surface.

While there are direct observations of peptide effects on bacteria by selected biophysical techniques [43,44], many studies were performed in membrane model systems. These include lipid bilayers, bicelles, and micelles [45,46]. Lipid bilayers are useful to establish peptide orientation by solid-state NMR spectroscopy. Bicelles are a membrane model between lipid bilayers and micelles. They can be used for both liquid and solid-state NMR studies depending on the size. However, for solution NMR studies, smaller micelles are preferred because rapid tumbling of such membrane-mimetic systems leads to high-resolution NMR spectra required for structural determination [47]. Sodium dodecylsulfate (SDS) and dodecylphosphocholine (DPC) are two commonly used micelles [45–50]. DPC has a lipid head group identical to phospholipids predominantly found in human cells, whereas SDS with an anionic head group mimics this aspect of bacterial membranes.

Because the major anionic lipids in bacterial membranes are PG, there is a desire to use a more relevant lipid. Based on a comparative study of a series of short-chain phosphatidylglycerols, the Wang laboratory selected dioctanoyl phosphatidylglycerol (D8PG) as a new bacterial

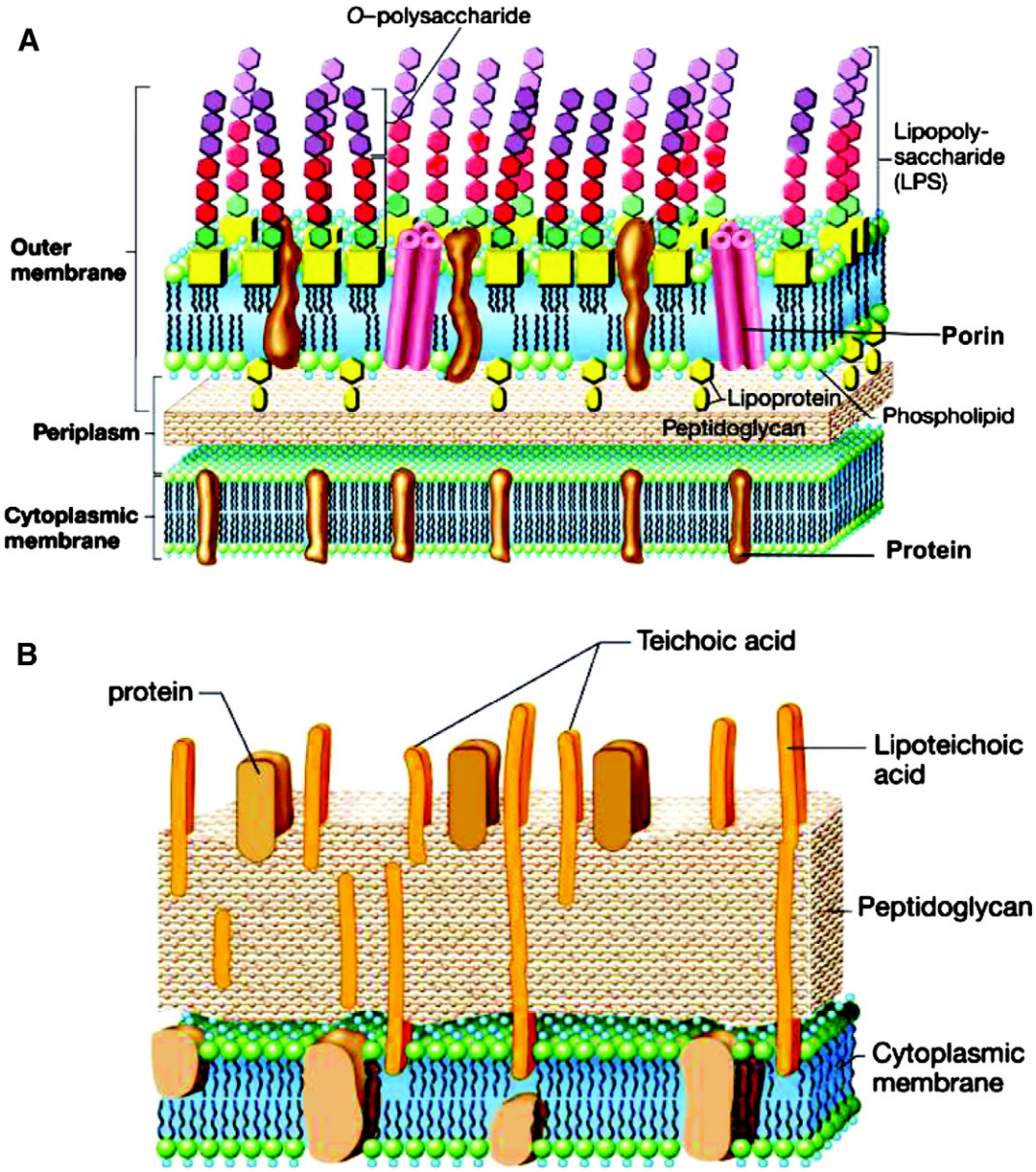

Fig. 2. A cartoon view of the membranes of (*A*) Gram-negative bacteria and (*B*) Gram-positive bacteria. The membranes of Gram-negative bacteria are composed of two layers: the outer membrane rich in LPS and the inner membrane rich in anionic PG. Gram-positive bacteria have a cell wall consisting of lipoteichoic acid and peptidoglycan and a cytoplasmic membrane.

membrane model for solution NMR studies [51–57]. This lipid offers multiple advantages. First, it has the same lipid head group as the major anionic lipid in bacterial membranes and should be biologically more relevant than SDS. Second, it appears to be slightly larger than the SDS micelle, making the surface less curved [56]. Third, a better NMR spectral dispersion has been observed in D8PG than in SDS for several peptides. The better spectral dispersion of the peptides in D8PG may be ascribed to the lipid head group. For example, the arginine side chain amide signals of LL-37 fragments are resolved in D8PG but not in SDS or DPC [57], opening a useful window for detection of peptide–lipid contact details *via* intermolecular NOESY experiments [58]. Fourth, subtle differences in helix formation of single-residue mutants are observed in D8PG but not in SDS [59]. As a consequence, D8PG is a unique bacterial membrane mimetic model that enabled us to determine the 3D structure and membrane surface location of an LL-37 peptide by using one sample and one experiment [54].

2.2. Three-dimensional structure of human LL-37

Typical evidence for membrane targeting of LL-37 is that peptides synthesized using all D-amino acids display similar antibacterial activity to the native LL-37 consisting of L-amino acids [60]. This justifies the structural determination in membranes. We chose anionic SDS micelles as a bacterial membrane mimetic for NMR studies because the sample can be stable for a sufficient time required to record a variety of NMR spectra. The first attempt to solve the 3D structure of LL-37 in the Wang laboratory was made in 2004. It was found that 2D ^{1}H NMR did not offer enough spectral resolution for LL-37 in complex with SDS micelles [61]. Further efforts were then devoted to the expression and production of isotope-labeled peptides [62], which enabled structural determination of LL-37 by 3D NMR spectroscopy [56]. In a 3D NMR spectrum [63], the NMR peaks initially crowded on one 2D spectrum were separated onto several dozen 2D planes along the third ^{15}N or ^{13}C dimension. The power of this 3D NMR method can be analogously understood because a book would become illegible had all the pages been printed onto a single page! Therefore, every effort was taken to optimize spectral resolution. The 3D structure of human LL-37 bound to SDS micelles is presented in Fig. 3A. This LL-37 structure possesses a long amphipathic helix (residues 2–31) followed by a disordered tail (residues 32–37) [56]. In the absence of peptide dynamic information, a flexible polypeptide region may be generated due to a limited number of NMR restraints or due to peptide dynamics [64,65]. The order-disordered structural pattern of LL-37 was validated by an independent measurement of the peptide ^{15}N backbone dynamics, which indicates that residues 2–31 are indeed rigid and only the C-terminal tail is mobile.

We should mention that the structure of LL-37 bound to DPC was also determined by using the traditional 2D ^{1}H NMR spectroscopy [66]. A helix-break-helix motif between residues 4–34 was found with both terminal residues flexible. Such a structural pattern found in DPC [66] differs from the ordered-disordered structural pattern determined in SDS micelles [56]. Importantly, only the LL-37 structure (Fig. 3A) determined in SDS micelles can be applied to the LPS and PG-bound states (below).

2.3. Structural basis of bacterial membrane binding of human LL-37

There is a strong desire to follow the action of antimicrobial peptides on bacteria. Transmission electron microscopy (TEM) can be used to view the effect of antimicrobial peptides on bacteria and some cell envelope damages or disruption have been observed [59]. On the other side of the coin, one can also follow the conformational states of the peptide spectroscopically. Before the peptide reaches the bacteria, circular dichroism (CD) studies showed that LL-37 did not have a defined structure (*i.e.* random coiled state) and became helical at neutral pH or in complex with membranes [67]. Using NMR spectroscopy, it is possible to precisely map the interactions of LL-37 with the major components of the bacterial outer and inner membranes [56]. This was accomplished using an isotope-labeled LL-37 [62]. Consistent with CD, the peptide is indeed disordered in water at pH 3.6. At pH 7, the peptide forms a tetramer [67,68] involving residues 1–36 [68].

Lipopolysaccharides (LPS) are a major component of the Gram-negative bacterial outer membrane (Fig. 2A). In complex with LPS, residues 1–31 of LL-37 are involved in binding, but not the C-terminal tail [56]. In the LL-37 structure, a hydrophilic S9 segregates the hydrophobic surface of the long helix into two domains (Fig. 4A). This two-domain structure explains the cooperative binding of LL-37 to LPS [69]. S9 appears to play a unique role in modulating peptide activity since a mutation of S9 to either A9 or V9 reduces the antibacterial activity of LL-37 against *E. coli* [55].

The establishment of the D8PG model also enabled us to determine the 3D structure of LL-37 bound to PG, which mimic the Gram-negative bacterial inner membrane (Fig. 2A). All the NMR data indicate that LL-37 bound to D8PG adopts the same structure as determined in SDS micelles [56]. It appears that the lipid head group or number of lipid acyl chains did not affect the structure in the case of LL-37. Hence, the ordered-disordered structure of LL-37 bound to SDS can be extended to the cases of both LPS and PG-bound states. This structure (Fig. 3A) forms the basis for understanding the interactions of LL-37 with bacterial membranes.

2.4. Direct NMR evidence for peptide–membrane interactions

In many cases, if not all, NMR studies focus on either the peptide portion (*e.g.* a full structural determination) or the membrane portion [50]. While solution NMR was applied to determine the structure of frog antimicrobial and anticancer peptide aurein 1.2, solid-state ^{31}P NMR was used to probe lipid vesicle structures [70–74]. Likewise using solid-state NMR, a single amino acid labeled peptide was used to determine the orientation of LL-37 on the lipid bilayer [74], whereas the natural ^{31}P probe was utilized to gauge lipid structure and motions [74,75]. However, a direct observation of the interactions between antimicrobial peptides and membranes is rare. We utilized solution NMR spectroscopy to provide direct evidence for the binding of the long amphipathic helix of LL-37 (Fig. 3A) to membranes [56]. Human LL-37 is a unique molecule with four aromatic phenylalanines, which are outstanding probes for peptide binding [51,54,70]. As an advantage of the use of protonated D8PG, we were able to observe the direct dipole–dipole interactions between the protons of peptide aromatic phenylalanines and the D8PG lipid. The four aromatic rings (F5, F6, F17, and F27) of LL-37 are well positioned along the peptide chain (Fig. 4B green). Thus, both helical domains in the long helix of the peptide are indeed involved in membrane binding. In addition, the same intermolecular NOESY spectrum of LL-37 in complex with D8PG also indicates direct interactions of arginines with PG.

2.5. Structures of LL-37 fragments determined by the improved 2D NMR method

Then, are all the arginines equally important or some more important than others? To facilitate our observation, we chose to use the major antimicrobial region of human LL-37 [21]. This major antimicrobial binding region was identified by NMR as illustrated in Fig. 3. Initially, LL-37 was cut into two halves: LL-12 (*i.e.* residues 1–12) and IG-25 (*i.e.* residues 13–37). While LL-12 has a very short helix at the N-terminus (Fig. 3B), a well-defined helical structure (residues 17–31) was observed in the central region of IG-25 bound to SDS (Fig. 3C) [61]. By trimming the C-terminal disordered region of IG-25, we obtained a 20 residue peptide. In addition, we swapped the positions between I13 and G14 since many natural

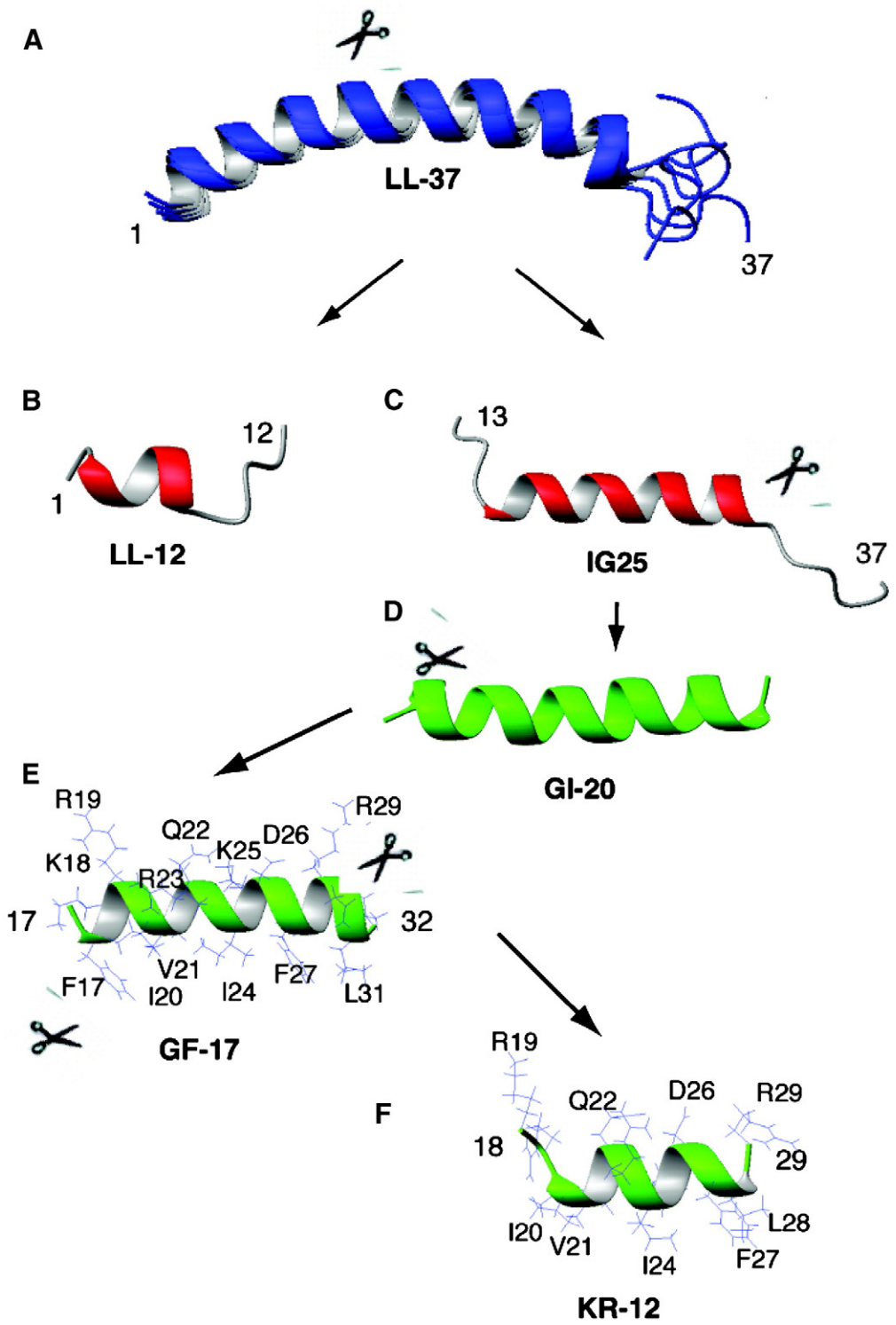

Fig. 3. Ribbon diagrams of the three-dimensional structures of human LL-37 and its fragments bound to membrane-mimetic micelles. (*A*) An ensemble of 5 structures of LL-37 is shown. The structured region of LL-37 superimposes well but not the disordered C-terminal tail. Also presented are NMR structures of LL-12 (*B*), IG-25 (*C*), GI-20 (*D*), GF-17 (*E*), and KR-12 (*F*). For clarity, only the side chains of GF-17 and KR-12 are shown and labeled. The structures of LL-37 (PDB entry: 2K6O), LL-12 (PDB entry: 2FBU), IG-25 (PDB entry: 2FCG), and GF-17 (PDB entry: 2L5M) were determined in the presence of SDS micelles [21,56,61], while the structures of GI-20 and KR-12 were determined in complex with D8PG [56,57]. In several cases including LL-37 itself, we found a similar structure for the peptide in SDS and D8PG [56]. Note the conformational changes at the C-terminus of LL-12 (*B*) and at the N-terminus of IG-25 (*C*) compared to the conformation of intact LL-37 (*A*).

antimicrobial peptides start with a glycine. The resulting peptide, GI-20, is entirely helical (Fig. 3D) in complex with D8PG or DPC micelles [54,57]. By trimming off both the disordered C-terminus and the less structured N-terminus of IG-25, we obtained a 16-residue segment. Again to better mimic natural antimicrobial peptides, a glycine was inserted at the N-terminus. This peptide, GF-17, is referred to as the major antimicrobial region of LL-37 [21]. GF-17 is entirely helical in the presence of SDS micelles (Fig. 3E).

In the structures of both GF-17 and GI-20, including the smallest antibacterial peptide KR-12 (Fig. 3F), R23 is located between the hydrophobic and hydrophilic interface (*i.e.*, interfacial region). This location can also be seen in the LL-37 structure, where only R23 is adjacent to

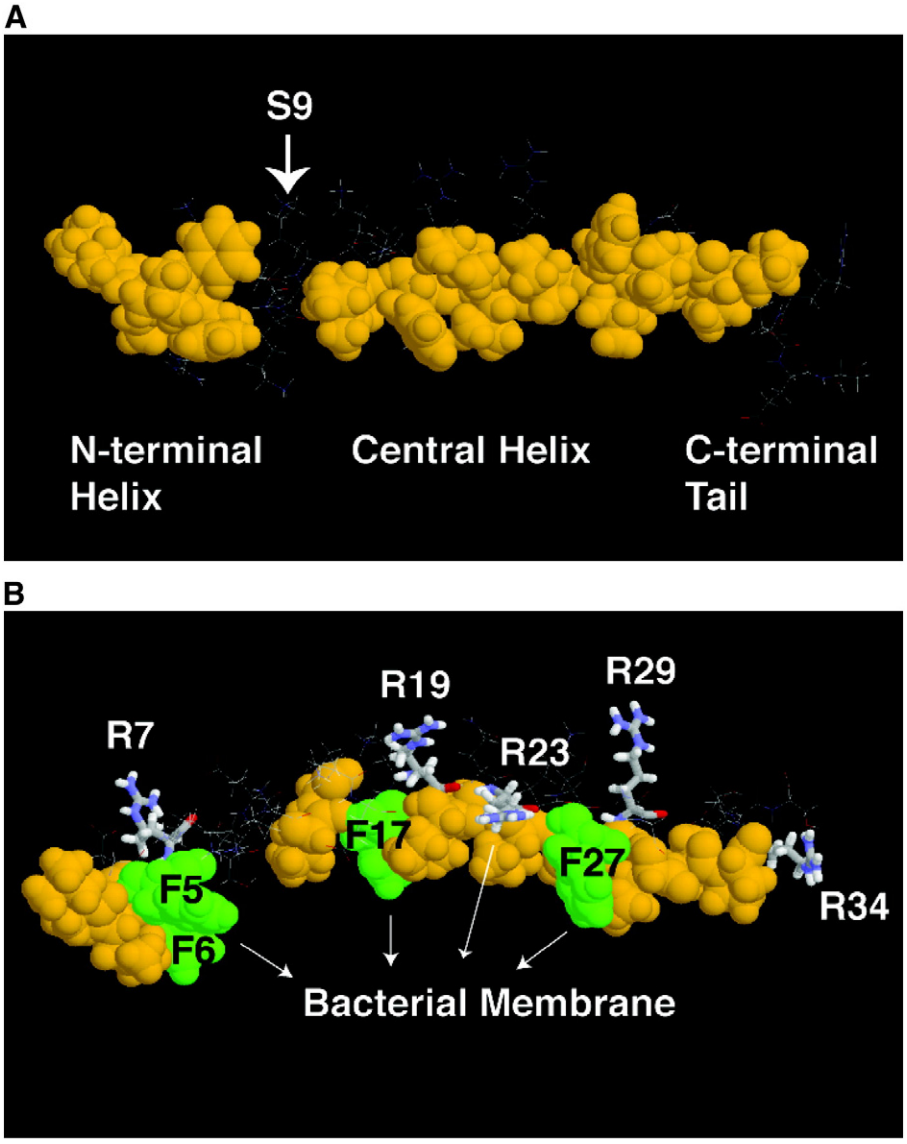

Fig. 4. The central helix stands out in the high-quality 3D structure of human LL-37 as the key antimicrobial region. (*A*) While the C-terminal tail does not participate in membrane binding, S9 splits the membrane-targeting hydrophobic surface (gold) of the long amphipathic helix (residues 2–31) into two domains: an N-terminal short helix and a long central helix. Such a structure explains the cooperative binding of LL-37 to LPS [69]. It also indicates that the central helix is the key antimicrobial region of LL-37, which is responsible for antibacterial, antibiofilm, and antiviral activities discussed in this article. (*B*) Identification of important side chains of LL-37 directly involved in membrane interactions by NMR. All of the four hydrophobic aromatic rings of F5, F6, F17, and F27 (green), as well as cationic R23 (stick), interact with phosphatidylglycerols. In this view, only the side chain of R23 is close to the hydrophobic surface (gold). It is thus an important interfacial basic side chain required for membrane interactions or binding to other anionic components such as DNA.

the hydrophobic surface (Fig. 4B). Interestingly, Arg–PG interactions were detected by solution NMR only for R23, but not for R19 or R29 in the NOESY spectra [54]. This R23 is essential for bacterial targeting because an alanine substitution reduced the antibacterial activity of GF-17 against Gram-negative *Escherichia coli* [21]. This agreement between biological and structural data underscored the importance of the interfacial arginine R23 (see also Fig. 4B).

In summary, our NMR structural studies led to (1) a high-quality 3D structure for intact LL-37, which uses residues 2–31 to interact with both LPS and PG [56]; (2) 3D structures for LL-12, KR-12, GF-17, GI-20, IG-25 (Fig. 3) [21,57,61], and LL-23 [55]; and (3) successful observation of direct interactions between arginines and PG as well as between aromatic phenylalanines and PG [54,56]. Structures of these LL-37 fragments were determined by using improved 2D NMR methods. In this improved method, additional NMR experiments are performed to enable the measurements of ^{13}C and ^{15}N chemical shifts at natural abundance (*i.e. without* isotope labeling). Such heteronuclear chemical shifts, not available in the traditional 2D proton NMR spectra recorded using unlabeled peptides, are utilized for structural refinement [70]. This method enables a more accurate definition of the structured regions of the peptides, especially under the situations when the NOE restraints are insufficient. The importance of this improved method has been highlighted in a recent review article [64]. In certain cases, this method can avoid the publication of misleading structure or dynamics. Hence, these high-quality 3D structures and atomic details for peptide–PG interactions laid a solid basis for us to discuss the structure–activity relationships of human LL-37 below.

3. Structural insight into antibacterial and antiviral activities of LL-37 peptides

Since 1995, the antibacterial activities of human LL-37 have been evaluated using multiple bacterial strains, including some resistant superbugs. It is noticed that antibacterial activity data can vary for the same bacterium. For example, LL-37 is active against *Staphylococcus aureus* ATCC 29213 [76], but is poorly active against methicillin-resistant *S. aureus*

(MRSA) USA300 [21]. The Lehrer lab found that LL-37 was only active against MRSA at a low salt concentration [77]. It seems that assay protocols, bacterial strains (including the mechanism of resistance), and assay conditions (*e.g.* pH, media, salts, serum) all matter in antimicrobial assays. Because of these variables, the goal of this section is to review antimicrobial activity data of LL-37 and its related fragments obtained by the same lab, using the same method, conditions and bacterial strains. Under such conditions, we may attribute the activity difference to peptide sequences and, whenever possible, explain peptide potency based on high-quality 3D structures of LL-37 and its fragments (Fig. 3).

3.1. Antibacterial and anti-biofilm activities of LL-37 peptides

Johansson et al. first compared LL-37 with N-terminally truncated fragments [67]. LL-37 is active against *E. coli* D21 with a MIC of 5 μM. In 5 mM Na_2SO_4, LL-37 is more active than FF-33 and SK-29 (sequences in Table 2). However, FF-33 is as potent as LL-37 in medium E. It seems that the very N-terminal four residues LLGD are dispensable in medium E. Subsequently, Shai and colleagues showed that LL-37 and FF-33 were equally potent in permeating membranes. Interestingly, the LL-37 oligomers were maintained in vesicles with a phosphocholine head group, but dissociated in PG-containing membranes, indicating that LL-37 works as a monomer [78]. Hence, the monomeric 3D structure of LL-37 determined in the presence of anionic D8PG or SDS well represents the active form. In this structure (Fig. 4B), the aromatic rings of F5 and F6 stack on each other as a result of aromatic–aromatic interactions. We propose herein that this F5/F6 aromatic–aromatic interaction may also play a role in LL-37 oligomerization, where a group of aromatic rings can cluster. This requires at least two copies of the LL-37 molecule in a parallel position in the tetramer. This speculation explains why further removal of F5F6 from the LL-37 sequence reduced the propensity of SK-29 in forming oligomers. Braff et al. found that N-terminal fragments such as RK-31 and KS-30 (Table 2) became more potent than LL-37 against Gram-positive, Gram-negative bacteria and *Candida albicans* [79]. Ciornei et al. also showed that removal of 2 or 6 residues from the N-terminal region of LL-37 had little effect on peptide activity [80]. Others found that both LL-31 and LL-32 exhibited a stronger killing effect than LL-37 [81,82]. All of these results suggest that the N-terminal short helix of LL-37 (Fig. 4A) is less important for antibacterial activity.

Several studies pointed at the importance of the central region for antimicrobial activity of LL-37. Sieprawska-Lupa et al. [83] identified FK-21 (Table 2), a C-terminal fragment of LL-37 derived from a protease degradation reaction. FK-21 displayed comparable minimal bactericidal concentrations (MBC) to LL-37 in killing *E. coli*, *B. subtilis*, *P. aeruginosa*, and *E. faecalis*. Nell et al. [84] scanned the LL-37 sequence with a window size of 22mer. Based on a comparison of a library of overlapping LL-37 peptides, they identified IG-24 (Table 2), a central fragment of LL-37, as the most promising segment in terms of antimicrobial and anti-LPS activity. Using an *in silico* approach, Sigurdardottir et al. identified a central fragment GKE-21 (Table 2), which is as potent as LL-37 in killing bacteria [85]. Based on the NMR technology dubbed TOCSY-trim, Wang was able to accurately map the major antimicrobial region of LL-37 to residues 17–32 [61]. The same region is also implicated in the structure of IG-25 when both flexible tails are cleaved (Fig. 3C). A resulting synthetic peptide GF-17 (Table 3) is highly potent against both *E. coli* and community-associated MRSA USA300 LAC [21]. Interestingly, this major antimicrobial segment (residues 17–32) discovered by NMR is contained within FK-21, IG-24, or GKE-21, explaining the antimicrobial potency of these fragments.

Also using a series of synthetic peptides, IG-19 (Table 2) was found to be the shortest fragment of LL-37 that exhibited antibacterial activity against *Burkholderia thailandensis* E264 [81]. The same peptide was also identified as the best immune modulating peptide in another study [86]. This IG-19 fragment is very similar to GI-20 (Table 3), which displays an optimal therapeutic index against HIV-1 [22]. These observations make sense, since GI-20, or a similar fragment IG-19, corresponds to the central helix of LL-37 (Fig. 4A).

Antimicrobial activity assays are usually conducted using planktonic bacteria cultivated under laboratory conditions (*e.g.*, rich media, 240 rpm, and 37 °C). This does not necessarily reflect commensal conditions or the microbial infectious states. In particular, bacteria frequently exist in biofilms, where many bacteria work together to create a tower-like structure. Such a bacterial community is more difficult to eradicate. The loss of potency of many traditional antibiotics demands more potent antimicrobials and antimicrobial peptides are potential candidates [1–4]. Recently, Nagant et al. compared anti-biofilm activity of LL-37 with its fragments against *P. aeruginosa* PAO1 [87]. They found that the N-terminally or C-terminally truncated fragments RK-31 and LL-31 (Table 2) were most potent. The central fragment RK-25 (residues 7–31) displayed both strong antimicrobial and anti-biofilm activity. The authors proposed that this fragment is the minimal sequence required for anti-biofilm activity. Feng et al. [88] evaluated antimicrobial and anti-biofilm activities of LL-37 and fragments against *A. baumannii*. The antimicrobial potency is in the following order: KS-30 > LL-37 > KR-20 > KR-12. While all these peptides can inhibit biofilm formation, only KS-30 was able to disrupt pre-formed biofilms. It is evident that both RK-25 (Table 3) and KS-30 (Table 2) peptides contain the central helix of LL-37 (Fig. 4A), explaining their anti-biofilm potency.

3.2. Antiviral activities of LL-37

There is also high interest in identifying antiviral peptides. Some antimicrobial peptides are known to have antiviral effects. In the APD database [8,9], 138 entries are annotated to possess antiviral activity as of October 2013. Human LL-37, but not a scrambled sequence, is potent against viruses [89]. For example, human cathelicidin could inhibit orthopox virus (vaccinia) replication both *in vitro* and *in vivo* [90]. LL-37 is potent against influenza virus A as well [89], probably by directly disrupting the viral membranes rather than *via* viral aggregation [91]. Crack et al. found a reduced viral load of varicella zoster virus (VZV) in infected keratinocytes and B cells due to the LL-37 expression [92].

In several cases, antiviral effects of both intact LL-37 and select fragments were tested. LL-37 was shown to inhibit HIV-1 replication in peripheral blood mononuclear cells (PBMC) [23,93]. Subsequently, Wang et al. evaluated anti-HIV activity of a panel of LL-37 fragments, and a central fragment, GI-20 (Table 3), showed the highest potency as well as therapeutic index. F17, helicity, and sequence order of LL-37 peptides are all important for inhibiting HIV-1 replication [22], consistent with its binding to the HIV-1 reverse transcriptase [94]. Currie et al. [95] found that LL-37 is inhibitory to respiratory syncytial virus (RSV) *in vitro* and early treatment (2 h after infection) is essential.

Table 2
Selected sequences of LL-37 and its fragments.

Name	Amino acid sequence	Ref.
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	[33]
FF-33	FFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	[67]
LL-32	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLV	[82]
LL-31	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNL	[81]
RK-31	RKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	[35]
KS-30	KSKEKIGKEFKRIVQRIKDFLRNLVPRTES	[35]
SK-29	SKEKIGKEFKRIVQRIKDFLRNLVPRTES	[67]
IG-25	IGKEFKRIVQRIKDFLRNLVPRTES	[61]
IG-24	IGKEFKRIVQRIKDFLRNLVPRTE	[84]
LL-23	LLGDFFRKSKEKIGKEFKRIVQR	[35]
KI-22	KIGKEFKRIVQRIKDFLRNLVP	[96]
FK-21	FKRIVQRIKDFLRNLVPRTES	[83]
GKE-21	GKEFKRIVQRIKDFLRNLVPR	[85]
KR-20	KRIVQRIKDFLRNLVPRTES	[35]
IG-19	IGKEFKRIVQRIKDFLRNL	[81]
LL-12	LLGDFFRKSKEK	[61]

Table 3
Useful LL-37 templates for peptide design.

Peptide	Amino acid sequence	LL-37 region	Activity	Refs
KR-12	KRIVQRIKDFLR (*Minimal antibacterial region*)	18–29	Anti-*E. coli*	[56]
FK-13	FKRIVQRIKDFLR (*The LL-37 core peptide*)	17–29	Minimal anti-HIV peptide	[61]
GF-17	FKRIVQRIKDFLRNLV (*Major antimicrobial region, core*)	17–32	Antibacterial (*e.g.* MRSA); anticancer	[21,61]
GI-20	GIKEFKRIVQRIKDFLRNLV (*The central fragment*)	13–32	Antiviral; immune modulation[a]	[22,54,86]
RK-25	RKSKEKIGKEFKRIVQRIKDFLRNL	7–31	Anti-biofilm	[87]

[a] A similar sequence IG-19 was reported for immune modulation [86].

Furthermore, antiviral effect is retained by a central fragment covering residues 12–33 (KI-22, Table 2), while both the N-terminal (residues 1–22) and C-terminal fragments (residues 17–37) were ineffective. These results all suggest the critical role of the central fragment in inhibiting the infection of viruses such as HIV-1 and RSV (see the central helix in Fig. 4A).

Antimicrobial peptides can usually work on viral envelopes. In this case, the LL-37 structure determined in membranes (Fig. 3A) can be useful to understand antiviral effects as well. However, there are also cases where antimicrobial peptides interact with a protein target. Human LL-37 appears to target HIV-1 reverse transcriptase [94]. It seems that the central region of LL-37 is sufficient for the inhibition of most of the viruses mentioned above. This central helix stands out in the 3D structure of LL-37—the N-terminal helix is separated from the central helix by a hydrophilic serine at position 9, while the very C-terminal tail is disordered and not required for membrane targeting (Fig. 4A). We propose that the helical structure of this central peptide (corresponding to GI-20) could be important for shutting down the viral machinery. This helical segment may also be expected to promote positive membrane curvature that would inhibit viral fusion [96]. In particular, HIV-1 viral proteins gp41 and gp120 can induce the formation of lipid rafts rich in cholesterol and sphingolipids that facilitate the viral fusion with host membranes. The interactions of LL-37 with such lipids could inhibit viral fusion [97]. Indeed, fusion of a peptide with such lipids enhances antiviral activity against enveloped viruses such as HIV-1 [98,99]. It should be pointed out that certain antimicrobial peptides such as human alpha-defensin HD-5 can directly neutralize non-enveloped viruses, including human adenoviruses, papillomaviruses, and polyomaviruses. For this action, select arginines and hydrophobic residues of the peptide are essential [100]. Since the central fragment of LL-37 has an important role in inhibiting various viruses, it constitutes an excellent template for developing antiviral therapies (Table 3), especially in cases where vaccines are not available or ineffective. Because of the significance of this central fragment of LL-37, further efforts were devoted to the understanding of the mechanism of action as well as functional roles of the cationic amino acids using GF-17 as a model (below).

4. Biophysical insight into membrane permeation, disruption and lipid domain formation by the major antimicrobial region of human LL-37

4.1. Membrane permeation

As shown in Fig. 2A, Gram-negative bacteria possess two layers of membranes: an outer membrane, containing LPS in the outer monolayer, and an inner membrane rich in PG. The total phospholipid composition of both membranes is shown in Table 1 (see also [101]). In general the membranes of Gram-negative bacteria are rich in phosphatidylethanolamine (PE) with variable amounts of the anionic lipids PG and CL. To follow peptide-mediated membrane permeation, the Lehrer group designed a dual reporter system for Gram-negative bacteria using *E. coli* ML-35p [69]. 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 outer membrane and is excluded from the periplasmic space. However, permeabilization of the outer membrane 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 this strain of *E. coli* lacks lactose permease, o-nitrophenylgalactose (ONPG) cannot traverse its inner membrane 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. Using this dual reporter system, we compared the membrane permeation ability of the single alanine variants of the five cationic amino acids in both the GF-17 and KR-12 peptide series. For GF-17, antimicrobial activity was reduced when either R23 or K25 was mutated to an alanine. Interestingly, the R23A peptide variant was unable to permeate either the outer or inner membranes of *E. coli*, identifying R23 as a critical cationic side chain for membrane permeation [21]. In contrast, the KR-12 peptide became more membrane permeable when either R23 or K25 was mutated to an alanine [59]. Correspondingly, these two KR-12 variants became more active (lower MIC values) against *E. coli*. It is evident that the membrane permeation ability of the peptide corresponds well to antimicrobial activity for both peptides. What was puzzling was the opposite effects observed in the KR-12 and GF-17 cases. Since GF-17, a major antimicrobial peptide of LL-37 (Table 3), was sufficiently hydrophobic, the loss of R23 had a drastic effect on peptide activity [21]. In the case of KR-12 (Fig. 3F), which corresponds to the smallest antimicrobial region of LL-37 (Table 3), the same substitution actually increased peptide hydrophobicity, making it a more potent analog [59]. This is an important finding, showing that the peptide length determines whether peptide activity rises or decreases when a cationic amino acid is changed to an alanine. Nevertheless, in both cases, the critical cationic amino acids for membrane binding and permeation are R23 and K25, which are located in the interfacial region of the amphipathic helices (Fig. 3). It seems that this observation applies primarily to Gram-negative bacteria because there are relatively small changes in antimicrobial activity against Gram-positive bacteria such as *S. aureus* when R23 or K25 was replaced [21].

4.2. Membrane disruption: carpet or pore formation?

Membrane-targeting peptides can permeate the bacterial outer membrane and reach the inner membrane. It is proposed that the action of peptides on membranes is sufficient in itself to cause lethality. Experimentally, membrane disruption can be viewed from TEM images and other imaging methods. To explain membrane disruption, a variety of models have been proposed. These include the carpet model [102], barrel-stave [103], and toroidal pore [104]. All these models point to the change in membrane structure in the presence of the peptides. In many cases the pores that are formed lead to movement of ions across

the membrane, resulting in the loss of membrane electrical potential [105,106]. Such damaging effects could be lethal, leading to bacterial death. Experimental evidence for the carpet model results when the peptide is oriented parallel to the plane of the membrane bilayers. For LL-37, supporting evidence for a surface location was obtained from the lipid bilayer model based on solid-state NMR as well as FT-IR studies [70,78]. These results agree with our finding from solution NMR studies as well that suggest a surface location [54,56]. Oren and Shai have suggested that LL-37 does not form a pore in a membrane [78]. However, others have obtained evidence that LL-37 can form a pore on membranes. It was suggested, based on effects of membrane curvature, that this peptide forms a toroidal pore [74]. It was also suggested that although LL-37 did not form a pore on its own, it did promote pore formation in cells expressing the P2X receptor [107]. Based on spectroscopic evidence, it was suggested that either a carpet or toroidal pore mechanism was possible for LL-37 [108]. Diffraction measurements, albeit at high peptide concentrations, show a trans-bilayer orientation of the peptide, consistent with pore formation [109]. Time-lapse fluorescence microscopy was suggested to be more consistent with a pore model [110]. All of these results are difficult to reconcile. The experimental conditions, including the modification of the LL-37 sequence, might have played a role here. These observations could suggest that LL-37 adopts different states depending on the type of membrane surface. We do not exclude the possibility that these two states represent different stages of peptide action on membranes. The carpet model emphasizes the initial state when the peptide is coating bacterial membranes. Subsequently, the peptide can transit to another state and cause either membrane lysis like a detergent or pore formation depending on the properties of the peptide, the nature of the membrane, the extent of hydration, and the conditions of growth. The curved structure of LL-37 (Fig. 3A) might be perfect for sealing the wall of a pore or micellation of bacterial membranes like a detergent.

Another approach to understand the mechanism of action of LL-37 is to utilize homologous primate cathelicidins. There appears to be a balance between cationic residues contributing to peptide binding to anionic lipids and the influence of charge on peptide properties such as aggregation. Tossi and colleagues proposed that peptides in the free state in solution are more likely to permeate the outer membrane and work on the inner membrane to kill bacteria, while those in the aggregated form tend to stay on membrane surface and mainly act to modulate immune functions. Being different in sequence, these homologous cathelicidins may work *via* different mechanisms. For example, atomic force microscopy (AFM) revealed a carpet-like mode of permeabilization for RL-37 and formation of more defined worm-holes for LL-37 [111].

Although lipid vesicles may be an over-simplified model compared to bacterial membranes, dye release from lipid vesicles may also yield some evidence for membrane damage (pore or micellization). GF-17 can lyse lipid vesicles but not KR-12 [112]. Then, what does KR-12 do to the membranes?

4.3. Membrane domain formation induced by LL-37 fragments

There are cases when the peptide does not pass through the membrane and does not lyse the membrane. Under such conditions, how could the growth of a bacterium be inhibited? There are antimicrobial peptides that coat bacterial surfaces [113], preventing bacterial replication. The Epand laboratory has shown the formation of lipid domains in the presence of cationic peptides or their mimicries, providing yet an alternative model to understand peptide action [114–120]. The fundamental force behind this can be ascribed to the attraction of anionic lipids toward cationic peptides, leading to the enrichment of anionic lipids around basic peptides. This peptide-induced lipid domain formation in the membrane could have a global impact on bacteria. For example, it would hinder bacterial signal transduction that requires PG for membrane targeting of amphitropic proteins such as IIA^{Glc} involved in *E. coli* glucose uptake [51,121]. It would also influence the normal functions of other membrane-bound proteins such as voltage-dependent potassium channels that also require proper Arg–PG interactions [122]. These effects of antimicrobial peptides would disturb the membrane physiology of bacteria, leading to cell growth inhibition or cell death [56]. It is relevant to mention that human LL-37 and its variants can also induce lipid domain formation in *C. albicans* [123].

Multiple techniques have been used to support the mechanism of lipid clustering. These have been recently discussed [101] and include DSC, ^{31}P NMR, ^{2}H NMR, freeze-fracture electron microscopy, AFM and the combination of AFM with polarized total internal reflection microscopy. KR-12 is a good model system for these experiments [112,115].

Next, we have also compared the lipid clustering capability of different LL-37 fragments [112]. Of particular interest is the finding that substituting 3 resides in GF-17 with the corresponding enantiomers to produce GF-17d3 [61], resulted in a peptide with greater lipid clustering ability. This peptide, as well as the KR-12 [56], has weaker ability to induce liposomal leakage and to exhibit bacteriostatic activity [124]. This may be a consequence of their lower hydrophobicity, preventing them from penetrating deeply into the membrane and perturbing it.

What types of basic amino acids are important for lipid clustering? Both GF-17 and KR-12 were utilized to answer this question. It seems all the basic amino acids play a role, although to varying degrees. Therefore, the two sets of lipid-clustering basic residues are not identical as a consequence of the change in peptide length. In both cases, however, basic residues on the hydrophilic face appear to be important. In addition, more flexible side chains appeared to be more important. In the case of KR-12, the basic amino acids that are more important in lipid clustering are K18 and R29. These residues are located at the terminal regions of the peptides (Fig. 3F) and can be more mobile than those located in the interface [59]. As a further support for the role of flexibility, the Epand laboratory found that, among numerous antimicrobials studied up to date, most flexible peptide mimicries also showed the largest degree of lipid clustering [106,125].

More generally, the formation of a lipid domain in the presence of cationic peptides is related to the reconstitution of the system into a different phase diagram. Therefore, additional non-peptide factors such as lipid chain length and packing density may come into play [126,127]. Membrane segmentation can generate defects and facilitate the insertion of peptides into the lipid bilayers, leading to pore formation.

4.4. New insights into the membrane perturbation potential model

It has been realized for a long time that both hydrophobic and basic amino acids play an essential role in determining peptide activity [1–4,10,78,102–105]. When a single parameter fails to correlate structure with activity, we found it useful to combine the hydrophobic and basic elements. Wang et al. [70] derived the membrane perturbation potential model based on a comparison of the 3D structures of a non-toxic bacterial membrane anchor and antimicrobial and anticancer peptide aurein 1.2, both containing two lysines. The bacterial membrane anchor sequence, with a short 3-turn helix (10 residues), was able to associate with anionic PG but not phosphocholines, verifying the important role of electrostatic interactions for preferential binding to bacterial membranes rich in PG [121]. Although both are helical peptides, the bacterial membrane anchor with a shallow hydrophobic surface is unable to deeply penetrate the membrane, thereby it is not toxic to bacteria. In contrast, aurein 1.2 has a broader hydrophobic surface, enabling deeper membrane penetration to kill bacteria [70]. Indeed, solid-state NMR studies indicate that aurein 1.2 acts *via* a carpet model [71–73]. Likewise, DFTamP1, a peptide designed based on the

database filtering tech, has a broad hydrophobic surface and is potent to kill MRSA USA300 [128].

Using peptides from the central antimicrobial region of LL-37 as models, we have learned the following: (1) The peptide sequence order is not important for antibacterial activity but essential for anti-HIV activity [22,129]. (2) Peptide length (related to mainly hydrophobicity) is clearly important for the antimicrobial activity of this helical peptide. While truncation of GF-17 into KR-12 reduced antimicrobial activity [56], an extension of the helical region of the bacterial membrane anchor by one turn made the peptide antibacterial [70]. (3) The aromatic phenylalanines are involved in membrane binding and they may show clear difference in micelle penetration depth [129]. (4) The continuity of the hydrophobic surface of an amphipathic helix is important for a deep penetration into membranes [55]. (5) Cationic amino acids of GF-17 appear to have multiple roles such as membrane binding, permeation, and domain formation [21]. Among the five basic amino acids, interfacial R23 plays an essential role in these membrane interactions, while cationic residues on the hydrophilic face tend to be more important in lipid clustering. (6) It is important that the peptide length determines whether the activity goes up or down when an interfacial cationic arginine is replaced by an alanine. For a more hydrophobic GF-17, the loss of R23 reduces peptide activity, whereas the peptide antibacterial activity increased in the case of less hydrophobic KR-12 [59]. These new results shed additional light on the membrane perturbation potential (MPP) model proposed earlier. The hydrophobic surface bordered by the interfacial basic residues defines the first action layer important for membrane binding, membrane permeation, and peptide activity, while non-interfacial basic amino acids on the hydrophilic surface could modulate other physical processes such as membrane domain formation. With the accumulation of data, it may become feasible to quantify the MPP as a measure of peptide membrane perturbation ability. This will form the basis for us to rationally design peptides with desired MPP.

There are also some antimicrobial peptides, however, with non-helical structures. For example, Pro-rich peptides and Trp-rich lactoferrins do not adopt a regular helical structure. In the case of a 15mer lactoferrin fragment, membrane interactions did not correlate with antimicrobial activity [130–132]. Likewise, alanine substitutions of lysines or the arginine of PAF26 (Ac-RKKWFW-NH_2) showed no effect on antifungal activity against plant pathogen *Penicillium digitatum*, although substitution of hydrophobic residues did [131]. It is notable that these peptides are proposed to cross cellular membranes and bind to internal targets. Such peptides, therefore, do not fit into the MPP model we highlighted above for membrane-targeting helical peptides. Interestingly, both helical and non-helical peptides could contain numerous aromatic residues in the sequence. While LL-37 contains four phenylalanines, PAF26 contains two Trp residues. The aromatic residues are known to prefer membrane interfaces [133–136]. Aromatic amino acids such as phenylalanines can also form the interface for protein–protein interactions [56].

5. Concluding remarks

Human cathelicidin LL-37 has been demonstrated to have antimicrobial activity against a variety of microbial pathogens, including bacteria, fungi, viruses, and parasites. It appears that LL-37 targets bacterial membranes (Fig. 2). The high-quality 3D structure of human LL-37 determined by 3D NMR spectroscopy (Fig. 3) reveals that it uses a long amphipathic helix (residues 1–31) to interact with the outer and inner membranes of Gram-negative bacteria, and the C-terminal tail (residues 32–37) is not required for membrane binding (Fig. 3A). The two hydrophobic domains of LL-37 are critical for binding LPS and explain its anti-septic effects. It is also established that both hydrophobic aromatic phenylalanines and interfacial cationic lysines and arginines of LL-37 participate in membrane binding (Fig. 4B). This amphipathic structure provides a solid basis for a membrane surface location at the equilibrated experimental conditions and may represent the initial state during bacterial killing. While acidic residues of LL-37 appear to control peptide aggregation as well as activity [137], our work also sheds light on possible roles of cationic amino acids in LL-37 fragments. In particular, the central cationic R23 in the central helix of LL-37 can directly interact with anionic PG (Fig. 4B) [54,56], consistent with its critical roles in membrane targeting and permeation.

It is pertinent to point out that, while bacterial membrane disruption is emphasized as the major mechanism of action of LL-37 in several studies [56,61,74,78,126,127], we do not exclude contributions from an alternative mechanism. It is demonstrated that the major antimicrobial region of LL-37 can directly associate with DNA based on gel shift assays (T. Lushinikova and G. Wang, unpublished). Once bacterial membranes have been damaged due to the action of LL-37, DNA binding could also occur in the same manner as observed for DNA-binding dyes [21]. R23 is proposed to be essential for DNA binding as well [138]. It is believed that binding of LL-37 to DNA or other negative components is responsible for the loss of antimicrobial activity in a cystic fibrosis (CF) lung [139]. In both cases, the binding of LL-37 to DNA may generate a new signal for the biological system. The LL-37/DNA complex may trigger part of the immune modulation in analogy to the neutralization of LPS by the same peptide. LL-37 may deliver DNA into host cells to be recognized by TLR-9 [140,141]. There are also other pathways for immune modulation. LL-37 can directly associate with host cell receptors such as formyl peptide receptor-like 1 (FPRL1) and P2X7 [142,143]. Elucidation of these novel mechanisms may hold the key to new strategies for treating infectious diseases.

There have been attempts to map the functions of LL-37 to defined peptide regions. It appears the N-terminal region of LL-37 is not important for antibacterial activity since the N-terminal fragment LL-23 showed activity only to susceptible bacterial strains [55]. However, LL-23 may have immune modulation roles. In particular, a change of S9 of LL-23 to A9 or V9 led to distinct chemokine release patterns. NMR studies revealed that both the N-terminal and C-terminal regions of LL-37 are involved in peptide aggregation [56]. This explains the fact that truncation of either the N-terminal or C-terminal 6–7 residues of LL-37 could increase peptide potency. It is clear now that the central helix of LL-37 is of primary importance in inhibiting microbes (Fig. 4A). A variety of fragments have been derived based on this central region (Table 3). GF-17, a peptide corresponding to the major antimicrobial region of human LL-37, is known to have activity against pathogenic superbugs such as MRSA and HIV-1, as well as cancer [21,22,61]. The minimal peptide sequence may vary depending on the type of antimicrobial activity (Table 3). KR-12 is sufficient to inhibit *E. coli*, whereas FK-13, the LL-37 core peptide, is the minimal sequence for inhibiting HIV-1 [22,56]. Furthermore, GF-17 is potent against MRSA USA300 [21]. However, an even longer fragment, RK-25, is suggested for the anti-biofilm activity against *P. aeruginosa* [87]. Therefore, there is a consensus that the central fragment of LL-37 is required for antibacterial, anti-biofilm, antiviral activities. Table 3 contains a collection of interesting LL-37 templates for developing novel therapeutic molecules for treating different infectious diseases. Alternatively, elucidation of other inducing factors and the mechanism of induction of LL-37 could offer a new avenue for treating infectious diseases [144].

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