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Review

LL-37, the only human member of the cathelicidin family of antimicrobial peptides

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Abstract

Antimicrobial peptides and their precursor molecules form a central part of human and mammalian innate immunity. The underlying genes have been thoroughly investigated and compared for a considerable number of species, allowing for phylogenetic characterization. On the phenotypical side, an ever-increasing number of very varied and distinctive influences of antimicrobial peptides on the innate immune system are reported. The basic biophysical understanding of mammalian antimicrobial peptides, however, is still very limited. This is especially unsatisfactory since knowledge of structural properties will greatly help in the understanding of their immunomodulatory functions. The focus of this review article will be on LL-37, the only cathelicidin-derived antimicrobial peptide found in humans. LL-37 is a 37-residue, amphipathic, helical peptide found throughout the body and has been shown to exhibit a broad spectrum of antimicrobial activity. It is expressed in epithelial cells of the testis, skin, the gastrointestinal tract, and the respiratory tract, and in leukocytes such as monocytes, neutrophils, T cells, NK cells, and B cells. It has been found to have additional defensive roles such as regulating the inflammatory response and chemo-attracting cells of the adaptive immune system to wound or infection sites, binding and neutralizing LPS, and promoting re-epthelialization and wound closure. The article aims to report the known biophysical facts, with an emphasis on structural evidence, and to set them into relation with insights gained on phylogenetically related antimicrobial peptides in other species. The multitude of immuno-functional roles is only outlined. We believe that this review will aid the future work on the biophysical, biochemical and immunological investigations of this highly intriguing molecule.
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Keywords: Antimicrobial peptide; LL-37; Cathelicidin; Solid-state NMR; Antibiotic; Membrane

Contents

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1. Introduction

1.1. Mammalian innate immunity

During the last two decades, piece by piece, a fascinating and still enigmatic strategy of vertebrates to deal with intruding microorganisms has been revealed. In tissues that first make contact with intruders, antimicrobial peptides (AMPs) are released from precursor proteins [1,2]. Their cationic and amphipathic properties make them resemble antimicrobial peptides that are excreted by prokaryotes to kill other microorganisms. In prokaryotes, AMPs are either synthesized in non-ribosomal pathways or cleaved from ribosomally generated precursors. Certain mammalian leukocytes, called granulocytes, contain granules that hold large amounts of AMP precursors. These precursors are cleaved into active AMPs upon secretion into phagosomes. Thereby, AMPs are brought into direct fatal contact with phagocytosed microorganisms.

However, direct antimicrobial action is not the only, and possibly not even the primary task of mammalian AMPs. This is suggested by the fact that peptides of innate immunity suffer losses in their antibacterial properties under serum and tissue conditions. Instead, they seem to fill important immunomodulatory roles in a complicated network of interactions between innate and acquired immunity [3–6].

In mammals, at least two distinct groups of antimicrobial peptides and corresponding precursors are found. Defensins form the group with most representatives. Their instances in human were covered in another review article by Dhople et al. in this special issue. Cathelicidin-derived AMPs form a second group of mammalian AMPs. Their common feature is the highly conserved cathelin domain that complements the antimicrobial peptide in the precursor. Some researchers, e.g., De Smet and Contreras [7], regard histatins as a distinct third group that has to be seen in close relationship to defensins and cathelicidins. A recent review article examines the role of antimicrobial peptides in the skin [8].

1.2. Cathelicidins

Cathelicidins form a distinct class of proteins present in the innate immunity of mammals [9,10]. Similar to defensins, they act as precursor molecules that can release an antimicrobial peptide after proteolytic cleavage. Their structural features clearly distinguish them from defensins. The discovery of cathelicidins commenced after the isolation of the antimicrobial peptide Bac5 from bovine neutrophils, and the realization that they are cleaved from inactive precursors [11–13]. Cloning of a cDNA coding for the precursor allowed to search for the gene [14]. Surprisingly, the search lead to the discovery of numerous genes. It was soon realized that those genes code for a whole family of related proteins [15].

The hallmark of the cathilicidin family of proteins is the presence of a highly conserved cathelin domain [9,16]. Cathelin was first found in pig leukocytes and characterized as an inhibitor of the cysteine proteinase cathepsin L, and was named after this property [17]. Amino acid sequence comparison locates cathelin to the cystatin superfamily of proteins [17–19], which comprises cystatin (cysteine proteinase inhibitor), kininogen, and stefin proteins [20]. The realization that the cathelin region is highly conserved among the cathelicidins of one species, as well as among different species, made direct genetic searches possible. For a number of species, the genes of their cathelicidins could be successfully located by sequence screening and cDNA-cloning, making use of the highly conserved nature of the cathelin domain. Once the gene is identified, evidence for the proprotein and the derived mature AMP can be directly sought for.

The cathelicidin proteins are characterized by a highly conserved N-terminal domain of about 100 amino acid residues. This 14 kDa cathelin-like domain is flanked by a signal peptide domain (approximately 30 residues long) on its N-terminus, and by an antimicrobial peptide region on its C-terminus. Fig. 1 gives the topology of the 18-kDa human cathelicidin hCAP18 as a prototype illustration for the similar topology of all cathelicidins [21]. The primary translation product is called the 'preprotein', since it contains a signal sequence as a prelude to the cathelicidin holo-protein. This signal peptide is cleaved off once it has fulfilled its purpose of targeting the cathelicidin to storage granules or to the exterior of the cell. The formation of two disulfide-bridges finalizes post-transcriptional processing. The cathelicidin holo-protein is then referred to as the 'proprotein', since it does not represent the active, but rather a storage form. Only after cleavage of the proprotein into the cathelin domain and the cathelicidin-derived AMP, does it unfold its host of activities.

Cathelicidin proteins have been extensively studied in mammals to date. However, cathelicidins were also recently found in chicken [22] and three species of fish [23–25]. Especially interesting is the case of hagfish, the oldest living member of

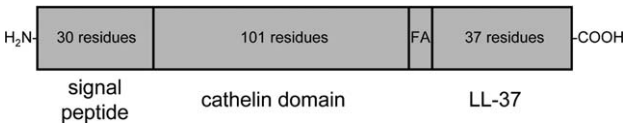

Fig. 1. A cartoon representation of the only human cathelicidin hCAP18.

jawless craniates. This species remarkably lacks essential components of adaptive immunity [26]. In hagfish, two cathelicidins are described [25]. The presence of cathelicidins in this very ancient species may indicate that cathelicidin genes developed early in phylogenesis and are present in most, if not all, higher organisms. In cattle, horse, pig, sheep, and goat, up to seven cathelicidins are found, each being the precursor for individual AMPs of very different structural properties. Among this multitude of structurally divergent AMPs, all of the named species have at least two α-helical AMPs derived from cathelicidin precursors. In human, only a single cathelicidin is found. This is in contrast to the situation for the cathelicidins of other species. It is also contrasting the (at least) 10 human defensins. The same situation of a single cathelicidin is found in rhesus monkey [27,28], mouse [29], rat [30], and guinea pig [31]. All these species are phylogenetically more closely related to human than the species named earlier. An exception is formed by rabbit, a further representative of rodents, in which a second cathelicidin called p15s is found in addition to CAP18.

Both Uzzell et al. [25] and Termén et al. [30], in connection to their studies on HFIAP and rCRAMP, respectively, performed sequence alignments between the signal and cathelin domains of their cathelicidins and all other known ones. Thereby, they could establish the phylogenetic ancestry of the cathelicidins. A concise graphical overview about the phylogenetic relationships and the cathelicidins found in each species is given by Zanetti [9]. The single 16 kDa human cathelicidin is denoted as hCAP18. Its name was chosen because of its close relationship to the cationic antimicrobial peptide found in rabbit, which has a molecular weight of 18 kDa and is therefore called CAP18. The gene for hCAP18 was described by three research groups independently [32–34]. Fig. 1 gives an overview of the topology of the primary translation product reported by these studies. The gene for the human cathelicidin hCAP18 is a compact gene of 1963 bp located to chromosome 3, and is composed of four exons [21,35]. Exons 1 to 3 code for the signal sequence and the cathelin domain, while exon 4 codes for the antimicrobial peptide, LL-37. Boman [3] presents the numerous upstream control sites for transcription factors that regulate the expression of the gene.

The cathelicidin proprotein is currently regarded as an inactive precursor that becomes active only upon proteolytic cleavage into cathelin domain and cathelicidin-derived AMP. Two observations may challenge that view: uncleaved proprotein is present in human plasma lipoproteins [36], and holoproteins in rabbit granulocytes are found to have synergy with bactericidal/permeability-increasing protein [37]. Elastin seems to be the most common peptidase to cleave cathelicidins [38,39]. In human hCAP18, however, protease-3 cleaves the proprotein [40]. Two research groups reported alternative processing procedures. Sorensen et al. [41] found seminal hCAP18 to be cleaved to ALL-38 by the prostate-derived protease gastricsin at vaginal pH. Murakami et al. [42] investigated LL-37 in sweat and found that it is further degraded by a serine-protease dependent mechanism to yield KR-20, RK-31, and KS-30. These fragments were found to possess increased antimicrobial activity. The authors conclude that further cleavage allows the organism to shift LL-37's action from signaling function towards antimicrobial effect. A hint at more subtle properties of cathelicidin cleavage comes from the mouse and rat cathelicidins, CRAMP and rCRAMP, respectively [29,30]. Although very similar in structure, they are cleaved at two different sites, resulting in AMPs of quite different length. This makes Termén et al. [30] speculate that either two alternative cleavage procedures may be present in one or both of the species, or that each species has developed an individual cleavage mechanism to respond to evolutionary pressure.

The structure of the cathelin domain freed from pig protegrin-3 was investigated in two different studies. Sanchez et al. crystallized the cathelin domain and determined its structure using X-ray crystallography [43], while Yang et al. found a very similar structure by means of NMR spectroscopy [44]. The structure consists of a four-stranded β-sheet and an α-helix spanning one side of the β-sheet. Since the very similar protegrin-1 is known to form a β-hairpin secondary structure [45,46], Sanchez et al. [43] speculate that in the uncleaved proprotein this β-hairpin extends the cathelin domain's β-sheet moiety into a six-stranded β-sheet. This model predicts the connecting loop region (between cathelicidin and protegrin-1 domain) to be on the protein surface, and thus to be fully accessible to the cleaving peptidase. After cleavage, the human cathelin domain possesses antimicrobial and protease inhibitory functions of its own [47]. However, the present contribution will now turn to its main focus on the AMPs that are cleaved off of the cathelin domain.

1.3. Cathelicidin-derived AMPs

In contrast to the highly conserved cathelin domain of the cathelicidins, the derived AMPs show highly differing sequences and secondary structures. For example, α-helical AMPs rich in Pro, Arg or Trp (indolicidin) and cysteine-bridged AMPs (dodecapeptide, integrins) have been reported. Gennaro and Zanetti [16] as well as Ramanathan et al. [48] presented overviews about the different structural classes of AMPs derived from cathelicidin holo-proteins. The present review focuses on LL-37, the only human cathelicidin-derived AMP. LL-37 belongs to the class of α-helical AMPs. This seems to be the common secondary structure in all organisms where only a single cathelicidin-derived AMP is found. LL-37 owes its name to its 37 amino acid overall length with the two leading residues being leucines. It does not contain any cysteine residues.

The initial report of the hCAP18/LL-37 gene [32] assumed the 39-residue C-terminal domain (termed FALL-39) to be the active AMP, based on the presence of a typical KR cleavage site [33,34]. Gudmundsson et al. [35] shortly afterwards used antibody binding to prove that LL-37, a peptide two amino acids shorter than FALL-39, is the matured AMP. Their study also found the peptide to be located in granulocytes. Two of the three groups first describing the hCAP18/LL-37 gene prepared the peptides FALL-39 and LL-37 synthetically, and showed them to be antimicrobially active [32,33]. Larrick et al. [35] additionally synthesized a truncated peptide with the C-terminus being shortened by 5 amino acids. This truncated peptide showed increased antimicrobial activity [33].

To present LL-37 within its structural context, we wanted to compare it to the sequences of other α-helical cathelicidin-derived AMPs found in closely related species, i.e., species where only a single cathelicidin is present. Therefore, we took the respective portion of the table given in Ramanathan et al. publication [48], and augmented it in three ways. First, we added the sequence of rCRAMP, a cathelicidin-derived AMP only recently described in rat, and predicted to be α-helical [30]. Moreover, we give both contradictory sequence determinations carried out for rhesus monkey [27,28]. For the sake of completeness, we also included the sequences of cathelicidin-derived AMP recently described in chicken [22] and three species of fish, namely rainbow trout [23,24], atlantic salmon [24], and hagfish [25,49]. Both hagfish AMPs were predicted to be α-helical by Zanetti [9]. The resulting compilation is presented as Table 1.

Table 1
A selection of cathelicidin-derived α-helical AMPs, chosen by phylogenic or structural similarity to LL-37

Peptide	Origin	Amino acid sequence	#AA	Ref.
LL-37	Human	LLGDFFRKSK–EKIGKEFKRI–VQRIKDFLRN–LVPRTES	37	[51]
rhLL-37[a]	Rhesus	identical to LL-37	37	[27]
RL-37[a]	Monkey	RLGNFFRKVK–EKIGGGLKKV–GQKIKDFLGN–LVPRTAS	37	[28]
CAP18	Rabbit	GLRKRLRKFR–NKIKEKLKKI–GQKIQGLLPK–LAPRTDY	37	[52]
CRAMP	Mice	GLLRKGGEKI–GEKLKKIGQK–IKNFFQKLVP–QPE	33	[29]
rCRAMP	Rat	RFKKISRLAG–LLRKGGEKFG–EKLRKIGQKI-KDFFGKLAPE-IEQ	43	[30]
CAP11[b]	Guinea pig	(GLRKKFRKTR–KRIQKLGRKI–GKTGRKVWKA–WREYGQIPYY–CRY)$_2$	43	[31]
Fowlicidin-1	Chicken	RVKRVWPLVI–RTVIAGYNLY–RAIKKK	26	[22]
Fowlicidin-2	Chicken	RVKRFWPLVP–VAINTVAAGI–NLYKAIRRK	29	[22]
Fowlicidin-3	Chicken	LVQRGRFGRF–LRKIRRFRPK–VTITIQGSAR-FG	32	[22]
rtCATH_1	Rainbow trout	RRSKVRICSR–GKNCVSRPGV–GSIIGRPGGG–SLIGRPGGGS–VIGRPGGGSP–PGGGSFNDEF–IRDHSDGNRF-A	71	[23,24]
rtCATH_2	Rainbow Trout	RRGKDSGGPK–MGRKNSKGGW–RGRPGSGSRP–GFGSGIAGAS–GVNHVGTLPA–SNSTTHPLDN–CKISP-Q	66	[24]
asCATH_1	Atlantic Salmon	RRSQARKCSR–GNGGKIGSIR–CRGGGTRLGG–GSLIGRLRVA–LLLGVAPFLL–DLSQINVMEI–AFA	63	[24]
asCATH_2	Atlantic salmon	RRGKPSGGSR–GSKMGSKDSK–GGWRGRPGSG–SRPGFGSSIA–GASGRDQGGT–RNA	53	[24]
HFIAP-1	Hagfish	GFFKKAWRKV–KHAGRRVLDT–AKGVGRHYVN–NWLNRYR	30	[25,49]
HFIAP-3	Hagfish	GWFKKAWRKV–KNAGRRVLKG–VGIHYGVGLI	30	[25,49]

In addition, the AMPs recently found in chicken and three species of fish are given.
[a] Two different studies reported two different sequences for a single cathelicidin gene and the derived AMP, but agree that only one cathelicidin gene is present.
[b] Homodimer via the C-terminal cysteine residue.

A second cathelicidin holoprotein, denoted p15s, is found in rabbit [37]. This holoprotein apparently does not undergo processing with the release of an active AMP. Lehrer's group has identified cathelicidins in goat. In addition to the published AMP chBac5 [50], a homologue of bovine Bac5, they found three other cathelicidins. These three sequences are deposited in the GenBank database under the GI codes 5139355, 5139357, and 5139359, but no information is available on the mature AMPs.

2. Sources and function of LL-37

LL-37 and its precursor, hCAP18, are found at different concentrations in very different cell and tissue types and body fluids. This fact is closely related to its double nature as peptide antibiotic and signaling molecule. The following paragraphs describe the differential expression pattern and summarize the current knowledge on LL-37's antimicrobial properties and its signaling action.

2.1. Places of gene expression

LL-37 was first described in leukocytes and testis, but was soon found in a large variety of cells, tissues and body fluids. Table 2 summarizes the sites of expression that were described for LL-37. The time-dependence of LL-37 gene expression in maturing neutrophils has found special interest [53,54]. Bülow et al. investigated the means by which immature hCAP18/LL-37 is sorted for storage in specific granules [55]. LL-37, as well as its proprotein, were also found bound to plasma lipoproteins [36,56,57]. Sorensen et al. suggested that lipoproteins may form an important reservoir for LL-37 [36]. Paulsen et al. did not find LL-37 expression in the nasolacrimal duct [58].

Included in Table 2 are reports of increased or decreased LL-37 production in numerous diseases. Upregulation is found more commonly than downregulation, indicating that LL-37 assists the immune system in fighting disease. Bacterial products were shown to increase LL-37 production in cultured sinus epithelial cells [59]. Likewise, human beta-defensin-2 increases the level of LL-37 expression in colon and breast epithelial cells [60]. In four cases, decreased levels of LL-37 are found in disease. In atopic dermatitis and chronic ulcers, this may be related to the causes of the disease [61–63]. Pathogenic microbes may actively reduce LL-37 production in enteric infection, thus ensuring their own survival [64]. Patients suffering from morbus Kostmann are devoid of LL-37 in granulocytes and saliva [65]. Unless treated with cytokine G-CSF, all patients die from bacterial infections within the first year of their life. Studies of xenograft models in mice established a connection between LL-37 and cystic fibrosis [66]. A recent review summarizes the role of LL-37 in lung infection and inflammation [67].

2.2. Antimicrobial and cytotoxic activity

LL-37 was initially recognized for its antimicrobial properties [51,107–109]. It exhibits a broad spectrum of antimicrobial activity against bacteria, fungi, and viral pathogens [70,75], with microbicidal activity varying against different species and

Table 2
Various cell types and tissues where hCAP18/LL-37 expression was reported, along with pathologies which are described to correlate with increased or reduced expression of hCAP18/LL-37

Type of cell, tissue, or body fluid	Reference
Leukocytes	[34,35,68–70]
Myelocytes, metamyelocytes	[70]
Bone marrow	[32]
Breast milk	[71,72]
Skin of newborn infants	[73,74]
Numerous squamous epithelia	[75]
Nail	[76]
Sweat	[77]
Wound fluid, blister fluid	[78]
Ocular surface epithelia	[79]
Synovial membranes	[80]
Nasal mucosa	[81]
Lung epithelia	[82]
Developing lung	[83]
Bronchoalveolar lavage fluid	[84]
Salivary glands	[85,86]
Saliva	[85]
Gingiva	[87]
Colon epithelium	[88]
Colon mucosa	[89]
Testis	[32]
Epididymis epithelium, spermatozoa	[90]
Seminal plasma	[90,91]
Vernix caseosa	[74,92–94]
Amniotic fluid	[92,93]
Upregulation in disease	
Keratinocytes in inflammatory disorders (psoriasis, lupus erythematosus, contact dermatitis)	[95–97]
Gastric epithelia, *H. pylori* infection	[98]
Inflamed synovial membranes	[80]
Chronic nasal inflammatory disease	[99]
Bronchoalveolar lavage fluid	
In cystic fibrosis	[100]
In sarcoidosis	[84]
Tracheal aspirates of newborn infants	[101]
Breast cancer	[102]
Keratinocytes in condyloma acuminatum and verruca vulgaris	[103]
Cholesteatoma	[104]
Downregulation in disease	
Atopic dermatitis	[62,63]
Chronic ulcer epithelium	[61]
Enteric infection	[64]
Neutrophils, acute myeloid leukemia	[105,106]
LL-37 is absent in granulocytes and saliva	
Morbus Kostmann	[65]

strains. Table 3 gives an overview of the antimicrobial data published on a number of Gram positive and Gram negative bacterial strains, as well as on the yeast *Candida albicans*. Antiviral action was investigated on two viruses. Only little viral inactivation by LL-37 was reported for the herpes simplex virus [110]. On the other hand, a reduction of virus replication was found for the vaccinia (smallpox) virus [111].

For *Candida albicans,* den Hertog et al. showed that the place of action is the cell wall and cell membrane [112]. These showed massive disruption in electron microscopy images. Remarkably, the same study found very different effects for the human AMP histatin 5, namely minor membrane defects but intracellular accumulation. López-García et al. found a correlation between membrane permeabilization and *C. albicans* growth inhibition [113]. In *Bacillus subtilis*, Pietiäinen et al. reported a complex stress response when the organism was subjected to subinhibitory concentrations of LL-37 [114]. For a comparison of the antimicrobial action of LL-37 with other human AMPs, readers are referred to an article reported by De Smet and Contreras [7], as well as on defensins by Dhople et al. in this special issue. The study of Sambri et al. presented a comparison of LL-37 and four other cathelicidin-derived AMPs with respect to their action against three pathogenic species of spirochaetes (a special type of bacteria showing flagellae and motility) [115].

Several studies reported the MIC values in high salt concentrations and under serum conditions, and found antimicrobial action to be noticeably reduced [51,107,118,119]. Similarly, the activity of LL-37 was reduced in the presence of a lung surfactant preparation [120] as well as in the presence of artificial tears [121]. The loss of antimicrobial activity that is observed for most AMPs in elevated salt concentrations, but is less

Table 3
Compilation of reported antimicrobial properties of LL-37

Organism	LL-37 activity	Reference
Gram positive bacteria		
Streptococcus		
Group A	1–16 μM	[116]
Group B	≥32 μM	[116]
Group C	16 μM	[116]
Staphylococcus aureus	>32 μM	[116]
Enterococcus faecalis	30 μg/ml	[82]
Staphylococcus epidermidis	7.6 μg/ml	[107]
Listeria monocytogenes	1.5 μg/ml	[107]
Enterococcus faecium	0.7 μg/ml	[107]
Lactobacillus acidophilus	19 μM	[28]
Bacillus subtilis	2.7 μg/ml	[107]
Bacillus megaterium	0.2 μM	[32]
Gram negative bacteria		
Escherichia coli	>32 μM	[116]
Pseudomonas aeruginosa	16 μg/ml	[82]
Actinobacillus actinomycetemcomitans	10 μg/ml	[109]
Salmonella typhimurium	3.5 μg/ml	[108]
Salmonella minnesota	0.2 μg/ml	[108]
Burkholderia cepacia	79 μg/ml	[107]
Capnocytophaga ochracea	11 μg/ml	[109]
Klebsiella pneumoniae	4.2 μg/ml	[108]
Proteus mirabilis	5.7 μg/ml	[107]
Stenotrophomonas maltophilia	1.9 μg/ml	[107]
Proteus vulgaris	2.5 μg/ml	[107]
Capnocytophaga sputigena	7.5 μg/ml	[109]
Capnocytophaga gingivalis	9 μg/ml	[109]
Salmonella serovar dublin	2.8–6.0 μM	[88]
Spirochaete		
Leptospira interrogans	144–225 μg/ml	[115]
Borrelia spp.	450 μg/ml	[115,117]
Treponema pallidum	450 μg/ml	[115,118]
Yeast		
Candida albicans	>20 μg/ml	[108]

pronounced in LL-37 than in other human AMPs, may be relevant in diseases of the lung. High ionic concentrations are generally found in body fluids of patients suffering from cystic fibrosis and other inflammatory lung diseases. In this context, gene transfer resulting in four-fold overexpression of LL-37 in a cystic fibrosis xenograft model succeeded to restore bacterial killing to normal levels [66], suggesting that LL-37 may protect against bacterial lung infections and showing a possible therapeutic strategy. Similarly, adenoviral gene therapy with hCAP-18/LL-37 was found effective in the treatment of infected burn wounds [122]. The reduced activity of LL-37 at high salt levels made the in vivo relevance of LL-37's microbicidal action disputable. However, Bals et al. [123] established a mouse model whose properties were consistent with in vivo antimicrobial activity. Nizet et al. prepared knock-out mice in which the gene coding for CRAMP, the mouse analogue of LL-37, was disrupted [124,125]. These animals showed severely deteriorated resistance to necrotic skin infections caused by Group A *Streptococcus*. In a later study, transgenic mice expressing the porcine cathelicidin PR-39 in addition to CRAMP proved to be better protected against bacterial skin infection [126]. Bowdish et al. prepared an analog of LL-37 which lacked antimicrobial activity, but which was still found to protect the mouse organism from infection [127].

In cultured keratinocytes, Erdag et al. found expression of antimicrobial peptides including LL-37 to be increased after application of interleukin-1α and interleukin-6, and found the treated tissue to be better protected against three bacterial species [128]. In gastrointestinal cells, LL-37 expression was induced by histone-deacetylase inhibitors [129]. In colonocytes, LL-37 expression is modulated by short chain fatty acids [130].

A number of studies described bacterial strategies to counter antimicrobial peptides. Bacteria of the upper respiratory tract were able to evade the antibacterial action of LL-37 by increasing the amount of phosphatidylcholine lipids in their membranes [131]. Similarly, the polysaccharide intercellular adhesin (PIA) joining *Staphylococcus epidermidis* into biofilms was shown to protect the bacterium from LL-37 action [132]. Common pathogenic bacteria inactivate LL-37 by degradation through proteinases [132–135]. In the case of *Pseudomonas aeruginosa,* Werthén et al. showed that a synthetic cationic polymer was able to inhibit these proteinases [136]. In addition to protecting proteinases, *Streptococcus pyogenes* also excretes a protein that interacts with the complement system and was additionally found to interfere with LL-37 action [137,138]. McPhee et al. described a two component regulatory system that regulates resistance in *Pseudomonas aeruginosa* [139]. Shafer et al. reported that an efflux pump effectively protects *Neisseria gonorhoeae* from the lethal action of LL-37 and protegrin-1 [140].

Decreased levels of LL-37 were found in enteric infection, possibly indicating a downregulation in expression that is caused by the invading pathogens [64]. Similarly, a downregulation of LL-37 in *Neisseria gonorrhoeae* infection was described by Bergman et al. [141]. Tzeng et al. investigated causes for the intrinsically high resistance of *Neisseria meningitidis* against cationic antimicrobial peptides, including LL-37 [142]. Samuelsen et al. found that strains of *Staphylococcus aureus* that had developed resistance to lactoferricin B showed related cross-resistance to other antimicrobial peptides, but not to LL-37 [143].

The in vitro cytotoxicity of LL-37 was addressed in two studies. Johansson et al. found LL-37 concentrations of 13–25 μM to be sufficient to make human leukocytes and T-cells nonviable [51]. Oren et al. found substantial lysis of red blood cells at similar concentrations of LL-37 [144]. These concentrations are surprisingly similar to the MIC values collected in Table 3 and characterize LL-37 as a comparatively unspecific antimicrobial agent, which might as well be referred to as a toxin.

Synergistic action between LL-37, β-defensins, and lysozyme was initially described by Nagaoka et al. [145] and later comprehensively investigated by Chen et al. [146] Evaluation of their combined action against *E. coli* and *S. aureus* in neutral as well as acidic milieus was performed. The synergistic effect found was strong enough to make the authors speculate that defensins may not be able to act as antibacterial molecules by themselves, but only in synergism with cathelicidins [145]. However, Singh et al. found only additive action when investigating LL-37 in combination with other antimicrobial peptides of the human airway surface liquid [147].

All of the studies on antimicrobial activity published to date used peptide prepared by solid-state synthesis. Only recently two groups developed vectors for expression of recombinant peptide in *E. coli.* Li et al. expressed the wild type sequence [148], while Yang et al. expressed GSLL-39 [149], a slightly elongated variant of LL-37 that showed identical antimicrobial properties. In our laboratory, we have successfully expressed and purified LL-37 from *E. coli* [150]. For details on the biological expression and purification, we refer to the article by Moon et al. in this special issue.

2.3. Lipopolysaccharide binding properties

From the point of its discovery, investigators also realized that LL-37 shows a strong binding affinity for lipopolysaccharides (LPS) [33,34,107]. This property is also known for rabbit CAP18 [151], and is physiologically relevant since LPS form the outermost membrane leaflet of Gram-negative bacteria. Released from those bacteria in sepsis, LPS is termed 'endotoxin' and causes adverse effects like septic shock by inducing the production of higher concentration of systemic pro-inflammatory cytokines. However, LL-37 can neutralize the biological activity of LPS by binding it with higher affinity [33,152–155]. Herasimenka et al. investigated the binding behavior of LL-37 with LPS from three lung pathogens [156]. By comparing LL-37 to three other AMPs, Rosenfeld et al. established a positive correlation between binding affinity and a peptide's ability to inhibit LPS-induced cytokine secretion [157]. Likewise, Zughaier et al. found LPS-induced macrophages to secrete markedly less tumor necrosis factor α (TNF-α) as well as reactive oxygen species (ROS) in the presence of LL-37 [158]. Bowdish et al. showed that LL-37 and indolicidin synergistically suppress LPS-induced secretion of TNF-α from macrophage cell lines [159]. Ciornei et al. investigated rat aorta in vivo and measured reduced production of nitric oxide in the presence of LL-37 [160]. Two studies addressed the structure of LPS-binding peptides: an NMR structure is available for sheep

SMAP-29 [161], as well as an X-ray crystal structure for a peptide from horseshoe crab [162]. Nagaoka et al. performed mutation studies to enhance the LPS-binding effect on mutated analogs of LL-37 [163]. By changing the hydrophobicity and cationic properties of an 18-mer peptide (K15 to V32), they were able to increment its ability to neutralize LPS. An in vivo study by Fukumoto et al. applied LL-37 to rats in which sepsis was induced by exposure to peritoneal LPS injection [164]. The study showed that LL-37 improved the course of sepsis, which can be explained by retained LPS-binding in vivo. However, the same study found higher doses of LL-37 to have adversary effects on the survival rates of the test animals, possibly related to the cytotoxic properties of LL-37.

2.4. Chemotactic activity of LL-37

Chertov et al. found LL-37 to chemoattract T-cell leukocytes [165], as well as mononuclear cells and neutrophils [166]. In addition, interleukin-8-stimulated neutrophils were found to release LL-37 [165], thus attracting even more leukocytes to sites of increased LL-37 concentration. As described above, increased levels of LL-37 are regularly found in inflamed or infected tissue. Interestingly, the chemotactic activity of LL-37 is not affected by the presence of serum [167], at the same serum concentrations that are known to inhibit its in vitro antimicrobial effects [51,107]. Dendritic cells form another class of leukocytes receptive to LL-37. Davidson et al. found LL-37 to profoundly influence the exact course of their maturation process, a process which forms the borderline between innate and adaptive immunity [168]. Scott et al. treated mice with intratracheal application of LL-37 and found increased production of the chemokine MCP-1, but no increase in the inflammatory mediator TNF-α [152]. They interpret this as evidence that LL-37 can block bacterial product-induced inflammation while simultaneously recruiting macrophages.

Mast cells, which form an important tissue-localized part of innate immunity, show degranulation upon stimulation with LL-37 [169]. Degranulation releases pro-inflammatory mediators, such as histamine and prostaglandins, into the surrounding tissue, thus increasing vascular permeabilization and promoting the infiltration of inflammatory cells. In addition, mast cell migration is induced by LL-37 [170]. Di Nardo et al. found that mast cells are not only susceptible to LL-37, but also produce the peptide themselves [171]. The effects of LL-37 on mast cells were reviewed by Niyonsaba et al. [172].

The signaling means by which LL-37 exerts its immunostimulatory effects are presently a very active and productive area of research. LL-37 was proven to activate at least three different receptors, namely FPRL-1 (formyl peptide receptor-like 1) [167,173], EGFR (epidermal growth factor receptor) [174], and $P2X_7$ [175]. For both FPRL-1 and $P2X_7$, a direct activation via specific binding is postulated, while for EGFR only transactivation was discussed. EGFR was found to be activated by the all-D analog of LL-37 [176]. This activation is stronger than the one by the wild type, probably due to increased resistance to proteolytic degradation. Likewise, LL-37 induces activation of extracellular signal-regulated kinase and p38 kinase signaling pathways in monocytes [177] and keratinocytes [178]. In a confocal microscopy study performed on fluorescently labeled LL-37, the peptide was found to be actively taken up into lung epithelial cells and trafficked to their perinuclear region [179]. This is in contrast to the situation in the target organism *Candida albicans*, where LL-37 could not be detected in the cytoplasm [112]. Bowdish et al. give an in-depth description of the detailed mechanisms of the immunostimulatory effects of LL-37 [127]. Lau et al. demonstrated that LL-37 induces apoptosis in cultured lung and airway epithelial cells, and in vivo in the murine airway [180]. Using cultured cells, LL-37 was shown to bind DNA plasmids and to target them to the cell nucleus in membrane raft dependent endocytosis [181].

2.5. Role of LL-37 in wound healing and angiogenesis

Increased levels of LL-37 were found in human skin after a wound had been inflicted [116], which returned to normal levels as the wound closed. A later study found re-forming epithelium migrating over the wound bed to be the primary place of LL-37 expression in wounds [61]. The same study found that inhibition of LL-37 by specific antibodies prevented wounds from healing, and found reduced levels of LL-37 in chronic ulcer epithelium. Other studies found LL-37 production to be increased by common growth factors [182], or suggest that LL-37 acts as a putative growth factor for epithelial cells [102]. Tokumaru et al. [183] showed that LL-37 induces the migration of keratinocytes, and established a connection to the transactivation of epidermal growth factor receptor. A review article by Borregaard et al. [184] stresses the necessity to coordinate the behavior of neutrophils and keratinocytes in wound healing, and the role that LL-37 plays as a signaling molecule in that context. Shaykhiev et al. found a similar stimulation for airway epithelial cells in the presence of LL-37 [185].

Angiogenesis is another interesting function of LL-37. Koczulla et al. found that application of LL-37 resulted in neovascularization in a tissue model as well as in a rabbit model [186]. Moreover, they showed the receptor FPRL-1 on endothelial cells to be the mediator of angiogenesis. An angiogenic role is also described for a cathelicidin-derived AMP in pig [187]. Remarkably, the porcine peptide is not a member of the α-helical class of AMPs.

3. Structural studies on LL-37

3.1. Amphipathic properties

The primary structure of LL-37, LLGDF–FRKSK–EKIGK–EFKRI–VQRIK–DFLRN–LVPRT-ES, was given along with other α-helical cathelicidin-derived antimicrobial peptides in Table 1. The sequence reveals the highly charged nature of the peptide. At physiological pH, 16 of its 37 residues are charged, namely 6 Lys and 5 Arg residues carry 11 positive charges, while 3 Glu and 2 Asp residues bear 5 negative charges. The resulting net charge at physiological pH is + 6. LL-37 assumes an α-helical secondary structure under physiological conditions. This behavior is found in all species that have a single cathelicidin. All

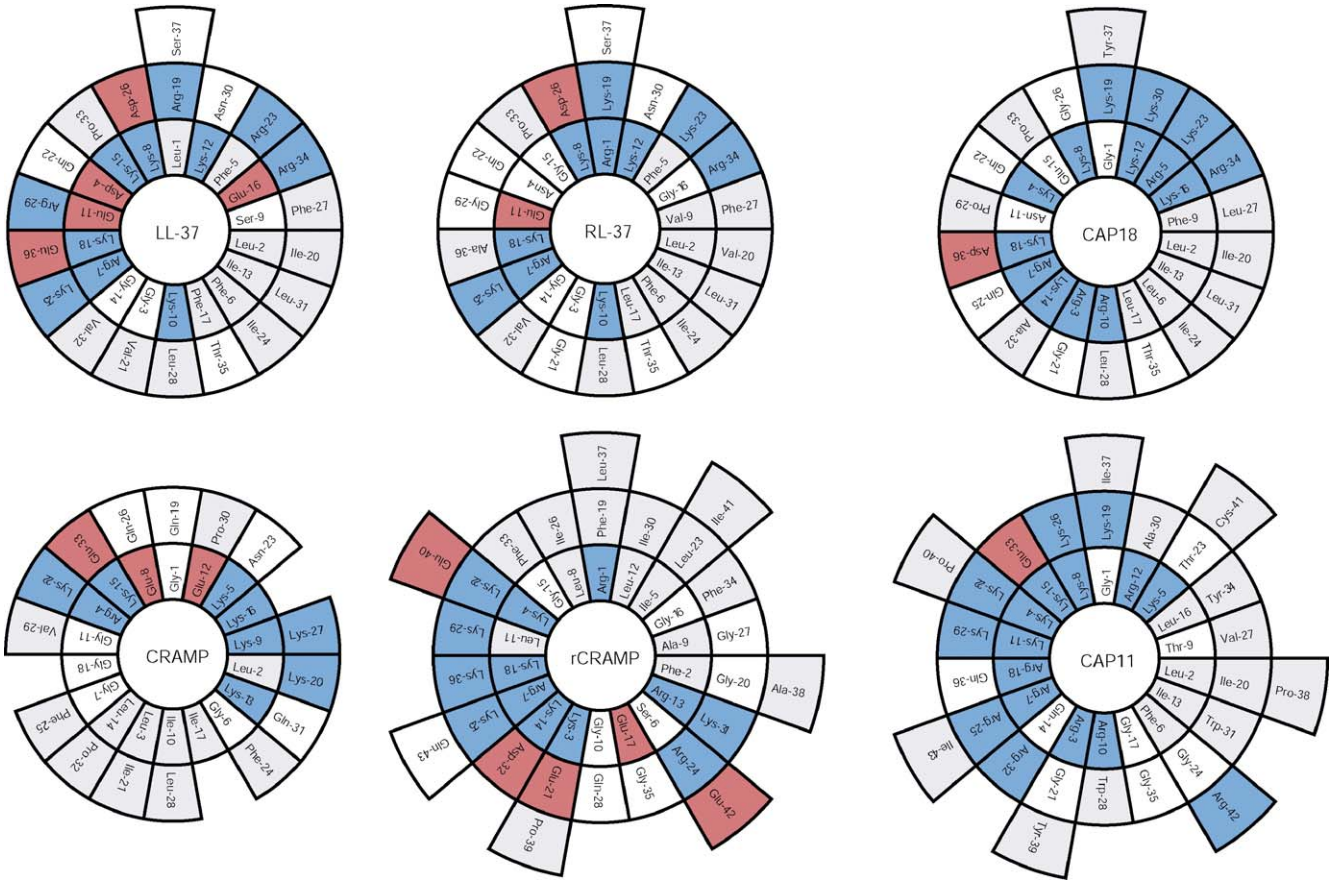

Fig. 2. Helical wheel projections of the cathelicidin-derived AMPs from species that are known to possess only a single cathelicidin. They are found in (top left to bottom right) human, rhesus monkey, rabbit, mouse, rat, and guinea pig. For sequences and references, see Table 1. Positively charged (blue), negatively charged (red), polar (white), and hydrophobic (gray) residues are highlighted.

currently known members of this group are shown in Table 1. For the same set of six AMPs, Fig. 2 gives the helical wheel projections. The projections show that all six peptides gain striking amphipathic features when assuming a helical structure. Amphipathicity is common to all antimicrobial peptides and allows them to interact electrostatically with anionic bacterial membranes. In each helical wheel diagram of Fig. 2, the hydrophobic residues (shaded gray) cluster in a certain portion of the helix perimeter. The charged and polar residues (shaded red, blue and white for negatively charged, positively charged, and polar residues, respectively) populate the opposite face of the helix. All six peptides show a 'hydrophobic angle' of approximately 160° of the helix circumference. A closer inspection of the human peptide, LL-37, reveals that in the N-terminal region, the amphipathic faces of the helix are less perfectly formed. This suggests that the N-terminus is less apt to form α-helical secondary structure, thus explaining the 20–30% random coil proportion found in CD spectroscopy (see next section).

Although CAP11 was shown to form covalently bound homodimers via its C-terminal cysteine residues, it still displays a strikingly similar helical wheel when compared to other peptides in Fig. 2. This indicates that CAP11's covalent dimer structure does not make it an exception. Instead, a non-covalent dimerization may be expected as an important common feature that is yet to be discovered in other systems.

Fig. 3 gives a three-dimensional model of the peptide in an assumed perfect α-helical conformation. The C-terminal region forms an aliphatic α-helix, which is the major recurring structural motif present in all known α-helical AMPs. This amphiphilic pattern does not extend through the whole molecule. Rather, the N-terminal region consists of stretches of hydophilic and hydrophobic residues, which close to form two "rings" or "strips" around the N-terminus (assuming this terminus is α-helical in structure). The cartoon representation shown in the lower half of Fig. 3 tries to capture this very abstract description of hydrophobic properties. A closer examination of the three-dimensional model

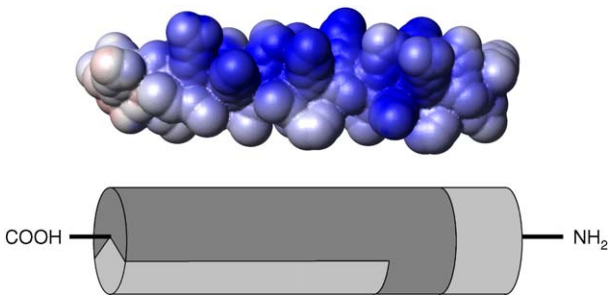

Fig. 3. A hypothetical ideal α-helical structure of LL-37. Surface representation and cartoon sketch for illustrating amphipathic properties. Note that the N-terminal region (right end) does not readily form an amphipathic α-helix.

reveals numerous pairs of charged amino acids in direct proximity. There are more pairs of oppositely charged residues, which are energetically favorable and may form salt bridges, than there are energetically unfavorable pairs of equally charged residues. The geometrical distribution of amphiphilic properties is represented very clearly in the 'helical net diagram' given as Fig. 1 of Johansson et al. [51].

3.2. Studies using circular dichroism spectroscopy

Initial CD spectroscopic studies were conducted in connection with the respective functional studies [32,107]. It was found that LL-37 is in random-coil conformation in pure aqueous solution, but adopts partly helical secondary structure in common structure-inducing environments [51,150]. These include trifluoroethanol and the presence of salts or lipid bilayer vesicles. In most studies, LL-37 was found to be only 70–80% α-helical, in good agreement with the more hydrophobic nature of the N-terminus, which cannot readily form an amphipathic helix.

The study of Johansson et al. [51] is the most comprehensive among CD spectroscopic work. Here, positively charged ions present in aqueous solution were found to be more apt to induce α-helical secondary structure than negative ions. In addition, antimicrobial testing was performed at the same conditions that were used for CD spectroscopy. It was found that conditions promoting α-helical structure are also making the peptide more effective in killing bacteria. Moreover, human serum was found to reduce the antimicrobial activity of L-37. Johansson et al. [51] observed cooperative behavior in CD experiments, indicating that α-helical LL-37 forms tetramers and even higher oligomers in solution. Oren et al. [144] confirmed this finding by chemical cross-linking in solution that proved the presence of dimers, trimers, and higher oligomers in a concentration-dependent distribution. This propensity of LL-37 to form α-helical aggregates in aqueous solution can be rationalized by the Hofmeister effect [188]. Here, the increasing ionic strength of the solvent makes it more and more unfavorable for the peptide to expose hydrophobic faces. Thus, an increasing salt concentration causes the peptide to form increasing amounts of secondary structure, to favor oligomeric assemblies where hydrophobic faces are hidden, and eventually to precipitate. The Hofmeister effect is commonly employed in processing of proteins, where precipitates are generated by "salting out" the protein. The Hofmeister effect as the driving force for LL-37 aggregation in the presence of salt is supported by the study of fragments of LL-37 lacking the hydrophobic N-terminus (see Section 3.5 below). These do not form α-helical structure in solution (unpublished data from our laboratory).

LL-37's properties to assume a secondary structure and form oligomers in aqueous solution is different from the ordinary behavior found in antimicrobial peptides, which typically assume a secondary structure only upon membrane binding [189]. Thus, the energetical advantage of forming a secondary structure upon binding may not be present in LL-37, hence LL-37 should bind less readily to membranes. So far, we described studies that were performed in aqueous or buffer solution. Next, we will discuss studies in the presence of lipid bilayers.

3.3. Behavior in model lipid membranes/vesicles

Oren et al. [144] employed the fluorescent properties of rhodamine- and NBD-labeled LL-37 analogs along with a number of other biophysical techniques to study LL-37 in zwitterionic POPC vesicles as well as in charged POPS/POPC vesicles. LL-37 was found to readily bind to the surface of both types of vesicles, and to induce comparative leakage in both vesicles. This is seen in close correlation with the comparable hemolytic and antimicrobial properties. In contrast, a study by Neville et al. [190] on lipid monolayers found LL-37 to insert into and disrupt DPPG monolayers, but to have virtually no effect on DPPC and DPPE monolayers. Using FTIR spectroscopy, Oren et al. [144] found that membrane-bound LL-37 has 85% α-helical content, while a small β-sheet or extended contribution was also reported. Polarized ATF-FTIR spectroscopy showed that the peptide lies on the membrane surface, thus giving no indication of a pore-forming mechanism of antimicrobial action as suggested by solid-state NMR experiments (see below).

Finally, fluorescence and FRET experiments indicated that LL-37 forms dimers or higher oligomers in zwitterionic POPC vesicles, while being monomeric in anionic POPC/POPS vesicles [144]. This oligomer formation in membrane environment is different from the aggregation reported for LL-37 in aqueous solution. There, the highly hydrophobic N-terminus of the peptide is thought to drive the formation of oligomeric aggregates based on physicochemical principles. In a membrane environment, however, this line of reasoning no longer holds true. Consequently, and considering the differing behavior in zwitterionic and charged membranes, the formation of membrane-associated oligomers is likely to bear functional significance.

The flat alignment of LL-37 on the surface of the lipid bilayer does not preclude the possibility of a dimer formation. Since the axial rotation of the peptide is not well characterized at present, it may align its hydrophobic profile monomerically with the hydrophobic properties of the lipid bilayer, as suggested by Oren et al. [144]. It may equally well form a dimer and insert as such into the hydrophilic headgroup region of the lipid bilayer, as reported for Magainin2 [191,192] and PGLa [193].

3.4. Studies using nuclear magnetic resonance spectroscopy

While studies on the three-dimensional structure determination of LL-37 are in progress in our laboratory, a structure of the corresponding CAP18-derived AMP in rabbit was determined using solution NMR experiments [52]. Since this structure was obtained in a strongly helix-inducing environment, namely 30% TFE solution, the resulting monomeric and fully α-helical structure does not carry any physiological meaning.

Our laboratory has conducted extensive solid-state NMR structural studies of LL-37 in model lipid bilayers [194]. LL-37 was synthesized with three isotope labels incorporated into the peptide backbone for the solid-state NMR experiments. Two carbon-13 nuclei were introduced in the carbonyl position of Leu-31, and into the C_α-position of Ala-13. Labeling of chemically distinct positions allowed for immediate assignment

of the resonances. The values of isotropic chemical shift observed for both labels showed the peptide to have α-helical structure in those positions, indicating that the α-helical region of the peptide extends at least from residue 13 to 31. A single nitrogen-15 isotope label in the amide group of Val-21 allowed the determination of the peptide's orientation within macroscopically aligned DMPC bilayer samples. It turned out that the helical part of the peptide lies on the bilayer surface, forming an angle of about 72° between the helical axis and the bilayer normal. A 1D dipolar shift experiment confirmed this result, which is also in agreement with the ATR-FTIR experiment of Oren et al. [144]. A 2D PISEMA experiment was performed and the measured $^{1}H–^{15}N$ dipolar coupling was in agreement with these results. Since a transmembrane orientation of the peptide was not observed for concentrations up to 7 mol% in our studies, the barrel-stave mechanism of membrane-disruption by the peptide can be ruled out [194].

In subsequent experiments, Henzler-Wildman et al. investigated the effect of temperature, peptide concentration, lipid composition, and the presence of ions onto LL-37's orientation [194]. Only minor changes were observed. In addition, ^{31}P-NMR was used to investigate the peptide-induced disorder on the lipid bilayer. In a variety of model bilayers as well as in *E. coli* lipids, LL-37 interactions with membranes have been very well characterized. Both solid-state NMR and differential scanning calorimetry (DSC) experiments suggested that perturbation is effected by the induction of positive curvature strain on lipid bilayers, which stabilizes non-lamellar phases, but was not found to cause isotropic phases such as micelles or cubic phase. This suggests that the peptide is immobilized in bilayers. The assumption of an immobilized peptide is in good agreement with the measured $^{1}H–^{15}N$ dipolar coupling from ^{15}N-labeled LL-37 using PISEMA (polarization inversion spin exchange at the magic angle) [195–197] experiments (unpublished) and ^{2}H quadrupole couplings from ^{2}H-labeled LL-37 embedded in multilamellar vesicles. In a consecutive study, Henzler-Wildman et al. [198] investigated the effect the peptide takes onto the hydrophobic core of the lipid bilayer. In DSC experiments, it was found that LL-37 suppresses the pre-transition in DMPC and DMPC:DMPG membranes, and makes the main phase transition less cooperative. The asymmetric shape of thermograms observed at a high LL-37 concentration may indicate the presence of peptide-rich and peptide-poor bilayer domains, as observed for PGLa [199,200] and pardaxin [201,202]. Solid-state ^{2}H-NMR experiments on chain-deuterated lipids enabled the measurement of the acyl-chain order parameter profiles in the presence of LL-37. Analyzing the order parameter profiles using the first-order mean-torque model, material properties of the lipid bilayer were extracted [150,198]. In summary, it was found that LL-37 disrupts acyl-chain packing and cooperativity. It inserts into the hydrophobic–hydrophilic interface of the bilayer. This insertion is sensitive to the lipid composition of the investigated bilayer. Cholesterol, a highly abundant component in mammalian membranes, was found to reduce the peptide-induced disorder in the membrane bilayer. Since cholesterol is known to make the bilayer rigid, the peptide-induced disorder particularly in the hydrophobic core of the bilayer is suppressed [198]. In summary, the solid-state NMR studies rule out the barrel-stave mechanism, the detergent-like mechanism of bilayer disruption, and the induction of non-lamellar phases such as hexagonal or cubic phases. Further, these results suggest a carpet/toroidal-type model to explain LL-37 action. A cartoon summarizing the structural findings of solid-state NMR and DSC experiments is given in Fig. 4.

3.5. Truncation and mutation studies

An increasing number of studies focus on the properties of fragments of LL-37. A fragment is usually denoted by the one-letter-code abbreviations of its two initial amino acids, followed by the number of residues it is composed of. Consider, as an example, a fragment lacking the initial eight residues in the N-terminus. Its sequence starts with the amino acids serine and lysine and consists of 29 amino acid residues. Therefore, its shorthand notation is SK-29. Increased antibacterial action was found for three cleaved fragments of LL-37 present in human sweat [42], as mentioned above. Johansson et al. [51], along with their studies of the wildtype peptide, investigated the two truncated variants FF-33 and SK-29. These lack 4 and 8 residues

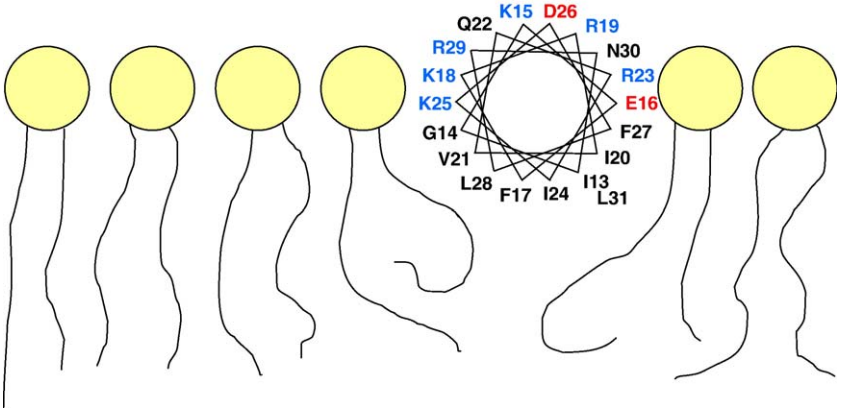

Fig. 4. Membrane-bound LL-37 resides in the bilayer interface region. Solid-state NMR experiments show that the peptide lies on the membrane surface [150,194]. The plot shows the helical wheel projection of the central region of LL-37 in the membrane interface and aligned according to its amphiphilic properties. In addition, the cartoon tries to capture the disorder that the peptide induces in the lipid acyl chain region as measured in ^{2}H solid-state NMR experiments, as compared to more ordered acyl chains in the absence of lipid (left) [198].

on the N-terminus, respectively, making the fragment more hydrophilic than the full-length peptide. Both fragments formed α-helices less readily in aqueous solution and were less effective in killing bacteria. Oren et al. [144] also worked on FF-33 in addition to LL-37, and found FF-33 to have identical antimicrobial properties, but two-fold less hemolytic potential. Moreover, they find that FF-33 does not dimerize in zwitterionic membranes and is far less effective in making these membranes permeable. They interpret this behavior as the reason for FF-33's reduced hemolytic activity. Isogai et al. [203,204] investigated the peptide fragment FR-27 as well as two mutants with increased hydrophobicity. They found increased antimicrobial activity and increased LPS-binding affinity in all their fragments. Remarkably, in a later study Okumura et al. [205] found that carcinoma cells from oral squamous cells undergo apoptosis when exposed to the fragment FR-27 and its two mutants. This does not happen in healthy cells, showing a possible way for a future therapeutic strategy. Nagaoka et al. investigated the antimicrobial [206] and LPS-binding [163] properties of the 18-mer fragment KE-18. In addition, they prepared two mutants with increased hydrophobic angle and increased polarity in the hydrophilic face. They found similar or increased antibacterial activity, marked hemolytic potential, and improved binding to LPS. Ciornei et al. [207] extended those studies to the fragments RK-31 and GD-35. While those fragments showed reduced hemolytic potential, the antimicrobially most potent mutant of KE-18 induced severe hemolysis.

Andersson et al. [208] found that structural motifs with heparin-binding affinity may confer antimicrobial properties to a peptide. The most comprehensive set of fragments was investigated by Braff et al. [176]. They prepared 14 different fragments as well as the wildtype peptide and its all-D analog. The declared aim was to identify the regions that are involved in immunostimulatory signaling, and to distinguish them from the regions responsible for antimicrobial action. The fragments KS-30 and RK-31 showed highest (increased) antimicrobial activity, while truncation of the central fragment EK-20 led to a sharp decline in antimicrobial activity. Antimicrobial activity did not correlate with stimulated IL-8 release from keratinocytes. On the contrary, immunostimulatory properties seem to reside in the N-terminal region of the peptide, which does not form an amphiphilic α-helix (see Fig. 3), while antimicrobial action seems to be borne by the amphiphilic C-terminal α-helix. Nell et al. prepared an equally comprehensive set of analogs, with the aim of finding the optimal fragment for pharmaceutical application [209]. In a mutant of the fragment IG-22, they find optimal antibacterial and LPS-binding properties at lower pro-inflammatory and cytotoxic activity. A far more complex genetic construct was used in a study by An et al. [210]. LL-37 was genetically fused to a receptor gene and the resulting construct was found to protect mice from challenges with tumor cells.

In our laboratory, we have synthesized a 21-residue amphipathic helical fragment (called RK-21) of LL-37. Structural and functional studies on this peptide have been completed (unpublished). This peptide does not form a helical structure in solution but forms a helical structure in a membrane environment. It has been shown to have a broad spectrum of antimicrobial activity and better selectivity than the native peptide. Hence, it seems to bear greater similarity with other antimicrobial peptides that form secondary structure only upon membrane interaction, depending on the composition of the targeted membrane.

4. Summary and outlook

Today, an increasingly complete picture of the role of human antimicrobial peptides keeps emerging. In human, as well as in all investigated mammalian organisms, antimicrobial peptides fill two clearly distinct roles. On the one hand, they show antimicrobial activity and are constitutively expressed in skin and epithelia to protect these covering tissues from invading bacteria. They are additionally utilized by white blood cells for the direct killing of microbes, and may help alleviate sepsis by their LPS-binding propensity. In a second, completely different role, they fill functions as signaling molecules on the borderline of innate and acquired immunity.

LL-37 is a specific human antimicrobial peptide for which all the aspects as mentioned above in a general context fully apply. LL-37 is the only representative of the class of cathelicidin-derived AMPs that is found in human. This review article has given an overview about the current knowledge of its antibiotic as well as immunoregulatory roles. Also, studies on the gene of the precursor cathelicidin protein, and the connected regulation of expression have been thoroughly mentioned. Studies on the cathelicidins of a number of species allows for phylogenetic comparison between these species. In summary, a large and ever-increasing amount of knowledge exists on the genetics and the antibiotic and immunoregulatory roles of LL-37. In contrast, insight into its structure is still very limited and relying on only a handful of studies. In lipid environment, LL-37 assumes α-helical structure and binds peripherally to the membrane. This is analogous to most other α-helical antimicrobial peptides. In zwitterionic membranes, LL-37 is found in oligomeric form. Our solution NMR experiments suggested that LL-37 behaves like a ~ 30 kDa protein in size due to oligomerization (data unpublished). Our ^{14}N (unpublished results) and PISEMA [195–198] solid-state NMR experiments on aligned bilayers containing LL-37 suggest that the oligomerization of the peptide depends on the composition of the membrane. Fig. 5 gives a graphical overview about the structural properties known for LL-37 in solution and lipid bilayer environments.

The formation of oligomers seems to be a recurring theme in the action of antimicrobial peptides [191–193,198,211,212]. In the case of LL-37 it could be speculated that the reported activation of three membrane receptor systems may be closely related to a dimer formation. Future solid-state NMR spectroscopic studies on selectively or uniformly isotope labeled LL-37 may help to further characterize the oligomeric state of LL-37 in solution and in lipid bilayers. The aptitude of the isotopic labeling scheme will be of critical importance for the success of such studies, since a vast multitude of oligomeric states can be readily imagined already for hypothetical dimers. Fig. 6 gives an illustration of the potential contribution that advanced methods of solid-state NMR can make to the structural characterization of LL-37 in lipid bilayers. The figure shows simulated PISEMA spectra of uniformly ^{15}N-labeled LL-37 in lipid bilayers aligned

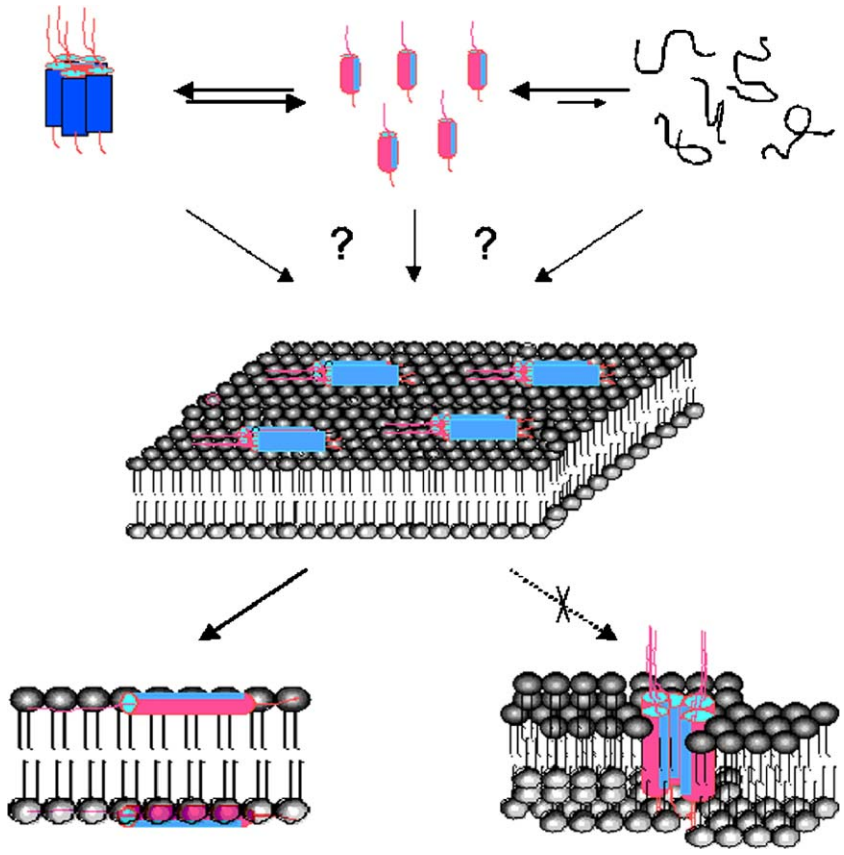

Fig. 5. A graphical summary of LL-37 folding in solution and membrane environments. In aqueous solution, LL-37 readily forms α-helical secondary structure and assembles into oligomers (top). It is not known whether binding to lipid bilayers proceeds via unordered, ordered or oligomerized structures. LL-37 binds readily to lipid bilayers, in zwitterionic bilayers it is reported to form oligomers, possibly dimers (middle). Mechanism of antibacterial action is illustrated in Fig. 4 [150,194,198]. A barrel-stave mechanism of action (bottom right) was ruled out by solid-state NMR experiments on aligned bilayers, leaving the carpet model (left) or a toroidal-type model to explain LL-37 action.

with the bilayer normal parallel to the external magnetic field of the spectrometer. The two spectra shown were calculated for two orientations of the molecule with respect to the magnetic field. The SIMMOL simulation package was used for the calculations [213]. The markedly different resonance patterns observed for both cases demonstrate the wealth of structural information that

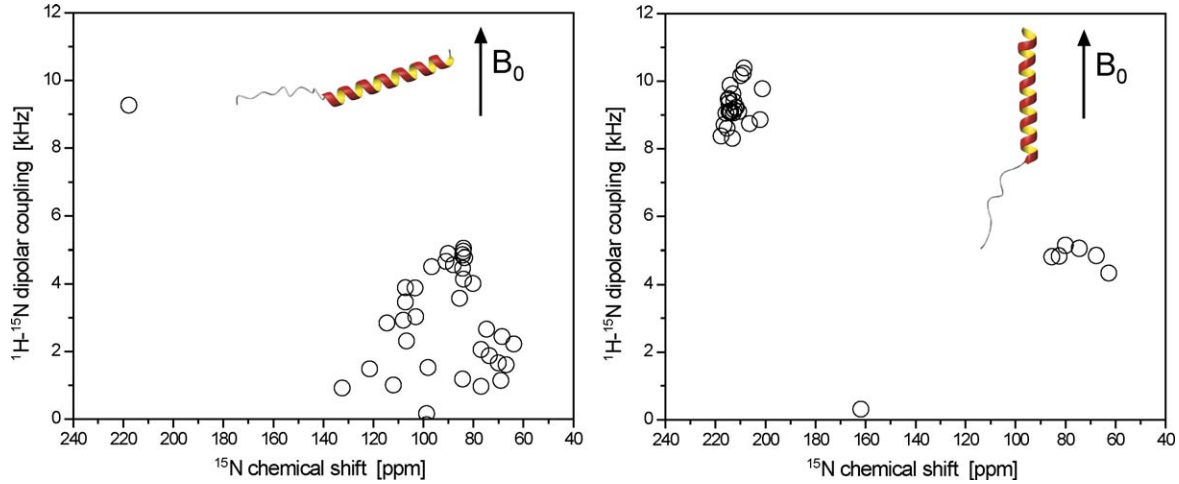

Fig. 6. ^{15}N-PISEMA spectra simulated for a hypothetical molecular model of LL-37 in which the C-terminal region is α-helical, while the N-terminus was distorted into a random conformation. The molecular models are shown as inset, the direction of the membrane normal was assumed to be identical to the direction of the external magnetic field, B_0. (Left) Spectrum calculated for a membrane surface-attached orientation as found by Henzler-Wildman et al. [150,194]. (Right) Spectrum calculated for a transmembrane orientation of the peptide in the lipid bilayer. Note that the spectra correspond to the concurring models of antimicrobial action shown in the lower portion of Fig. 5. Spectra were calculated using the SIMMOL simulation package [213].

is present in each PISEMA spectrum. With the availability of recombinant LL-37 (see the study by Moon et al. in this issue), these solid-state NMR experiments are now feasible.

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