

The Role of Cathelicidins in the Innate Host Defenses of Mammals

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Abstract

The cathelicidin peptides comprise one of several families of antimicrobial peptides that are found in neutrophils and epithelia as components of the early host defenses of mammals against infection. All cathelicidin family members are synthesized and stored in cells as two-domain proteins. These are split on demand to produce a cathelin protein and an antimicrobial peptide. Accumulating evidence indicates that both the cathelin portion and the C-terminal peptide exert biological activities connected with host protection. This review presents an overview of the structure and biology of cathelicidins and discusses recent progress in cathelicidin research with emphasis on the functional properties and role in host defense of the human cathelicidin hCAP18/LL-37. Although investigators initially concentrated their attention on antibiotic activity, it is becoming clear now that LL-37 is a multifunctional molecule that may mediate various host responses, and thus represents an essential component of the innate immune system in humans.

Introduction and historical background

The cathelicidins comprise a large number of precursors of antimicrobial peptides that typically contain a conserved N-terminal sequence ('cathelin' domain) and a C-terminal antimicrobial domain of varied sequence and length (Fig. 1). The C-terminal peptides express antibiotic activity after they have been cleaved from the holoprotein, and current evidence indicates they may contribute to host defense by both direct pathogen inactivation and by other biological activities.

The discovery of cathelicidins can be traced back to the isolation of a disulfide-containing cyclic dodecapeptide during studies of the antimicrobial activity of bovine neutrophil lysates (Romeo *et al.*, 1988). The identification of the dodecapeptide was soon followed by the purification of two additional neutrophil antimicrobial peptides that were designated bactenecins (after the latin words '*bacterium necare*'). Bac5 is a C-terminally amidated peptide of 43 amino acid residues with a polycationic sequence characterized by a repeated proline motif; Bac7 is a 60-residue proline-rich peptide with distinct amino

acid sequence and proline motif (Gennaro *et al.*, 1989). Cloning of the cDNA of Bac5 from a bovine myeloid cDNA library (Zanetti *et al.*, 1993) was instrumental in showing that despite their diversity, dodecapeptide, bactenecins and yet other structurally unrelated antimicrobial peptides (Agerberth *et al.*, 1991; Selsted *et al.*, 1992; Kokryakov *et al.*, 1993) were in fact closely related members of a single protein family. This became apparent during a molecular cloning step that was intended to amplify a Bac5 cDNA fragment from bovine bone marrow mRNA by pairing an oligo-dT primer with a forward primer based on a cDNA sequence upstream from the peptide coding region (Zanetti *et al.*, 1993). Although the template sequence was thought to be unique to Bac5 mRNA, a variety of additional transcripts were co-amplified using these primers. Each cDNA predicted a distinct peptide sequence that was attached to a propiece showing 75–87% sequence identity to the corresponding Bac5 propiece (Zanetti *et al.*, 1995). The diverse C-terminal peptides turned out to be either previously isolated or novel antimicrobial peptides. A molecular cloning strategy was then devised based on the highly conserved N-terminal propiece, to enable the identification of further members of this novel protein family in other species as deduced from cDNA. Newly discovered cathelicidin family members were designated after the putative C-terminal antimicrobial domain, by using acronyms (e.g., CRAMP for 'cathelin-related antimicrobial peptide' or BMAPs for 'bovine myeloid antimicrobial peptides'), one-letter symbols of key amino acid residues followed by the number of residues of the antimicrobial domain (e.g., LL-37), or referring to other specific peptide features. Sequences corresponding to this domain served as templates for chemical synthesis of peptides that were then functionally characterized.

The N-terminal propiece of approximately 11 kDa (99 to 114 residues) is the hallmark of cathelicidins (Zanetti *et al.*, 1995). It generally shares higher than 70% sequence identity to cathelin, a protein that was isolated from porcine neutrophils as an inhibitor of cathepsin L (cathe-L-in is an acronym for *cathepsin L inhibitor*) (Kopitar *et al.*, 1989) prior to the initial recognition of the cathelicidin family. As will be discussed later, the structural features of porcine cathelin suggested it was a member of the cystatin superfamily of cysteine proteinase inhibitors (Ritonja *et al.*, 1989).

Definition and structural diversity

The term 'cathelicidins' was proposed in 1995 to acknowledge the evolutionary relationship of the novel protein family to cathelin (Zanetti *et al.*, 1995) and it is used to denote holoproteins that contain a cathelin-like sequence and a cationic antimicrobial domain.

The mature antimicrobial peptides (Table 1) will be referred to here as cathelicidin peptides. The mammalian cathelicidin peptides display a wide repertoire of

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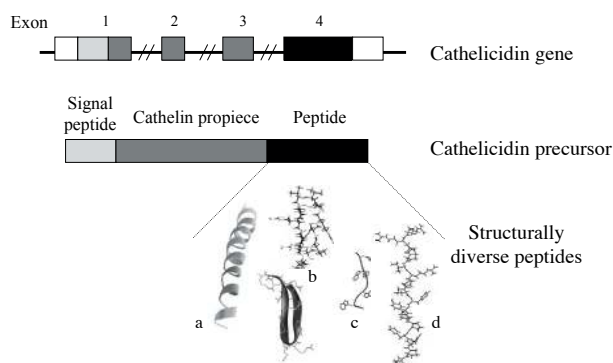


Fig. 1. Schematic representation of the gene and protein structure of cathelicidins. The mature cathelicidin peptides include α -helical (a), cysteine-rich (b), tryptophan-rich (c) and proline-rich (d) peptides.

structures and include linear and cyclic molecules (reviewed in Gennaro and Zanetti, 2000). No obvious homology is generally shared with other antimicrobial peptide sequences and the diversity in size and sequence within this family appears to be remarkably higher than within any other antimicrobial peptide family. Peptides that assume α -helical conformations in environments mimicking biological membranes are most common and either one or several peptides of 23 to 40 amino acid residues within this structural group have been found in every mammal examined. These peptides however are quite distinct from the α -helical peptides described from insects, amphibians and fish (Tossi *et al.*, 2000). Other linear cathelicidin peptides include an extended 13-mer named indolicidin, which contains 3 regularly spaced proline residues and an unusually high proportion of tryptophan (5 Trp) and a few proline-rich peptides ranging in size from 39 to 80 amino acid residues that show different types of proline repeat motifs in their sequences, namely, PR-39, Bac5, Bac7 and prophenins. Structural studies of PR-39 and Bac5 have indicated a polyproline II helix as the probable biologically active conformation of these peptides. Cyclic peptides include the protegrins and dodecapeptides. Protegrins (PG-1 through PG-5) are a 5-member group of 16–18 residue peptides characterized by two intrachain disulfide bonds and amidated C-termini. NMR spectroscopy of PG-1 indicates a two-stranded antiparallel β -sheet structure, with strands connected by a β -turn that results in an amphipathic conformation with a central hydrophobic region and two hydrophilic ends. The two-cysteine-containing dodecapeptide was suggested to be a 12 residue loop stabilized by a disulfide bond although mass spectrometric and SDS-PAGE analyses of the native molecule are rather consistent with a dimeric peptide stabilized by intermolecular disulfides. Further functional and structural information on cathelicidin peptides can be obtained from Gennaro and Zanetti, 2000 and from the AMSPdb database at <www.bbcm.units.it/~tossi/antimic.html>.

Antimicrobial activity

Cathelicidin peptides display typical features of antimicrobial peptides, including a net positive charge at neutral pH, due to a predominance of basic residues in their sequences, and an overall amphipathic topology.

When tested for *in vitro* antimicrobial activity, most peptides rapidly kill a wide range of microorganisms at micromolar and sub-micromolar concentrations, with a generally broad spectrum but significant differences in specificity and potency (reviewed in Gennaro and Zanetti, 2000, Zanetti *et al.*, 2002). *In vitro* assays have proven a valuable means to assess antimicrobial features, albeit with an inevitable degree of variability in results generated by different laboratories, mainly due to the use of different methodologies (i.e., microbiological dilution assays, inhibition zone assays in solid media, fluorescence assays to monitor microbial death) and/or differences in the reagents (use of different microbial strains or different magnitudes of bacterial inocula, composition of incubation media). Furthermore, *in vitro* screening methods usually consider the activity of individual molecules, whereas several peptides and other biological substances that may cooperate to mutually increase the antimicrobial efficacy are present *in vivo* (Nagaoka *et al.*, 2000; Yan and Hancock, 2001; Zarembek *et al.*, 2002; Dorschner *et al.*, 2003; Midorikawa *et al.*, 2003). For these reasons, the results obtained *in vitro* may not fully reflect the contribution of these molecules to microbial killing *in vivo*.

With few possible exceptions (proline-rich peptides and the loop form of dodecapeptide) the killing mechanism is mediated by disruption of the integrity of bacterial membranes (Gennaro and Zanetti, 2000). The α -helical peptides SMAP-29 and BMAPs, from sheep and cows, respectively, and the two-disulfide bridged protegrins from pigs are amongst the most rapid and potent. Their spectra of activity encompass Gram-negative and Gram-positive strains, including clinically relevant and multi-drug resistant bacteria, fungal species, parasites and enveloped viruses (Saiman *et al.*, 2001; Zanetti *et al.*, 2002; McGwire *et al.*, 2003; Giacometti *et al.*, 2003a). Conversely, those showing proline-rich sequences are predominantly active against Gram-negative organisms in physiological salt concentrations (Gennaro *et al.*, 2002). Another important feature of these peptides is their capacity to bind and neutralize bacterial endotoxin *in vitro* (Wiese *et al.*, 2003), which may account for the ability of exogenously administered peptides to protect *in vivo* against sepsis (Giacometti *et al.*, 2003b; Warren *et al.*, 2003).

Distribution

Species distribution

Cathelicidins have been found in every mammal that has been examined, with substantial interspecies variation in the number of different family members. Only one has been described in humans (Agerberth *et al.*, 1995; Cowland *et al.*, 1995; Larrick *et al.*, 1995), monkeys (Bals *et al.*, 2001; Zhao *et al.*, 2001), mice (Popsueva *et al.*, 1996; Gallo *et al.*, 1997), rats (Termen *et al.*, 2003), rabbits (Larrick *et al.*, 1991), guinea pigs (Nagaoka *et al.*, 1997) and dogs (GenBank accession number NM_001003359). Three members have been identified in horses (Scocchi *et al.*, 1999). The corresponding mature peptides in these species are invariably of the α -helical type. Pigs (Storici and Zanetti, 1993a and 1993b; Storici *et al.*, 1994; Zhao *et al.*, 1994; Zanetti *et al.*, 1994; Strukelj *et al.*, 1995; Tossi *et*

selective pressures from microbial pathogens, suggesting that cathelicidin peptides may evolve rapidly under the pressure of the microbial flora and species-specific environmental pathogens.

In addition to one typical cathelicidin, rabbits (Levy *et al.*, 1993) and mice (Moscinski and Hill, 1995) also contain distantly related family members, denoted p15s and B9, respectively. Functional *in vitro* studies of rabbit p15s have shown that, unlike typical cathelicidin components, these proteins do not undergo processing with release of an active peptide and are not microbicidal *per se* (Zarembek *et al.*, 2002). However, they exhibit antibacterial synergy with both the bactericidal/permeability-increasing protein (BPI) and the rabbit cathelicidin peptide CAP18 (Zarembek *et al.*, 2002). This activity, together with their location in the secondary granules of neutrophils, points to a role of p15s in potentiating neutrophil antimicrobial activity following degranulation.

A few cathelicidin-related sequences have recently been identified in other vertebrates including chicken (Lynn *et al.*, 2004), rainbow trout (GenBank accession number AX812046), hedgehog skate (*Leucoraja erinacea*) (GenBank accession number CV222467) and the primitive vertebrate Atlantic hagfish (*Myxine glutinosa*) (Basanez *et al.*, 2002, Uzzell *et al.*, 2003). Those from hagfish are the most ancient and their propiece shows very limited sequence similarity to the mammalian cathelin propiece. However there are a few distinctive features that place the hagfish molecules within the cathelicidin protein family. For instance, the spacing of the four characteristic cysteine residues in the propiece is fairly well preserved, and a conserved F-x-[IV]-x-E-T-x-C-x(10)-C stretch that includes 2 of 4 cysteine residues of the proregion is present in the hagfish as well as the skate, chicken and trout sequences, and in most of the mammalian cathelicidins.

Tissue distribution

All mammalian cathelicidins were initially recognized as neutrophil-specific constituents. Their relative abundance in these cells varies between and within species. The amount of the hCAP18/LL-37 in neutrophils, for instance, is 0.627 μg protein per 10^6 cells (Sorensen *et al.*, 1997a) whereas the bovine cathelicidins Bac5 and Bac7 account for at least 2 μg each per 10^6 cells in the bovine neutrophils (Zanetti *et al.*, 1991). Based on comparative Northern analysis of bovine bone marrow cells, substantially

lower amounts of BMAPs are present in the same cells (unpublished observations).

The cathelicidin storage organelles in neutrophils are secretory granule subsets (Zanetti *et al.*, 1991; Nagaoka *et al.*, 1997; Sorensen *et al.*, 1997b) that can be readily mobilized upon inflammatory or infectious stimuli and rapidly discharge their contents extracellularly (Zanetti *et al.*, 1991; Panyutich *et al.*, 1997; Sorensen *et al.*, 2001) or in phagocytic vacuoles (Zanetti *et al.*, 1991; Sorensen *et al.*, 2001). However, hCAP18/LL-37 is produced in other types of leukocytes, in skin and in various epithelia (Table 2), as will be discussed later in this chapter. Mouse CRAMP (mCRAMP) also has a broad tissue distribution. In addition to myeloid cells, mCRAMP is produced in the skin, testis, spleen and digestive tract of mice (Gallo *et al.*, 1997) and rat CRAMP (rCRAMP) is detected in the thymus, lung, oral cavity, digestive tract and testis (Termen *et al.*, 2003) (Table 2). In those mammals, where multiple cathelicidin genes are present, there appear to be differences in the pattern of expression of distinct family members, which could be connected with a diversified biological role of these molecules. For instance, RT-PCR of bovine cells and tissues indicates that the genes encoding proline-rich cathelicidin peptides are expressed in bovine marrow cells and lymphoid organs (Tomasinsig *et al.*, 2002) and those encoding α -helical and cysteine-containing peptides are further detected in tongue, mammary gland, vagina and testis (Tomasinsig, unpublished observations) (Table 2).

Genes and biosynthesis

Gene structure

Cathelicidin genes are approximately 2 kb in size and show a conserved four-exon/three-intron organization (Zhao *et al.*, 1995a,b; Gudmundsson *et al.*, 1995; Gudmundsson *et al.*, 1996; Larrick *et al.*, 1996; Scocchi *et al.*, 1997; Huttner *et al.*, 1998). Exons 1 to 3 specify the signal peptide and the cathelin propiece and the exon 4 region contains the processing site, the antimicrobial domain-coding sequence and the 3'UTR (Fig. 1). Generally, the 5' moieties of cathelicidin genes are much more related in sequence than the regions corresponding to the antimicrobial peptides. In mammals where more than one gene is present, conservation of the 5' region is higher from intra- than interspecies comparisons at both the exon and intron levels. Porcine gene pairs, for instance, can reach higher than 99% sequence identity in the region encompassing the 5' UTR, exons 1 to 3 and

Table 2. Cell- and tissue-specific expression of selected cathelicidin peptides

Cathelicidin	Expression	
	Constitutive	Inducible
LL-37	Immature neutrophils, monocytes, mast cells, NK, B, T cells, newborn skin, epithelia of the respiratory, digestive and reproductive tracts, eccrine and salivary glands	Keratinocytes (inflammation, injury, infection), intestinal epithelial cells (dietary substrates, cell differentiation), synovial membrane and nasal mucosa (inflammation)
CRAMP	Immature neutrophils, spleen, respiratory and intestinal epithelia, newborn skin, testis	Keratinocytes (injury, infection)
PR-39	Immature neutrophils, lymphoid organs (piglets)	Bone marrow cells (bacteria, LPS, IL-6, retinoic acid)
Bac5	Immature neutrophils, lymphoid organs	Peripheral neutrophils (bacteria, LPS)
Dodecapeptide and BMAPs	Immature neutrophils, lymphoid organs, tongue, mammary gland, reproductive tract	Not determined

introns 1 to 3 (Zhao *et al.*, 1995b), most likely reflecting the occurrence of specific gene conversion events that contribute to the sequence homogeneity in this region.

Chromosomal localization

The conserved structural organization of the genes and the high sequence identity of the 5' regions support the concept that all mammalian cathelicidins arose from the same gene precursor. Further evidence for a common origin is provided by adjacent chromosomal location of multiple cathelicidin genes in cattle (Castiglioni *et al.*, 1996), sheep (Huttner *et al.*, 1998) and pigs (Zhao *et al.*, 1995a,b), as revealed by genomic sequencing and chromosomal mapping studies. Eleven cathelicidin genes cluster to the same region on bovine chromosome 22q24, thereby defining a locus that has been referred to as CATHL@ (Castiglioni *et al.*, 1996; Scocchi *et al.*, 1997), and an eight-member gene cluster has been mapped to sheep chromosome 19 (Huttner *et al.*, 1998). The number of distinct genes (Gudmundsson *et al.*, 1995; Zhao *et al.*, 1995a,b) and cDNAs identified in pigs indicates that the porcine cathelicidin family is comparable in size to that of cattle. In striking contrast with the high abundance of human beta defensin (hBD) genes (Scheetz *et al.*, 2002), only one cathelicidin gene (*CAMP*) has been detected in humans. *CAMP* has been localized to human chromosome 3 (Gudmundsson *et al.*, 1995), which is homologous to mouse chromosome 9 and pig chromosome 13, where the genes encoding mCRAMP (*Cnlp*) and PR-39 have been mapped respectively in mice and pigs.

Biosynthesis and activation

Detailed studies of the biosynthesis and biological processing of the neutrophil cathelicidins have been performed in humans (Sorensen *et al.*, 2001), cows (Zanetti *et al.*, 1990) and pigs (Panyutich *et al.*, 1997). The biosynthetic window for these molecules in bone marrow cells is restricted to the myelocyte/metamyelocyte stage of neutrophil maturation. Similar to defensins, cathelicidins are synthesized in a propeptide form including signal peptide, propiece and C-terminal peptide (Fig. 1), but unlike the neutrophil α defensins, which are fully processed to mature peptides before storage in the azurophil granules (Yount *et al.*, 1999), cathelicidins are targeted as propeptides to the specific granules (Cowland *et al.*, 1995; Nagaoka *et al.*, 1997; Sorensen *et al.*, 1997b) or to a distinct granule subset present in the bovine neutrophils called large granules (Zanetti *et al.*, 1990). No constitutive expression of cathelicidins has been detected in adult mammals following release of neutrophils into the bloodstream. The neutrophil granule storage form of cathelicidins is not microbicidal (Scocchi *et al.*, 1992; Zaiou *et al.*, 2003) because an additional processing step, involving cleavage in the region that joins the cathelin and the C-terminal domain, is required to unmask the antimicrobial activity. This processing step is mediated by elastase in cows and pigs. The requirement of elastase for the bovine and porcine cathelicidins has been determined based on *in vitro* (Scocchi *et al.*, 1992; Panyutich *et al.*, 1997) and *in vivo* (Cole *et al.*, 2001) processing studies and has been confirmed by the presence of elastase-specific

cleavage residues at the peptide/cathelin boundary in the propeptide sequences (Zanetti *et al.*, 1995). In humans, activation of neutrophil-derived hCAP18/LL-37 is carried out by a different serine protease, i.e., proteinase 3 (Sorensen *et al.*, 2001), as will be described in more detail later. Both elastase and proteinase 3 are conveniently stored in a separate compartment, the azurophil granules, which prevents unwanted intracellular processing of cathelicidin molecules to active antimicrobial peptides in resting neutrophils. Processing studies of the bovine (Zanetti *et al.*, 1991) and porcine (Panyutich *et al.*, 1997) cathelicidins have shown that the mobilization of secondary/large and azurophil granules leads to coincident release of the granule contents either in the extracellular milieu (Zanetti *et al.*, 1991; Panyutich *et al.*, 1997) or in phagocytic vacuoles (Zanetti *et al.*, 1991) and this in turn results in rapid and efficient generation of functionally active cathelicidin peptides, further indicating that the biological processing mediated by the neutrophilic serine proteases is an effective means to control peptide activation.

Regulation of cathelicidin biosynthesis

Besides expression in the neutrophil precursors, some cathelicidin genes show constitutive and/or inducible expression in various cells and tissues (Table 2). Their tissue-specific expression is regulated by microbial, inflammatory and developmental stimuli. The molecular mechanisms of gene regulation, however, are largely unexplored.

The hCAP18/LL-37 gene is induced in keratinocytes exposed to *S. aureus* (Midorikawa *et al.*, 2003) and the CRAMP-encoding gene is upregulated in mouse skin in response to subcutaneous inoculation of Group A *Streptococcus* (Dorschner *et al.*, 2001). Induction of PR-39 and protegrin gene expression in porcine bone marrow cells (Wu *et al.*, 2000) and of Bac5 gene expression in bovine peripheral neutrophils (Tomasinsig *et al.*, 2002) has been observed following cell activation with bacteria or purified LPS.

Both the hCAP18/LL-37 and mCRAMP genes are upregulated in response to sterile incision of the skin (Dorschner *et al.*, 2001). Interestingly, only insulin-like growth factor I (IGF-I), a molecule involved in tissue regeneration after wounding, among other growth factors (TGF- α , TGF- β 1, bFGF) and proinflammatory cytokines (IL-1 β , IL-6, TNF- α), elicits expression of hCAP18/LL-37 in cultured human keratinocytes (Sorensen *et al.*, 2003a), suggesting a role for this growth factor in hCAP18/LL-37 expression upon injury. Conversely, hBD-2 and hBD-3 are induced in cultured keratinocytes by IL-1 β and TGF- α , respectively, but not by IGF-I (Sorensen *et al.*, 2003a), possibly indicating that these cells respond to different stimuli with distinct patterns of antimicrobial peptide expression. As opposed to hBDs (Liu *et al.*, 2002; Liu *et al.*, 2003; Tsutsumi-Ishii and Nagaoka 2003), no evidence has been produced to date for direct induction of hCAP18/LL-37 by proinflammatory cytokines. Both hCAP18/LL-37 and hBD2 genes are underexpressed in atopic dermatitis (Ong *et al.*, 2002), but only the defensin defect has been explained in a recent report based on insufficient amounts

of TNF- α and INF- γ in this disease (Nomura *et al.*, 2003). The reason for the under-representation of hCAP18/LL-37 still needs to be explained.

Cathelicidin synthesis is also connected with the state of cell differentiation. This is illustrated by human colon epithelium that shows constitutive expression of the hCAP18/LL-37 gene coincident with epithelial cell differentiation (Hase *et al.*, 2002; Schaubert *et al.*, 2003). Notably, no changes in the expression levels of this gene are observed in inflamed colon (Hase *et al.*, 2002) or in colonic epithelial cell lines stimulated by IL-1 α , TNF- α , LPS, PMA or other proinflammatory mediators such as IFN- γ , IFN- α and IL-6 (Hase *et al.*, 2002; Schaubert *et al.*, 2003). Expression of hCAP18/LL-37, however, is stimulated by butyrate and other dietary short chain fatty acids that are known to induce differentiation of human colon cells (Schauber *et al.*, 2003). The possible involvement of the MEK-ERK intracellular signal pathway is suggested by the observation that MEK inhibition blocks hCAP18/LL-37 induction by butyrate, while enhancing butyrate-dependent cell differentiation. Conversely, inhibition of MAP kinase p38 does not affect butyrate-stimulated hCAP18/LL-37 induction, but inhibits butyrate-dependent epithelial differentiation (Schauber *et al.*, 2003), thus suggesting that butyrate-induced epithelial cell differentiation and hCAP18/LL-37 expression in colon are mediated by distinct signaling pathways that are both activated by butyrate.

The mCRAMP and PR-39 appear to be regulated developmentally (Gallo *et al.*, 1997; Wu *et al.*, 1999), since the CRAMP transcript is detected early during embryogenesis and PR-39 is expressed in bone marrow and in various organs in piglets up to the first post-natal days and only in marrow cells afterwards.

Inducible expression of PR-39 by interleukin 6 or retinoic acid is observed in porcine bone marrow cells (Wu *et al.*, 2000), in keeping with the presence of consensus sites/response elements for nuclear factors dependent on these signals in the gene promoter region. Indeed, putative consensus sites for binding to transcription factors involved in hematopoiesis, inflammation and acute phase reaction, such as nuclear factor-interleukin 6, nuclear factor- κ B, acute phase-response factor and gamma-interferon response element, are present within a few hundred basepairs in the 5' flanking sequences of effectively all sequenced cathelicidin genes, indicating the potential to respond to signals from inflammatory and infectious stimuli. However, in general, very little information is available on the intracellular mechanisms involved in cathelicidin gene regulation. A deeper understanding of this aspect of the biology of cathelicidins is essential to clarify the role of cathelicidins in health and disease.

The cathelin domain: an unsolved puzzle

Several reports indicate that although devoid of independent antimicrobial activity, unprocessed cathelicidins (i.e., the propeptides) may increase the antimicrobial potency of other (poly)peptides and/or exert separate host defense activities. Some of the bovine cathelicidin propeptides, for instance, inhibit *in vitro* the activity of cathepsin L, as will be discussed shortly, and studies of the bovine

cathelicidin proBac7 indicate that this propeptide is a chemoattractant *in vitro* for monocytes, with a dose-dependent, bell shaped response and optimal activity at 10^{-9} M (Verbanac *et al.*, 1993). Although reported much earlier than similar activities in other peptides, the molecular mechanism of this chemotactic activity has not been investigated in depth, although it certainly involves the cathelin portion of proBac7 since the mature Bac7 peptide has no effect on the migration of monocytes (Verbanac *et al.*, 1993). Unprocessed rabbit cathelicidin proCAP18 exhibits antibacterial synergy with antimicrobial peptides and polypeptides and displays endotoxin-neutralizing activity independent of the presence of other defense molecules at sub-nanomolar concentrations. The latter activity cannot be attributed to the C-terminal peptide domain since the propeptide is 100-fold more potent, on a molar basis, than the mature peptide CAP18 (Zarembert *et al.*, 2002). These findings strongly suggest that structural determinants within the cathelin domain of cathelicidins can make contributions to host defense independent of the C-terminal domain. Evidence for an autonomous function of cathelin has been provided by the reported ability of the recombinant hCAP18 cathelin domain to inhibit bacterial growth *in vitro* (Zaiou *et al.*, 2003). An interesting implication of this finding is that the holoprotein hCAP18/LL-37, which does not display autonomous antimicrobial properties, is processed to generate two distinct antimicrobial peptides, cathelin and LL-37. Recombinant cathelin is active against *S. aureus* and *S. epidermidis* (MIC of 32 μ M), which are resistant to LL-37 under the same *in vitro* conditions, and so the two peptides seem to exert complementary antibiotic activities (Zaiou *et al.*, 2003). It remains to be clarified how human cathelin exerts this bactericidal effect. The mechanism of action likely differs from that of classical antimicrobial peptides in that the cathelin sequence has no net positive charge and its overall structure does not match that of typical antimicrobial peptides.

In addition to a possible direct antimicrobial activity, human cathelin inhibits the activity of cathepsin L *in vitro* (Zaiou *et al.*, 2003). This feature is shared with the bovine propeptides (Verbanac *et al.*, 1993; Storici *et al.*, 1996) and can be explained based on structural considerations. The cathelin domain has a sequence of approximately 100 residues, including four cysteine residues at conserved positions that form two disulfide bonds in the 1-2, 3-4 arrangement. Besides high levels of identity among cathelicidin family members, a moderate (less than 40%) sequence similarity is shared with members of the cystatin family of cysteine proteinase inhibitors, both at the protein (Ritonja *et al.*, 1989; Storici *et al.*, 1996) and the gene (Scocchi *et al.* 1997; Zanetti *et al.*, 2000) levels. Furthermore, the X-ray structure of the cathelin domain of the porcine cathelicidin protegrin-3 shows an overall fold similar to that of chicken cystatin (Sanchez *et al.*, 2002), and although significant differences in the detailed structures, particularly in the vicinity of loop regions, have been revealed by the corresponding solution structures (Yang Y. *et al.*, 2003), these differences do not affect the global cystatin-like fold. Based on these considerations, a certain degree of functional similarity between the two families is reasonably expected. Like human cathelin,

cystatin members display *in vitro* antibacterial activity that is independent of cysteine proteinase inhibition (Blankenvoorde *et al.*, 1998; Hamil *et al.*, 2002).

The degree of evolutionary divergence between the cystatin and cathelicidin families can be inferred from studies of the cystatin-like activities of cathelicidins. Bovine cathelicidin components inhibit the activity of cathepsin L *in vitro*, but do not affect the activity of papain, although both papain and cathepsin L are highly susceptible to cystatins (Verbanac *et al.*, 1993; Storici *et al.*, 1996). Also, the concentrations required for cathepsin L inhibition are much higher for cathelicidins (K_i values in the 10^{-7} to 10^{-8} M range) than for cystatins (K_i values of 10^{-10} to 10^{-12} M), likely due to the absence in cathelicidins of important determinants required for binding to cathepsin L, such as a conserved Gly at the N-terminus and the QXVXG binding motif of cystatins. These differences have raised an issue concerning the *in vivo* relevance of the inhibitory effect of cathelicidins. Cathelin levels compatible with *in vitro* inhibitory concentrations can be reached at inflammatory sites, due to massive recruitment and activation of neutrophils and subsequent extracellular release and processing of cathelicidins. This could lead to local accumulation of cathelin to levels sufficient for protection from the destructive potential of cathepsin L. This enzyme is a major secretion product of macrophages and recent evidence indicates that epithelial-derived β defensins and the antiprotease/antimicrobial protein SLPI are among the molecules that are susceptible to its proteolytic effects (Taggart *et al.*, 2003). The inhibitory properties of extracellularly released cathelin suggest that this protein is well suited to prevent antimicrobial peptide degradation by cathepsin L, once it is released in the extracellular milieu during inflammation.

Further roles have been suggested for the cathelin propiece, mostly based on structural considerations. The sequence is generally negatively charged and may thus interact with the positively charged C-terminal peptide. The resulting configuration could have implications for folding of the polypeptide or may help in preventing unwanted membrane interactions prior to granule mobilization. These putative activities, however, are not supported by experimental evidence and the validity of these hypotheses remains to be tested.

Although the exact role of the cathelin propiece is still debated and the *in vitro* effects of cathelin await further characterization and validation in animal models, the strong evolutionary pressure for sequence conservation is a clear indication of an essential role of this domain.

The human cathelicidin hCAP18/LL-37

Humans and primates each contain only one cathelicidin family member, with 86% overall sequence identity (68% in the 37-residue peptide region) between Rhesus and man (Zhao *et al.*, 2001).

The human cathelicidin was independently identified in 1995 by three groups; it was deduced from myeloid bone marrow cDNA (Agerberth *et al.*, 1995; Cowland *et al.*, 1995; Larrick *et al.*, 1995) and isolated from neutrophils (Cowland *et al.*, 1995). The name hCAP18 refers to the predicted mass of the full-length polypeptide (approximately 18 kDa) and the cationic character of the

C-terminal sequence (Cationic Antimicrobial Peptide) and implicitly recognizes its similarity to the rabbit cathelicidin CAP18. The alternative designation "FALL-39" was used originally on the assumption that Phe-Ala-Leu-Leu (FALL) were the N-terminal residues of the putative antimicrobial domain. The name was then changed to LL-37 when the antimicrobial peptide was isolated from neutrophils and found to consist of 37 residues, with Leu-Leu at the N-terminus (Gudmundsson *et al.*, 1996). The term hCAP18 is currently accepted to indicate the propeptide, whereas LL-37 denotes the peptide liberated from the holoprotein.

Sites of expression

hCAP-18/LL-37 is produced in immature neutrophils (Sorensen *et al.*, 1997) and in various blood cells populations that are involved in inflammatory and immune responses, including NK cells, $\gamma\delta$ T cells, B cells, monocytes (Agerberth *et al.*, 2000) and mast cells (Di Nardo *et al.*, 2003), as well as in hematopoietic cell lines and human leukemia cells (Yang Y.H. *et al.*, 2003). In addition, this gene is widely expressed in skin, in the squamous epithelia of the airways, mouth, tongue, esophagus, intestine, cervix and vagina (Frohm Nilsson *et al.*, 1999; Bals *et al.*, 1998; Hase *et al.*, 2002), in sweat and salivary glands (Murakami *et al.*, 2002a, b) and in epididymis and testis (Agerberth *et al.*, 1995; Malm *et al.*, 2000; Hammami-Hamza *et al.*, 2001). The polypeptide is secreted in human wound fluid (Frohm *et al.*, 1996) and airway surface fluid (Bals *et al.*, 1998) and is detected in intriguingly high amounts (85 μ g/ml on average) in seminal plasma (Andersson *et al.*, 2002). Additionally, hCAP18/LL-37 has been detected at approximately 20 μ g/ml in tracheal aspirates of newborns (Schaller-Bals *et al.*, 2002), and in vernix caseosa and skin during the perinatal period (Marchini *et al.*, 2002; Dorschner *et al.*, 2003). The synergistic antimicrobial activity of LL-37 and hBD-2 and efficient killing of Group B *Streptococcus*, an important neonatal pathogen (Dorschner *et al.*, 2003), suggest that these peptides provide vicarious defense functions during development of cellular immune response mechanisms in the newborn period. It is interesting to note that hCAP18/LL-37 and other human antimicrobial peptides, including β -defensins, often show overlapping tissue distribution. In some cases, different regional distributions and induction signals have been described (Hase *et al.*, 2002), suggesting that these peptides occupy distinct functional niches and may be called into play in response to distinct stimuli within the same tissue.

Processing

Investigations of the physiological processing of the myeloid-derived hCAP18 indicate that the propeptide is cleaved to generate the antimicrobial peptide LL-37 in exocytosed material from neutrophils (Sorensen *et al.*, 2001). Conversely, cleavage is not detected in phagocytic vacuoles, despite both hCAP18 and azurophil granule proteins being detected inside the vacuoles and despite the fact that all known azurophilic serine proteases, including elastase, proteinase 3 and cathepsin G, can cleave hCAP18 *in vitro*. Experimental evidence has shown that among these enzymes, proteinase 3 is solely responsible for processing the propeptide after exocytosis

(Sorensen *et al.*, 2001). This result correlates with the presence of a proteinase 3-compatible (Ala-Leu) cleavage site, as deduced from cDNA, between the cathelin portion and the antimicrobial domain of hCAP18. Instead, a typical (Val-Arg) elastase cleavage site is predicted at the cathelin/peptide boundary of most porcine and bovine cathelicidins. These findings suggest that cathelicidins are processed by distinct enzymes in related species. Further studies of the human cathelicidin have shown timely cleavage of epididymal-derived hCAP18 in seminal plasma by the prostate-derived protease gastricsin (pepsin C) in the presence of vaginal fluid at low pH, generating a cathelin protein and a functionally active ALL-38 (38 amino acid residues) (Sorensen *et al.*, 2003b). These studies provide evidence that cathelicidins are processed differently in different physiological contexts within the same organism. Since activation of the prostate-derived gastricsin requires the low pH of the vagina, processing of epididymal hCAP18 to ALL-38 likely takes place after sexual intercourse and may represent a mechanism to prevent infection after coitus. This hypothesis is further supported by the presence of various other antimicrobial proteins and peptides in the male reproductive tract (Yenugu *et al.*, 2003), including defensin members (Com *et al.*, 2003). Cystatin family members are also found in the male reproductive tract (Shoemaker *et al.*, 2000; Hamil *et al.*, 2002) and have been suggested to modulate cathepsin activities related to sperm development and maturation. The epididymal-derived cystatin 11, for instance, binds the surface of sperm (Hamil *et al.*, 2002), as does hCAP18/LL-37 (Malm *et al.*, 2000). In this context, since hCAP18/LL-37 is expressed in testis (Agerberth *et al.*, 1995; Hammami-Hamza *et al.*, 2001) and epididymis (Malm *et al.*, 2000) and is released in seminal plasma (Malm *et al.*, 2000), it would be interesting to investigate the potential of human cathelin to modulate the activity of cathepsin L, which is known to play an important role in spermatogenesis (Wright *et al.*, 2003).

It is not known whether other proteases may be involved in processing of hCAP18 in the numerous tissues where hCAP18 and/or the mature LL-37 have been detected.

The role and fate of hCAP18/LL-37 have proven elusive in some instances. Relatively high levels of uncleaved hCAP18 are present in plasma (approximately 1.2 $\mu\text{g/ml}$, Sorensen *et al.*, 1997a), where the propeptide circulates bound to lipoproteins through the antimicrobial domain (Wang *et al.*, 1998; Sorensen *et al.*, 1999). Based on their association with hCAP18, the plasma lipoproteins have been suggested to be a reservoir of LL-37 in blood, but the precise destination and metabolic fate of the lipoprotein-associated hCAP18/LL-37 has not been elucidated. The presence of uncleaved hCAP18 in blood may be explained by the need for protection from potential cytotoxic effects of mature LL-37. Alternatively, the cathelin domain may protect the peptide from proteolytic degradation in protease-rich media, although both the mature LL-37 and the holoprotein have been shown to be degraded *in vitro* and *ex vivo* in human wound fluid by elastase-producing *P. aeruginosa* (Schmidtchen *et al.*, 2002). Most intriguing is the persistence of uncleaved hCAP18 in infection/inflammatory settings, since

one should definitely expect to detect the biologically active form in these environments. The holoprotein, for instance, is found in the phagocytic vacuoles of activated neutrophils (Sorensen *et al.*, 1997b), in wound and blister fluids (Frohm *et al.*, 1996) and in psoriatic skin (Ong *et al.*, 2002). The accumulation of uncleaved propeptide in these settings lends credence to the functional significance of this molecule and encourages further functional studies of the propeptide.

Antimicrobial and cytotoxic activities

The antimicrobial peptide LL-37 corresponds to the 37 residue C-terminal region of hCAP18 and shows a net charge of +6 at neutral pH. It belongs to the group of membrane-active, amphipathic α -helical peptides with wide spectrum activity. Under laboratory assay conditions, this peptide inhibits the growth of a variety of Gram-negative (*P. aeruginosa*, *S. typhimurium*, *E. coli*) and Gram-positive (*S. aureus*, *S. epidermidis*, *L. monocytogenes* and vancomycin-resistant enterococci) species in the micromolar and sub-micromolar range of peptide concentrations (Turner *et al.*, 1998). LL-37 is active against clinically important strains of Gram-negative uropathogens (*E. coli* HU734, *P. aeruginosa* AK1, *K. pneumoniae* 3a) (Smeianov *et al.*, 2000), periodontal (*Actinobacillus actinomycetemcomitans*, *Campylobacter*, *Porphyromonas* and *Prevotella* spp.) (Tanaka *et al.*, 2000; Isogai *et al.*, 2003) and common wound pathogens (Group A *Streptococcus*) (Dorschner *et al.*, 2001). The high amounts of LL-37 detected in airway epithelial cells during infection (Schaller-Bals *et al.*, 2002) and especially in psoriatic skin lesions (approximately 304 μM , Ong *et al.*, 2002), suggest that the concentrations of LL-37 that are effective *in vitro* are fully compatible with those found *in vivo*. Furthermore, the *in vitro* antimicrobial activity is enhanced in the presence of α or β defensins (Ong *et al.*, 2002; Nagaoka *et al.*, 2000) further suggesting that these peptides synergize under *in vivo* conditions to form an efficient barrier against microbial invasion. Unlike most defensin members, LL-37 is active against several bacteria in high salt media (up to 150 mM NaCl) (Turner *et al.*, 1998; Nagaoka *et al.*, 2000), supporting its capacity to function under a variety of physiological conditions. Fungal species of *Candida* appear to be resistant to LL-37 (Turner *et al.*, 1998) but tripomastigotes of the protozoan parasite *T. cruzi* and peripheral blood leukocytes are susceptible *in vitro* to 13–25 μM peptide concentrations (Johansson *et al.*, 1998), indicating that LL-37 is not highly selective to prokaryotic cells. The cytotoxic activity is inhibited by the presence of serum (Johansson *et al.*, 1998) suggesting that serum components provide a physiological mechanism to protect blood cells from potentially harmful effects of the peptide.

Mechanisms of antimicrobial activity

Whereas most α -helical antimicrobial peptides show a transition from an unordered state in aqueous solutions to an α -helical structure in membrane-mimicking conditions, LL-37 adopts an α -helical structure in physiological buffer conditions and remains helical upon association with the membrane (Johansson *et al.*, 1998). The stability of the helix has been attributed to peptide concentration-

dependent aggregation induced by ionic and hydrophobic interactions that are favored by the presence of negatively charged residues and a short hydrophobic N-terminal region in the LL-37 sequence (Oren *et al.*, 1999). The extent of helicity correlates with the antibacterial activity of LL-37 against both Gram-negative and Gram-positive bacteria (Johansson *et al.*, 1998), indicating that this conformation is an important requirement for activity. The helical LL-37 kills target organisms by disrupting membrane integrity (Turner *et al.*, 1998). Biophysical measurements using model systems (Johansson *et al.*, 1998; Oren *et al.*, 1999; Henzler Wildman *et al.*, 2003) indicate that the membrane disruption mechanism involves orientation of the amphipathic helix parallel to the surface of both anionic and zwitterionic bilayers. The peptide accumulates on the membrane in a carpet-like fashion and induces leakage (Oren *et al.*, 1999; Henzler Wildman *et al.*, 2003), possibly through formation of toroidal peptide-lipid pores, as suggested by combined solid state NMR and differential scanning calorimetry studies (Henzler Wildman *et al.*, 2003) that show a peptide-induced positive curvature strain in lipid bilayers. LL-37 is less disruptive in this study to lipids with choline headgroups, consistent with a protective role for choline headgroups against the membrane perturbing effects of LL-37 (Henzler Wildman *et al.*, 2003). This observation supports bacterial resistance studies indicating that addition of choline headgroups to lipopolysaccharide in the outer membrane contributes to resistance of *H. influenzae* to killing by LL-37 (Lysenko *et al.*, 2000).

Other activities

Recent work has brought into the spotlight the multifunctional nature of LL-37 (Table 3). In addition to direct microbial killing, LL-37 binds free LPS *in vitro* with high affinity (Turner *et al.*, 1998), sharing this property with many other antimicrobial peptides, and inhibits LPS-induced cellular responses such as release of TNF- α , nitric oxide and tissue factor, PGE₂, MCP-1 and MIP-2 (Hirata *et al.*, 1994; Larrick *et al.*, 1994; Larrick *et al.*, 1995; Ohgami *et al.*, 2003). Additionally, LL-37 prevents macrophage activation by lipoteichoic acid and noncapped lipoarabinomannan (Scott *et al.*, 2002), suggesting that it binds various bacterial components *in vivo*.

There are clear data to show that LL-37 also interacts with host cells to mediate various host defense responses (Table 3 and Fig. 2). LL-37 is chemotactic *in vitro*, inducing selective migration of human peripheral blood monocytes, neutrophils and CD4 T cells (Agerberth *et al.*, 2000; De Yang *et al.*, 2000) with a dose-dependent, bell shaped response at optimal concentrations ranging from 10⁻⁵ to 10⁻⁷ M. Whereas the antimicrobial and cytotoxic activities are mediated by membrane perturbation and are serum-

sensitive (Johansson *et al.*, 1998), the chemotactic activity of LL-37 is largely unaffected by serum and depends on binding to formyl peptide receptor-like 1 (FPRL1), a G protein-coupled receptor (De Yang *et al.*, 2000). The latter finding suggests that LL-37 contributes to immune responses by participating in the recruitment of inflammatory and immune cells that express functional FPRL1. However, as the chemotactic concentrations of LL-37 are higher than those of classical chemokines, cellular recruitment by LL-37 may be active only when a threshold LL-37 concentration is reached following upregulation of the gene in epithelial cells and/or massive release from accumulating neutrophils.

LL-37 has been reported to attract rat peritoneal mast cells with an optimal concentration of 5 μ g/ml, apparently through a receptor/s other than FPRL1, and to induce histamine release and intracellular Ca²⁺ mobilization in these cells (Niyonsaba *et al.*, 2003). These activities of LL-37 highlight the potential involvement of this peptide in mast cell recruitment at inflammatory sites, where these cells are known to contribute to site-specific inflammation by phagocytosing opsonized bacteria and through neutrophil recruitment. LL-37-induced degranulation of mast cells leads to release of inflammatory mediators including histamine, which favors neutrophil infiltration into inflamed tissues by increasing vascular permeabilization. Investigations of the effects of LL-37 on mast cells however have been limited to rat mast cells and require confirmation using human mast cells.

Further evidence for involvement of LL-37 in modulating host cell responses comes from microarray-based studies indicating that LL-37 alters transcriptional responses in the human lung carcinoma cell line A549 and the murine macrophage cell line RAW 264.7. Numerous genes, including antiinflammatory and proinflammatory genes, are up/down-regulated in these cells in response to 50–100 μ g/ml of LL-37. Among the genes that are up-regulated are those encoding chemokines and chemokine receptors (Scott *et al.*, 2002), suggesting that LL-37 may indirectly amplify cell recruitment through modulation of gene expression in target cells.

A regulatory function of LL-37 in the antimicrobial and inflammatory response of the airway epithelium is also suggested by the finding that LL-37 activates primary bronchial epithelial cells and the lung carcinoma cell line NCI-H292, possibly via activation of a cell surface metalloproteinase that would then process membrane-anchored epidermal growth factor receptor (EGFR) ligands. These in turn would activate the EGFR, leading to activation of the extracellular signal-regulated kinase (ERK)1/2 and gene transcription (Tjabringa *et al.*, 2003).

In addition to its capability to augment host defenses via these activities, LL-37 could play a role in repair of

Table 3. Biological activities of selected cathelicidin peptides

LL-37	PR-39	BMAP28
Antimicrobial activity Binding and inactivation of LPS Chemotaxis of blood cells Activation of epithelial cells Histamine release from mast cells Induction of angiogenesis Modulation of gene expression Re-epithelialization of skin	Antimicrobial activity Chemotaxis of neutrophils Inhibition of NADPH oxidase activity Induction of angiogenesis Inhibition of IkBa degradation Induction of syndecan expression Inhibition of macrophage apoptosis	Antimicrobial activity LPS binding and inactivation Apoptosis of hematopoietic and fibroblast-derived cells

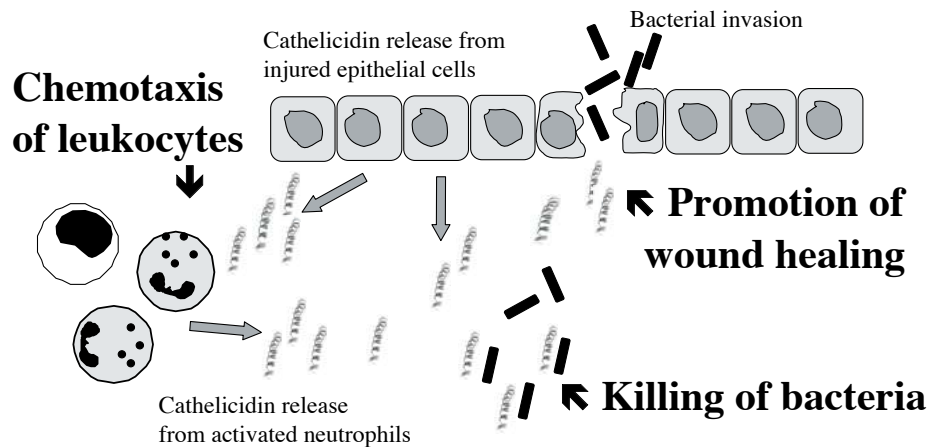


Fig. 2. Biological activities of the human cathelicidin LL-37 at epithelial surfaces. hCAP18 is synthesized and released from epithelial cells in response to microbial or physical injury. The holoprotein is processed to produce the α -helical peptide LL-37 and the cathelin propiece. LL-37 participates in the recruitment of neutrophils and other circulating cells at sites of infection. Release of hCAP18 from the cytoplasmic granules of activated neutrophils results in increased local concentrations of LL-37 and cathelin. LL-37 (and possibly cathelin) contributes to killing of invading pathogens and promotes repair of damaged tissue by inducing re-epithelialization and angiogenesis.

damaged tissue by promoting reepithelialization of healing skin (Heilborn *et al.*, 2003). This is suggested by high levels of LL-37 expression in human skin upon wounding (Dorschner *et al.*, 2001; Heilborn *et al.*, 2003), the ability of LL-37-specific antibodies to inhibit reepithelialization in a dose-dependent manner and by the decreased levels of LL-37 in chronic ulcers (Heilborn *et al.*, 2003). LL-37 could contribute to cutaneous wound vascularization as well, since application of exogenous LL-37 results in angiogenesis both in the chorioallantoic membrane assay and in a rabbit hind-limb model of ischemia (Koczulla *et al.*, 2003). The ability of this peptide to stimulate proliferation of cultured HUVECs and to cause endothelial sprouting from hamster aortic rings suggests that the angiogenic effect depends on direct activation of endothelial cells, a process that appears to be mediated by interaction of LL-37 with FPRL1 expressed on endothelial cells (Koczulla *et al.*, 2003).

Role in host defense

Additional studies are required to understand how the multiple activities of LL-37 impact host processes. However, several different lines of evidence already converge to indicate a crucial role of this peptide *in vivo* (reviewed in Nizet and Gallo, 2003).

Increased production of LL-37 has been observed in skin and mucosal epithelia in response to local and systemic infections as well as in inflammatory disorders and other skin pathologies (Frohman *et al.*, 1997; Dorschner *et al.*, 2001; Conner *et al.*, 2002; Schaller-Bals *et al.*, 2002; Ong *et al.*, 2002; Paulsen *et al.*, 2003; Kim *et al.*, 2003; Jung *et al.*, 2003), providing circumstantial evidence for a role of LL-37 in such conditions. Particularly, LL-37 and hBD-2 are upregulated in psoriatic skin but not in atopic dermatitis, which may render an explanation for the fact that patients with atopic dermatitis, but not psoriasis, suffer from frequent skin infections (Ong *et al.*, 2002). Similarly, decreased levels of hCAP18/LL-37 have been associated with decreased protection against infection. This is well illustrated by the correlation between LL-37 deficiency in neutrophils from patients with Morbus

Kostmann, a severe congenital neutropenia, and frequent occurrence of infections and chronic periodontal disease in these patients (Putsep *et al.*, 2002). The involvement of LL-37 in prevention of oral bacterial infections is further supported by the ability of this peptide to efficiently kill common periodontal pathogens (Tanaka *et al.*, 2000; Isogai *et al.*, 2003).

It is revealing that bacteria themselves can cause depletion of LL-37. Indeed, expression of hCAP18/LL-37 is downregulated in gut biopsies of patients with *Shigella* infections as well as in *Shigella*-infected monocyte and epithelial-derived cell lines (Islam *et al.*, 2001), suggesting that this may be a virulence feature of bacteria to compromise the intestinal innate defenses.

Augmentation of LL-37 by exogenous administration is a useful experimental strategy to assess capability of LL-37 to protect from microbial infection, as demonstrated using a genetic approach based on adenoviral-mediated hCAP18 gene transfer into respiratory epithelia (Bals *et al.*, 1999a,b). Overexpression of recombinant hCAP18 in cystic-fibrosis (CF) respiratory epithelial cells reverses the CF-specific bacterial killing defect (Bals *et al.*, 1999a). Furthermore, transfer of the hCAP18 gene into mouse airways results in decreased bacterial load and decreased inflammatory response in *P. aeruginosa*-infected mice (Bals *et al.*, 1999b). In addition, systemic overexpression of this gene protects mice from septic death (Bals *et al.*, 1999b), which may partly reflect the high LPS-neutralizing capacity of LL-37 (Larrick *et al.*, 1995; Kirikae *et al.*, 1998; Turner *et al.*, 1998; Scott *et al.*, 2002).

Activities and role in host defense of non-human cathelicidins

Emphasis has been given in this review to hCAP18/LL-37 with respect to its human host. This should not overshadow the substantial contribution of non-human cathelicidins to the appreciation of the function and biology of this protein family in mammals. Furthermore, the activities of cathelicidin peptides have stimulated intensive studies aimed to exploit their therapeutic potential for the benefit of humans.

The last part of this review briefly summarizes the results of several key experiments that highlight the contribution of non-human cathelicidin peptides to *in vivo* host protection and describes potential effects of two such peptides on mammalian cells (see Gennaro and Zanetti, 2000 and Zanetti *et al.*, 2002 for a comprehensive account of structural and activity studies of the cathelicidin peptides).

Role in host defense (CRAMP, protegrins, SMAP-29)

Mice have a single cathelicidin denoted CRAMP that is encoded by the *Cnlp* gene. The absence of other cathelicidins with similar functions has facilitated studies aimed at verifying the contribution of the CRAMP peptide to host defense. Targeted gene disruption with generation of mice null for the *Cnlp* gene has been quite informative in this respect, leading to an understanding of the role of CRAMP in skin infection (Nizet *et al.*, 2001). Homozygous loss of *Cnlp* results in much larger and persistent skin lesions following infection by Group A *Streptococcus* (GAS) than in wild-type mice (Nizet *et al.*, 2001). Moreover, mast cells derived from knockout mice show significantly lower amounts of endogenous CRAMP and decreased ability to kill GAS, as compared with wild-type mice (Di Nardo *et al.*, 2003). The protective effect of CRAMP against GAS has further been confirmed by generating a CRAMP-resistant GAS mutant and showing that wild-type mice infected with CRAMP-resistant GAS exhibit larger lesions than those infected with the parent GAS strain (Nizet *et al.*, 2001). A corresponding function of LL-37 in human skin protection is reasonably expected, since murine CRAMP and human LL-37, albeit different in sequence, have similar amphipathic α -helical structures and antimicrobial activity spectra.

A different approach, based on application of a neutrophil elastase-specific inhibitor (NEI) to prevent the maturation of cathelicidin peptides in a porcine skin wound model (Cole *et al.*, 2001), has convincingly delineated the contribution of protegrins in the antimicrobial defenses of pigs. Lack of mature protegrins under these conditions leads to inefficient clearance of bacteria from the wound, and addition of exogenous protegrin or excess elastase to NEI-inhibited wound fluid restores the antimicrobial activity of the fluid (Cole *et al.*, 2001). The effectiveness of PG-1 in preventing bacterial colonization in porcine skin wounds is further indicated by the marked reduction in the colony forming units after exogenous application of PG-1 to porcine skin wounds infected with *Pseudomonas aeruginosa* (Ceccarelli *et al.*, 2001).

Clear evidence for host defense capacity of SMAP-29 has come from the demonstration that intratracheal administration of this ovine peptide in a lamb pneumonia model induced by *Mannheimia haemolytica* reduces the bacterial concentration in bronchoalveolar fluid and in consolidated pulmonary tissues, as well as the severity of the lesions in the lungs (Brogden *et al.*, 2001).

The potent and broad spectrum antimicrobial activity of SMAP-29 and protegrins and their ability to protect from infection *in vivo* have prompted a conspicuous number of studies aimed to assess their potential clinical development as novel anti-infective agents (Zanetti *et al.*, 2002).

Effects on host cells (PR-39, BMAP-28)

Both the porcine PR-39 and the bovine BMAP-28 have been characterized for biological activities on mammalian cells (Table 3). PR-39 is a peculiar peptide and may contribute to the innate defenses of pigs with multiple activities. It belongs to the structural class of peptides that display tandemly repeated proline motifs in their sequences and have a non-lytic mechanism of antimicrobial activity (Gennaro *et al.*, 2002). No visible membrane damage is detected in killed bacteria following treatment with PR-39 (Shi *et al.*, 1996a). Instead, PR-39 causes inhibition of bacterial protein and DNA synthesis (Boman *et al.*, 1993). These events may be mediated by specific recognition of intracellular target(s), as indirectly suggested by the ability of a peptide corresponding to the N-terminal 1–15 sequence of PR-39 to rapidly enter mammalian cells without permeabilizing the plasma membrane and to bind cytosolic proteins containing Src homology 3 (SH3) domains (Chan and Gallo, 1998). Indeed, PR-39 shows a wide range of effects on mammalian cells, suggesting its participation in various biological processes. PR-39 is chemotactic for neutrophils at 0.5–2 μ M (Huang *et al.*, 1997), impairs the production of reactive oxygen species via inhibition of the phagocyte NADPH oxidase complex (Shi *et al.*, 1996b) and inhibits LPS-induced apoptosis of macrophages (Ramanathan *et al.*, 2003), suggesting that it may contribute to the regulation of inflammation. In addition, PR-39 acts as a signal to induce the synthesis of syndecan-1 and -4 in cultured mesenchymal cells (Gallo *et al.*, 1994). Members of the syndecan family of cell surface heparan sulfate proteoglycans are involved in wound healing (Bernfield *et al.*, 1992). In this respect, PR-39 is thought to function as a signaling molecule in wound repair. Furthermore, this peptide has been described as a putative regulator of angiogenesis and as an inhibitor of the ubiquitin-proteasome pathway-mediated degradation of $\text{I}\kappa\text{B}\alpha$ (Li *et al.*, 2000; Gao *et al.*, 2000), thereby affecting the expression of genes controlled by NF- κ B, a transcription factor with pleiotropic functions in immunity.

Most of these effects may be connected with the ability of PR-39 to enter cells and to bind SH3 domain-containing proteins. The biological relevance of these findings obviously relates to the pig and possibly to bovids, where structurally similar proline-rich peptides have been identified. The presence of related peptides in other mammals, including humans, has not been demonstrated. However, there could be a therapeutic interest in the multiple activities of PR-39 concerning, for example, the potential for treatment of diseases such as ischemia-reperfusion injury (Korthuis *et al.*, 2000).

The bovine BMAP-28 is an α -helical cathelicidin peptide of 27 residues with an amidated C-terminus. This peptide may contribute to host defense by direct antimicrobial effects and by modulating the inflammatory/immune response. In addition to killing a wide range of bacteria and fungi *in vitro* at the low sub-micromolar range of peptide concentrations (Skerlavaj *et al.*, 1996), BMAP-28 is toxic to cultured blood cells, causing cell membrane permeabilization and apoptosis in a variable proportion of the cells, with higher toxicity to hematopoietic tumor cell lines than normal leukocytes and to activated than resting human lymphocytes (Risso *et al.*, 1998). The

apoptotic effect likely depends on peptide-mediated induction of the mitochondrial permeability transition pore (MPTP), as suggested by the ability of BMAP-28 to target mitochondrial membranes and to induce cyclosporine-inhibited decreases in the inner mitochondrial membrane potential and release of cytochrome *c* (Risso *et al.*, 2002). Although the *in vivo* relevance of these effects has not been established, the high susceptibility of proliferating lymphocytes suggests that this peptide may be implicated in the resolution of inflammation and could contribute via this mechanism to prevent excessive tissue injury caused by release of enzymes and other toxic cellular components.

Conclusions and future trends

The cathelicidin family was identified over a decade ago based on the presence of a conserved cathelin domain in every family member. The importance of peptides belonging to this family has since gained increased recognition as their role, both as endogenous antibiotics and as effector molecules of the innate immune system, has been defined. In-depth studies of the human cathelicidin suggest wide expression of this gene in human tissues that are involved in early protection against infection, and a significant contribution of LL-37 to host defense is indicated by increased susceptibility to infection in disease-associated or experimentally-induced peptide deficiencies. The potency and effectiveness of both human LL-37 and non-human cathelicidin peptides is demonstrated by their capacity to protect from infection after exogenous administration or by overexpression in animal models. Furthermore, their ability to interact and synergize *in vitro* to enhance the antimicrobial effects in experiments reflecting *in vivo* conditions has given insight into the full potential of the complex mixture of substances found at infection sites, strongly suggesting that the combined effort of different peptides greatly increases the global antibiotic action. Finally, the capacity of LL-37 to modulate or elicit various responses connected with host defense, such as chemotaxis of inflammatory and immune cells, activation of early defense cells and promotion of wound healing, indicates a high level of integration of this effector molecule with other innate immune mechanisms.

Despite substantial progress in our knowledge of the function of the human cathelicidin, several important issues remain to be clarified. Our understanding of the multiple activities of LL-37 unrelated to direct microbial killing is still incomplete and further *in vitro* and *in vivo* studies are needed to determine how these effects globally impact host processes. It is important to dissect the mechanisms that control the tissue-specific expression of the hCAP18/LL-37 gene, also in view of developing novel therapeutic strategies intended to potentiate endogenous production of this molecule. It is evident that hCAP18 is released as a two-domain protein and is cleaved to give two distinct molecules, both of which have been detected in physiological settings. To fully understand the functional potential of the human cathelicidin gene, we need a precise understanding of the *in vivo* role of the cathelin domain, which is yet unclear.

Finally, a large body of data indicates that both the human and non-human cathelicidin peptides are a

promising source of lead compounds for the development of a novel class of anti-infective drugs. The rapid emergence of bacterial strains that are multiply resistant to conventional antibiotics urges an intensification of preclinical and clinical studies of these peptides in order to address what appears to be a major current threat to human health.

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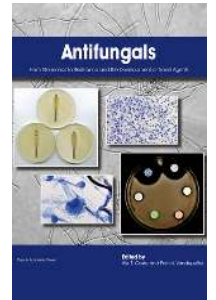
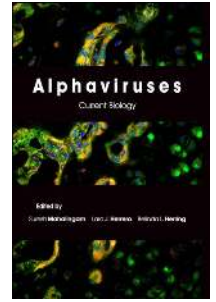
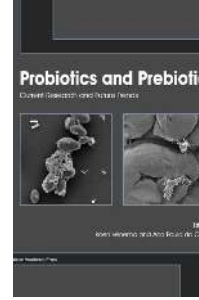
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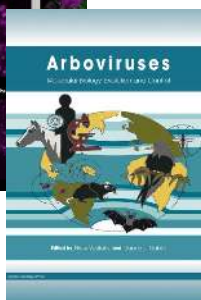
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