

# Antimicrobial Peptides: Cooperative Approaches to Protection

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**Abstract:** Reports of cationic antimicrobial peptides (CAPs) have become standard fare in research literature. But with several hundred peptides described to date, the investigator who tries to navigate the proposed models of their activity is only treated to a generous serving of incongruencies. Rather than acting in isolation as antimicrobial molecules, CAPs also may synergize with other molecules of innate immunity and modulate both innate and adaptive immune systems, thus providing a link between the various mechanisms that result in host protection.

**Key Words:** antibiotic, biodiversity, cationic antimicrobial peptide (CAP), fish, genomics, microbicidal, synergy.

## DEFINITIONS AND SCOPE

For the purposes of this review cationic antimicrobial peptides (CAPs) are defined as short, positively-charged peptides in which hydrophilic and hydrophobic residues are spatially well-separated. While this definition may appear broad, it reflects the latest trend in the field. Originally, these molecules were described as “short lytic peptides” but it soon became apparent that not all peptides were lytic. Hence, the descriptions “antimicrobial peptides” or “cationic antimicrobial peptides,” which have been widely used since then. Recently, Hancock has proposed that these molecules be renamed “host defence peptides” in recognition of the fact that several peptides which appear to offer the host protection from infections do not appear to have direct inhibitory activity against bacteria under *in vivo* conditions [1].

Primary translation products as well as those resulting from secondary processing (post-translational modifications or cleavage from larger molecules such as histones [2-4], hemocyanin [5], neuropeptide precursors [6], ribosomal proteins [7], and RNases [8]) are considered in this review. It is becoming apparent that insight into the role of peptides in the host can be gained not only by examining peptide activities and the regulatory controls that govern the expression of peptide genes, but also by examining the impact of post-translational processing and cellular context.

## OCCURRENCE AND PATTERNS OF EXPRESSION

CAPs occur wherever there is life. Bacterial species use them to combat against each other, and complex eukaryotic organisms utilise them to protect themselves from local infections. Generally, the more evolutionarily sophisticated the organism, the less reliant it is on innate immune defenses including CAPs, and the more dependent it is on the adaptive immune system. Having said that, humans still have a rich repertoire of peptides available to them both at

the site of infection and systemically, involving both constitutive and infection-induced expression (see [9,10]). The impact of CAPs in larval or developmentally immature animals that do not possess a functioning antibody-based immune system is particularly important. Transcripts for pleurocidin and hepcidin have been detected as early as five days posthatch in larval winter flounder [11,12] and, in both cases, different genes are expressed at different stages of development. The synthesis of enteric defensins has even been detected in human fetal intestine at 13.5 weeks of gestation [13].

Marine organisms rely heavily on their innate defenses and produce an amazing diversity of antimicrobial compounds in their efforts to maintain beneficial symbionts as well as fight the various harmful microbes in the surrounding aqueous environment. CAPs have recently been shown to be encoded by multigene families in mollusks, crustaceans and teleost fish (for review, see [14]). By examining the antimicrobial activities of the CAPs encoded by the different family members, either alone or in combination, and their expression and/or co-expression in various tissues, we can begin to understand how these peptides act to kill microbes and protect the host. Our own work on the expression of two families of CAPs in winter flounder [12,15] suggests intricate spatial (tissue-specific) and temporal (developmental stage-specific) patterns of peptide expression. In addition, CAP expression has been demonstrated in circulating fish immune cells that can migrate to sites of infection and exert their effects there [16,17].

Not only are many CAPs present at different sites and at different stages of development, but they often occur together with each other and with other innate defense effectors [18], and the expression of some peptides can be induced by pathogen challenge. Synergistic activities have been demonstrated among lysozyme, histone H1 and pleurocidin [19], and between defensins and cathelicidins [20].

While the diversity of species in which CAPs are encountered clearly indicates that they are ubiquitous in nature, the range of tissues and expression patterns within a

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single organism seems to indicate a complex role beyond the ability to directly damage bacteria.

### GENETIC ORGANIZATION, EVOLUTION AND REGULATION OF GENE EXPRESSION

CAPs known to be encoded by multigene families include cathelicidins [21], cecropin [22], apidaecins [23], dermaseptins [24], defensins [25], hepcidins [12] and pleurocidins [11]. In two cases, pleurocidins [15] and defensins [26,27], the genes encoding different family members are conserved both in structure and sequence and are organized in tandem clusters, indicative of evolution by gene shuffling, duplications and horizontal transfer [28]. Although the signal sequence and propeptide are extremely well-conserved, the exons encoding the mature peptide show significant variability, in keeping with the need of innate defences to adapt to different pathogens or perform specialized functions [27,29]. The number of non-synonymous substitutions is higher than the number of synonymous substitutions within the portion of the gene encoding the mature peptide, a feature consistent with positive selection pressure [27]. Furthermore, this selection appears to have favoured substitutions resulting in altered charge rather than polarities and volumes [29]. The high degree of sequence similarity between different gene family members, including within the introns, indicates the relatively recent expansion of this family. In addition, the presence of clearly recognizable pseudogenes within these clusters indicates that the genes are evolving rapidly and that they have only recently degenerated into inactive pseudogenes. It is interesting to note that many other genes involved in vertebrate host-defence have arisen by gene duplication and been subject to positive selection e.g. immunoglobulin, T cell receptor and major histocompatibility complex genes [30].

Control of expression of the various gene family members under different physiological conditions such as developmental stage, tissue-specific environment or during a disease episode [12,15,31] by different transcription factors will result in a cocktail of CAPs effective against a variety of pathogens and exerting different immunomodulatory effects on the host. Some defensins are induced by proinflammatory cytokines [32] and binding sites for transcription factors involved in expression of immune-related genes have been found upstream of several kinds of CAPs. For example, C/EBP and NF-IL6 motifs [33] have been found upstream of cathelicidin genes, AP2 and NF/IL6 motifs upstream of defensins [13], and NF/IL6, activator protein AP1, -interferon and OCT1 motifs upstream of pleurocidin genes [15]. Consensus binding sites for NF- $\kappa$ B have been found upstream of several genes encoding CAPs [34,35] and NF- $\kappa$ B is required for pathogen-stimulated transcription of the human beta defensin 2 gene [36]. Inhibition of CAP gene expression by glucocorticoids, known suppressors of immunity, is achieved through the induction of I $\kappa$ B expression, which binds to NF- $\kappa$ B and prevents its translocation to the nucleus and subsequent induction of gene expression [37].

The dependence of peptide expression on the presence of specific bacteria or their products is a particularly good

example of the tight controls exerted on the expression of some peptides. For example, the presence of the flagella filament protein FliC of *Salmonella enteritidis* has been shown to induce the expression of hBD-2 mRNA in Caco-2 cells [38,39], while *Helicobacter pylori* containing the cag pathogenicity island has been shown to induce the expression of the same antimicrobial peptide in the MKN45 human gastric cell line [36, 40]. In both cases, the induction was mediated by NF- $\kappa$ B. Interestingly, it has recently been shown that *Salmonella enterica* serovar Typhimurium decreases the expression of alpha defensins in Paneth cells [41] thus gaining a survival advantage in this milieu. The phoP regulon as well as the type III secretion system located on the *Salmonella* pathogenicity island 1 were required for this activity. This further illustrates the high level of sophistication involved in controlling peptide expression in the course of bacterial infections.

Many CAPs are encoded as preproteins containing an N-terminal signal peptide and an anionic propeptide that is cleaved by specific proteases to yield the mature active peptide. This propeptide can act both as a chaperone to ensure correct folding of the mature CAP sequence and as an inhibitor, preventing activity until cleavage [42,43]. Such proteases have been identified in a number of cases: activation of enteric defensin peptides by matrilysin (matrix metalloproteinase MMP7) [44-47], and activation of human cathelicidin hCAP18 by proteinase 3 [48]. In addition, the motif RXX/RR used by subtilase-like preprotein convertases [49,50] is found upstream of several types of CAPs, including hepcidin [12], and this family of proteases has been shown to mediate processing of neuropeptide precursors into peptides with antibacterial properties [51]. Matrilysin expression in human and mouse epithelial tissues is induced by bacterial exposure [52], particularly flagellin [53]. Thus, control of expression of these processing proteases can have a significant impact on the amounts of active CAP present in specific tissues under different conditions.

Post-translational modifications, such as the formation of disulphide bonds, C-terminal amidation, N-terminal pyroglutamic acid formation and, less frequently, glycosylation play an important role in the stability and activity of some CAPs. In addition, multiple isoforms of a peptide can be derived by amino-terminal truncation, in the hepcidins for example [54]. This confirms that the particular suite of active peptides in a tissue may be dictated at the level of post-translational modification.

### IN VITRO STUDIES OF ACTIVITIES AGAINST BACTERIA

Early research on antimicrobial peptides focused on their mechanisms of antimicrobial action and this has become a more complex field as the diversity of known CAPs has increased. Naturally-occurring CAPs were originally described to form a limited range of structures: (A)  $\beta$ -sheets stabilized by two to three disulphide bridges, (B) amphipathic  $\alpha$ -helices formed upon membrane contact, (C) extended structures (again formed upon membrane contact) that can be rich in tryptophan, proline and/or histidine, and (D) loops formed by a disulphide bridge. In terms of the separation of hydrophilic and hydrophobic residues,

amphipathic structures comprising a hydrophilic, positively charged face and a hydrophobic face have been known for quite some time, and a “double wing” structure with a hydrophobic centre and two areas of positive charge flanking it has been described more recently [55,56]. However, it is now known that the spectrum of structures includes other variants with at least eight sub-classes of  $\alpha$ -sheet peptides having been reported in plants alone [57]. Some CAPs from those classes have amidated C-termini, which appears to improve their antimicrobial activity. In addition, a range of unusual synthetic structures have been constructed or are being considered with the goal of enhancing their therapeutic potential. These include cyclic D-L- $\alpha$ -peptides [58], D-enantiomers (to see if turn of the helix affects activity) [59], retropeptides (to see if residues in reverse order and hence the direction of peptide bond affect activity) [59], or even peptidomimetics. This wealth of structures inevitably leads to a variety of ways in which peptides interact with their targets or potential targets.

Generally, an attempt was made to fit these structures into models of peptide interactions with bacterial membranes. The first (outermost) bacterial membrane encountered by peptides acting on Gram-negative bacteria is the lipopolysaccharide (LPS)-containing outer membrane. While many peptides have been shown to bind bacterial LPS [60] and at least two models for self-promoted uptake of the peptide across the outer membrane have been proposed [61], there are controversial reports regarding the correlation between the extent of LPS binding and peptide efficacy. Previous work in which we were involved as well as our recent data on pleurocidins suggest that the extent of LPS binding ability is neither directly nor inversely proportional to peptide activity [62]. Correlation between intrinsic resistance to peptides and LPS binding ability has, however, been suggested in the case of protegrins [63]. In addition, binding of the endotoxin portion of LPS is of consequence to the host, as will be discussed below.

Once the outer membrane has been breached, the peptide activity is proposed to be dependent on its ability to interact with bacterial cytoplasmic membranes. It seems that there are at least three variables which will influence the mode of peptide interaction with the membrane: (A) the sequence of the peptide itself, (B) the specific bacterial membrane (shown through studies of binding of peptides to membranes of different compositions, [64]), and (C) the concentration of the peptide at the membrane [64,65].

By studying the formation of channels in lipid bilayers, depolarization of bacterial membranes using the fluorescent dye diSC<sub>3</sub>(5), uptake of fluorescently labeled peptides by bacterial cells, binding and permeabilization of model membranes (lipid monolayer, liposomes) by peptides, or various combinations and variations of the above, several models of peptide action on the membrane have been proposed. In one scenario, referred to as the barrel-stave model, channels are formed across the cytoplasmic membrane leading to the leakage of cell content and bacterial death [66]. In another scenario, known as the carpet model, general destabilization and collapse of the membrane occurs upon interaction with peptides [67]. Finally there is evidence

that in some cases peptides can enter the cell ultimately leaving the membrane intact [62,68,69].

An entirely new field of possibilities opens when the potential intracellular activities of the peptides are considered. It has been shown in gel-retardation assays, e.g. [70], that peptides will bind DNA, rather indiscriminately. Protegrin PR-39 passes through membranes and kills bacteria by stopping DNA and protein synthesis [71]. On the opposite side of the spectrum, Otvos has shown through competitive binding techniques an interaction with specific molecules (DnaK) and inhibition of specific activities [68]. While this may well be the case, it is hard to imagine that short, highly-charged peptides would not bind nucleic acids. Indeed, with some peptides (butorin) being derived from histones [72] we can safely assume that some indiscriminate binding would take place in most instances. Inhibition of intracellular processes by sublethal concentrations of two insect peptides, cecropin A and apidaecin, has been demonstrated using microarrays [73] and assays of protein synthesis [74]. An altogether different possibility, in which peptides would induce bacterial autolysis, was proposed by Ginsburg and largely ignored by the mainstream CAP community. Dr. Ginsburg in fact drafted and published a letter questioning the lack of references to his work [75].

Based on data available to date it appears that the variables listed above could potentially alter the course of peptide encounter with a bacterial membrane and that each variable is subject to multiple independent events. For instance, the folded structure of the peptide could change in response to a reducing, oxidizing, amphipathic or aqueous environment. Moreover, bacterial mutants producing altered phospholipids could arise [76,77], decreased peptide expression could be caused by invading bacteria [78], and peptide concentration at the membrane could change in the presence of protein-degrading enzymes [79] or as a result of variable extents of LPS binding [80]. The mode of peptide action on bacteria would then likely depend on the transitory state of these variables, as discussed above.

In the light of the argument presented above, we contend that each peptide can adapt to playing many roles aimed at killing bacteria depending on the environment, underscoring the cooperative nature of peptide action.

## IN VITRO STUDIES OF ACTIVITIES IN HOST

CAPs have significant impacts on signaling and chemotaxis of immune cells and are able to exert their effects at concentrations well below those necessary for microbial killing [81]. Human neutrophil-derived  $\alpha$ -defensins enhance phagocytosis by mouse macrophages, promote activation and degranulation of mast cells, increase IL-8 transcription and production leading to recruitment of neutrophils at sites of inflammation, increase TNF- $\alpha$  and IL-1 proinflammatory cytokines, decrease production of the anti-inflammatory cytokine IL-10 by monocytes and regulate complement activation (see review by Yang *et al.* [82]). Some cathelicidins, also present in neutrophil granules, are chemotactic for neutrophils, monocytes, T cells and mast cells [83,84] and can cause degranulation of the latter [85] and subsequent migration of dendritic cells. Defensins are potent inhibitors of phospholipid/Ca<sup>2+</sup> protein kinase C [86],

which participates in membrane-based signal transduction pathways. This activity has important implications for neutrophils and other lymphocytes.

As mentioned above, CAPs have been shown to bind LPS. One consequence of LPS binding is that the endotoxin is prevented from binding the LPS-binding protein (LBP) [87] thus making LPS unavailable for interaction of the LPS-LBP complex to its receptor, CD14. CD14 normally activates a TLR4-mediated pathway that leads to the secretion of pro-inflammatory cytokines (such as TNF- $\alpha$ ) by macrophages. This results in reduced inflammatory response, and prevention of sepsis [88,89].

Interestingly, however, it appears that modulation of host immune responses and other effects exerted by CAPs can also occur directly. Receptors have been implicated in the chemotactic activity of antimicrobial peptides, with CCR6 being the receptor for human beta defensin 2 [90], FPRL1 being the receptor of LL-37 [91,92], and TLR4 being the receptor for MBD2 [93].

Neutrophils are known to contain many antimicrobial molecules in their granules (including myeloperoxidase, bactericidal permeability-increasing protein, azurocidin, defensins, cathelicidin, elastase, cathepsins and proteinase-3), and the composition of the granules changes as the neutrophils mature [94]. The induction of IL-8 in human lung epithelial cells by neutrophil defensins is finely balanced by their interaction with elastase and cathepsin G, although their antibacterial activity is unaffected by them [95]. Once again, the subcellular context in which a given CAP occurs dictates its ability to defend against infection.

CAPs are able to provide a link to the adaptive immune system by a variety of mechanisms, including stimulating production of immune mediators and signaling molecules [90,94]. Neutrophil CAPs are chemotactic for human monocytes, T cells and immature dendritic cells and can cause the differentiation of monocytes into macrophages and bring about the maturation of dendritic cells [81,96]. Eosinophils also contain CAPs in their granules one of which, eosinophil-derived neurotoxin (EDN), is chemotactic for immature and mature dendritic cells but not dendritic cell precursors [8]. Defensins have been shown to act as *in vivo* adjuvants, enhancing antigen-specific humoral and cellular immune responses [97]. They can also enhance apoptosis in macrophage cell lines and activated lymphocytes, thus removing infected cells from the host [98, 99]. Defensins can inhibit the production of immunosuppressants such as adrenal glucocorticoids [100] by blocking the adrenocorticotropin receptor [101], thereby enhancing the adaptive immune response.

Although the precise role of CAPs in the immune system of fish and invertebrates has not been determined, their presence in hemocytes of shrimp and mussels [102,103] and circulating eosinophilic granule cells of fish [16, 17] indicates that they may perform a similar role in innate immunity as the neutrophil-derived CAPs of mammals. In addition, the induction of fish hepcidin by infection with a bacterial pathogen [12] and the presence of motifs involved in regulation of immune system upstream of pleurocidin genes [15] may indicate roles beyond simply bacterial

killing. For example, hepcidin performs an additional role in iron homeostasis [104], and the presence of NF-IL6, AP-1, -IFN, OCT1 and GAAA motifs upstream of pleurocidin genes suggests the involvement of these CAPs in modulation of host defense.

Another important role of CAPs is in wound-healing and tissue repair through their inhibition of proteases involved in tissue degradation and by promoting angiogenesis. PR-39, a member of the cathelicidin family, can modulate production of syndecans in wounds [105]. CRAMP-deficient mice fail to vascularize post-injury, and indeed CRAMP or its human version LL-37 are required for angiogenesis [106]. The involvement of CAPs in the various aspects of healing and wound repair is exemplified by the ability of human neutrophil defensins to induce A549 lung epithelial cell proliferation via an EGF receptor-independent MAP kinase signaling pathway [107] and their ability to enhance lung epithelial wound closure in NCI-H292 cell cultures via an EGF receptor-dependent pathway [108].

Overall, it appears that the immunomodulatory as well as the antimicrobial activity of a given CAP within the host may be context-dependent and vary depending on which cells or tissues are involved, whether cells displaying appropriate receptors are present, and on the local concentration of CAPs in tissues e.g. low concentrations normally found in epithelial cells (immune watchdog) vs high concentrations when induced by infection (signaling and chemotaxis) [89].

## **IN VIVO STUDIES OF ACTIVITIES IN HOST**

Most *in vivo* studies have been aimed at testing whether exogenous CAPs, added as pre-made proteins or as transgenes, would protect the animal in a given model from infection and/or sepsis, or testing whether CAPs would aid in wound healing. Variables such as the method of administration and peptide dose have been tested for different peptides in a number of animal model systems against various outcomes. The most advanced and systematic studies have been those that led to clinical trials (for review, see [109]). Overall, many peptides have been shown to have protective, anti-sepsis, or wound-healing properties when tested in animal and clinical models, although in most cases the range of studied effects was usually limited to the single outcome of interest.

In contrast, relatively few studies have been aimed at elucidating the effects of ablating peptides in the native organism environments [110,111]. The loss of mBD-1 in the Moser mouse study [111] resulted in delayed clearance of *Haemophilus influenzae* from lung, while the loss of the human version of the peptide, hBD-1, tested by Goldman in a human bronchial xenograft model [110], resulted in the loss of some antimicrobial activity of the airway surface fluid. In addition, CRAMP-deficient mice have been shown to be more susceptible to Group A streptococcus infections [112].

The authors believe that further characterization of knockout animals such as the mBD-1 deficient mouse [110], and gain-of-function animals such as catfish bearing the cecropin transgene [113] will be the most revealing experiments for determining the scope of CAP effects in the

host because a comprehensive picture of the whole biological system can be obtained.

## CO-OPERATIVE ACTIONS IN HOST PROTECTION

Even a well-defined, specific property of a peptide can have multiple consequences. For example, as described above, CAPs bind bacterial LPS to various extents. This in turn will have a dual role in direct killing of bacteria (crossing outer membranes) and in modulating the inflammatory response in the host. In fact, this observation alone supports our contention that peptides coordinate various means of protection against infections.

Perhaps the best example in support of our argument is the cathelicidin LL-37. This peptide has direct antimicrobial effects, many host immunomodulatory effects and wound healing effects as described above, it is expressed in several tissues as well as being secreted to the airway, it is produced by humans, and it is part of the species-spanning cathelicidin family with a very close related peptide, CRAMP, present in mice. We contend that the reason why only LL-37 has received so much research attention (71 papers on Medline as of Nov 4, 2003) and has been shown to have so many activities is not because other peptides do not have them, but because LL-37 is human, non-disulphide bonded, and relatively easy and inexpensive to synthesize in sufficient quantities for experimental studies.

A dogma in studying important components of our immune system is that their malfunctioning would result in a clinically recognizable disease. For this reason researchers associated with Dr. Hans Boman, one of the founders of the antimicrobial peptide field, have developed interest in the "Kostmann patients" [114] from the Överkalix parish in northern Sweden. While the periodontal disease encountered in Kostmann patients may indeed be related to the absence of the antimicrobial peptide LL-37 in the saliva, some would consider this to be a disappointingly obscure disorder given the prevalence and proposed importance of antimicrobial peptides.

However, as is evident from the developments presented, there may be a great redundancy in the peptide repertoire of each organism, not altogether unexpected given the proposed importance of the peptides, and this may in fact account for the lack of significant disease phenotypes. This redundancy is reflected not only by multiple peptides performing the same function but also by a single peptide performing a variety of functions.

## CONCLUDING REMARKS

Review of the current literature suggests that each CAP may adapt to playing many roles in protecting the host, depending on the specific context in which it occurs. The context can be a specific tissue, a specific time point in the development of an organism, a specific time point in the course of an infection, the presence of receptors, the presence of processing or protein modification enzymes, or the presence of other messenger or effector molecules, including other CAPs. Antimicrobial peptides of innate immunity thus play a key role as immunity bodyguards, arranging and deploying protective measures against infection and

orchestrating our various defense strategies against invading pathogens.

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