Chicken antimicrobial peptides: biological functions and possible applications

Albert van Dijk

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Antimicrobiële peptiden van de kip:
biologische functies en mogelijke toepassingen

(met een samenvatting in het Nederlands)

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Chapter 1

Introduction

Food safety and feed conversion ratio are of great importance in the production of foods of animal origin. The increased population sizes of farm animals, clustering of food animal production units and the intensive global transport of live animals and animal products facilitate the spread of zoonotic pathogens. Moreover, farm animal health is severely affected by gastro-intestinal infections that occur frequently under large sized farming conditions. Thus, strategies aimed to improve farm animal health may have impact on both animal and public health. In addition, improvement of intestinal health will lead to lower costs since animals with impaired intestinal health have a reduced appetite and/or diarrhea, resulting in a reduced nutrient uptake and, therefore, negatively affect the feed conversion ratio. Additionally, the following immune response may trigger muscle wasting by increasing catabolism to fulfill the excessive need for amino acids necessary to produce immune response effectors, such as cytokines and antibodies (3, 25).

To promote growth, farm animal feed has been supplemented with subtherapeutical doses of antibiotics, so-called growth-promoting antibiotics (GPAs), since the mid 1940's (5). During the first three decades of their use in feed, mean increases of body weight ≥8% for penicillin and tetracyclins were reported (9). However, nowadays the magnitude of these effects is marginal, due to selective breeding, improved feed formulations and improved hygienic conditions in animal husbandry. Furthermore, the mechanisms involved in GPA-mediated enhanced growth are still under debate. Foremost, GPAs are thought to inhibit sub-clinical infections (8), thereby preventing illness and thus maintaining the feed conversion ratio. Other proposed modes of action are: suppression of carbohydrates and fat malabsorption; improved nutrient utilization by inhibiting the growth of normal GI tract flora; reduction of growth-depressing microbial metabolites, such as ammonia, aromatic phenols and bile degradation products, and enhanced nutrient uptake through the thinner intestinal wall in GPA-fed animals (8).

A more important point of concern was the relative ease at which microorganisms had demonstrated to be able to transmit antibiotic resistance genes via the exchange of transposons or plasmids and the possible transmission of this resistance to human pathogens. It became such a major concern that it resulted in a total European ban in 2006 on the use of antibiotics as a feed additive to promote growth (22). A consequence of the ban is that pathogens, suppressed by the use of GPAs, can now reemerge. Because GPAs were almost entirely aimed at gram-positive bacteria (28), it can be expected that gram-positive bacteria, such as *Clostridium perfringens* will increasingly become a problem in the poultry sector.

Regardless of a possible global ban on the use of GPAs, the many disadvantages involved with their use make it mandatory to search for alternative strategies to increase food safety and to promote growth in food animals. Not surprisingly, the body's natural defense mechanisms is now one of the focuses, in particular those of the digestive tract. The gastrointestinal (GI) tract comprises the largest mucosal surface in the body, and is in direct contact with the external

environment. A healthy GI tract harbors a wide variety of residential "non-pathogenic" and potential pathogenic microorganisms displaying complex symbiotic and competitive interactions (26). A disturbance in this balance could facilitate outgrowth of pathogenic microbiota, which will depress animal growth by competing with the host for nutrients and by producing toxic metabolites resulting in increased turnover of gut mucosa (26). Therefore, several strategies aim at shifting this delicate balance in the favor of beneficial microbiota by stimulation and/or activating growth of these subpopulations of intestinal microbiota via prebiotics, probiotics, organic acids, enzymes or herbs (26).

Research on host resistance against infectious microorganisms and the adaptation of these microorganisms to treatments, has been mainly focussed on the adaptive immune response, which is highly specific and vital for the final clearance of invading pathogens. However, a 48 to 72 hour gap exists between the onset of infection and a sufficient clonal expansion of B and T cells required for an adequate adaptive immune response. In addition to their functions in immunological barrier homeostasis at interfaces between body and environment, innate immune effectors enable the host to bridge this temporal gap in the host immune defense. The innate immune system is the phylogenetically oldest mechanism of defense and comprises: ***i.*** physical and chemical barriers, such as epithelia, mucus and antimicrobial substances (1, 4, 10, 12, 16, 17), ***ii.*** blood proteins, including complement and other mediators of inflammation (14, 19, 23), ***iii.*** phagocytes and natural killer cells (15, 24) and ***iv.*** cytokines that regulate and coordinate the activities of innate immune cells (1, 2, 13).

In addition to their role in local chemical barriers, antimicrobial peptide (AMP) expression may be locally up-regulated or induced via pathogen-associated molecular pattern (PAMP) recognizing receptors. Importantly, several antimicrobial peptides have demonstrated to link innate to adaptive immunity by recruitment of adaptive immune effector cells via chemotaxis (29). In mammals, the importance of antimicrobial peptides has been well demonstrated for skin-related innate immune defence (18) and evidence is mounting that disturbance of the antimicrobial peptide expression equilibrium is linked to the persistence of chronic disease, including atopic dermatitis, psoriasis, Crohn's disease and cystic fibrosis (7, 11, 20, 21, 27).

In stark contrast, little is known about the repertoire and biological functions of avian antimicrobial peptides. Avian tissue-specific AMP expression and the regulation thereof may largely reflect that found for mammalian species. Hence, modulation of antimicrobial peptide expression, for instance by dietary stimulation, could be an alternative strategy to improve health and welfare of animals as well as promote growth.

When I started my thesis work only a few antimicrobial peptides had been found in chicken and turkey heterophils (6, 10, 30). Therefore, we endeavored to identify novel chicken antimicrobial peptides and to examine their biological roles. **Chapter 2** gives an overview of the current status of knowledge regarding avian antimicrobial peptides with the emphasis on defensins and chicken cathelicidin

CMAP27. In **Chapter 3** the localization, broad-spectrum antimicrobial activity and mode(s) of action of one of the β-defensins we discovered by *in silico analysis*, Gallinacin-6 (Gal-6) are reported. In **Chapter 4** the discovery of a novel chicken cathelicidin-like peptide is described. In **Chapter 5**, the localization of CMAP27 in peripheral blood cells and digestive tract tissues in *Salmonella*-challenged and healthy animals is reported. In **Chapter 6**, the putative roles of CMAP27 in skin-related innate immunity against opportunistic skin pathogens and feather-degrading microorganims, the possible role of the uropygial gland therein, and the involvement of CMAP27 in wound-repair are presented. **Chapter 7** provides a summary of the results described in this thesis and its discussion.

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Chapter 2

The Role of Antimicrobial Peptides in Birds

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1. INTRODUCTION

Antimicrobial peptides are found throughout the animal, plant, fungi, and prokaryote kingdoms (6, 25, 66, 146, 149, 200, 226, 258). They are defined as peptides having less than 100 amino acid (aa) residues, enriched in hydrophobic and cationic aa residues, although anionic and neutral antimicrobial peptides do occur, with broad spectrum antimicrobial activity have been described (78). During evolution, their synthesis, localization and antimicrobial activity spectrum have been adapted constantly to benefit their host. To compete for nutrients, many bacteria produce small antibacterial peptides, so-called bacteriocins, that inhibit the growth of other microorganisms in the same habitat but do not interfere with their own growth (6). Plant defensins, on the other hand, have a high fungicidal activity that reflect the relative importance of fungal pathogens in the plant world (19). In mammals, antimicrobial peptides belonging to the defensin and cathelicidin superfamilies are highly represented, and are constitutively or inducibly expressed in blood cells and epithelial cells lining the mucosal surfaces of the respiratory, digestive and urogenital tracts (8, 229, 234, 256). In addition, antibacterial properties have also been attributed to peptides that were previously not known to be immune-related, such as ribosomal (220) and histone-related (55, 104, 166, 178) peptides, the latter of which are an import effectors in skin innate immune defense of fish. In birds, in addition to several β-defensins and cathelicidins, other types of antimicrobial peptides have been found, such as LEAP-2 (132) and AWAK, the latter of which exhibits both anti-protease and broad spectrum antibacterial activity (152, 222). In the following sections the current status of knowledge regarding avian defensins and cathelicidins is described, with the emphasis on localization, putative functions, biosynthesis and regulation, and where necessary, in relation to their mammalian counterparts.

2. AVIAN ANTIMICROBIAL PEPTIDES

2.1. DEFENSINS

Defensins are a family of small cationic peptides with broad spectrum antimicrobial activity against bacteria, fungi, protozoa and enveloped viruses (257). In addition to their direct antimicrobial activities, immunomodulatory properties have also been demonstrated. Defensins can promote adaptive immunity by selective recruitment by chemotaxis of monocytes (217), T lymphocytes (27), immature dendritic cells (249) and mast cells (153) to sites of inflammation. Furthermore, they are able to induce histamine release from peritoneal mast cells (12) and to enhance macrophage phagocytosis (56, 95).

Anti-inflammatory properties have also been attributed to defensins, such as inhibition of formylpeptide receptor mediated chemotaxis of polymorphonuclear leukocytes (75) and the binding of bacterial endotoxins (141). In addition, defensins may enhance wound repair by inducing fibroblast (145) and epithelial cell proliferation (1, 2, 145). In vertebrates, three different defensin subfamilies (α, β and θ) exist, differing in disulfide bridge pairing and positioning of their conserved six cysteine residues, Cys1-Cys6, Cys2-Cys4, Cys3-Cys5 for α-defensins versus Cys1-Cys5, Cys2-Cys4, Cys3-Cys6 for β-defensins (200). θ-defensins are highly similar to α-defensins, but truncated by a stop codon corresponding to the 4th residue on the carboxyl side of the 3rd cysteine of full-length α-defensin and form a 18 amino acid (aa) cyclic molecule by intermolecular disulfide bridge pairing and binary peptide ligation of the excised nonapeptides (118). Functional θ-defensins seem to be exclusively expressed in primate neutrophils and monocytes (215), as they have been found in Old World monkeys, lesser apes and in orangutans , but not in humans (pseudogenes only) or New World primates (29, 151). α-defensins have been isolated from leukocytes of rabbit (199), rodents (42) (rat, guinea pig and hamster) and primates (214) (human and rhesus macaques), but are completely lacking in mouse neutrophils (43). In addition, α-defensins have been found in human monocytes and NK cells (23, 133) and in intestinal Paneth cells located in the crypts of Lieberkühn of humans and mice (170) and, more recently, by sequence analysis in horses (127). The observation that α- or θ-defensins have not been found in phylogenetically much older vertebrates, such as birds and fish, suggests that all defensin subfamilies must have evolved from an ancestral β-defensin gene by duplication and diversification (202).

The β-defensin repertoire varies greatly between species and between individuals within a single species (7-9). For example, *in silico* analysis revealed ~30 putative human β-defensin genes and ~45 β-defensins in mice (192), although some of

these genes may turn out to be pseudogenes, such as the human DEFB109p gene (202). Several β-defensins have been described for avian species, in particular for galliformes. Harwig *et al.* (83) isolated three β-defensin-like peptides (so-called gallinacins) from chicken leukocytes, Gal-1 and its isoform Gal-1α and Gal-2. Five novel bactericidal peptides were purified from avian heterophil granules; two chicken heterophil peptides (CHP-1 and -2) and three turkey heterophil peptides (THP-1,-2 and -3) (48). The CHP-1 amino acid sequence is identical to that of Gal-1 with a C-terminal Trp_{39}-Arg_{39} substitution, whereas CHP-2 is similar to Gal-1α, but lacking the last five C-terminal amino acid residues. The first epithelial avian β-defensins, chicken Gal-3 and its turkey homologue gallopavin-1 (GPV-1), were discovered in tracheal tissue by RT-PCR (264) using primers based on the Gal-1 and THP-1 cDNA sequences published earlier by Brockus *et al.* (18). *In silico* analysis of chicken expressed sequence tags (EST) databases by Lynn *et al.* (130), revealed seven additional chicken β-defensins, Gal-4 to Gal-10. At the same time, Xiao *et al.* (245) discovered 10 novel chicken β-defensins genes using a combined EST (Gal-4 to Gal-12) and chicken genome database search approach, of which several sequences were identical to those described by Lynn *et al.* In addition, two additional chicken β-defensins were found by genomic database mining, designated Gal-11 and -12 (88), of which Gal-11 was found to be identical to an earlier reported gene (Gal-13), but lacking the 29 amino acid residue post-piece.

Due to the overlapping avian β-defensins nomenclature described in this section, for practical purposes, the nomenclature of chicken β-defensin and related sequences from other avian species in the following sections of this chapter is based on the recently proposed update of the avian β-defensin nomenclature by Lynn and colleagues (Table 1) (131).

2.1.1. *Genomic organization.*

At least 14 chicken β-defensin genes are located in a ~86.0 kb single β-defensin cluster on chromosome 3q3.5-q3.7 (131, 245). Chicken β-defensin genes consist of four exons, with the exception of the AvBD12 gene where the last two exons have fused (245) (Fig. 1). Defensins are synthesised as inactive precursors, i.e. prepropeptides, consisting of a short signal peptide, an 'anionic' propiece and mature peptide. The 1st exon corresponds to the 5'UTR region, the 2nd exon encodes the signal peptide and part of the propiece, while the remaining part of the short propiece and majority of the mature peptide are encoded by the 3rd exon. The remaining part of the mature peptide and the 3'UTR region are encoded by the 4th exon (Fig. 1). The chicken 3q3.5-q3.7 β-defensin locus appears to have evolved by a series of gene duplications, followed by substantial divergence of the exon(s) encoding the mature peptide with substantial

"positive selection", i.e. involving mutations that disproportionately favor the selection of charged aa residues. This is further emphasized by the finding of phylogenetically more conserved genes, such as cathepsin B and HARL2754, in close proximity of chicken, mouse and human β-defensin clusters (245) and suggests that vertebrate β-defensins originate from a single ancestral gene.

Table 1. Nomenclature of avian β-defensins and cathelicidins (131).

Designation	Designation	Synonyms/ References	Genbank
Chicken	AvBD1	Gal-1/1α (83); CHP-1 (48)	AAB30584
	AvBD2	Gal-2 (83)	AAB30585
	AvBD3	Gal-3 (264)	Q9DG58
	AvBD4	Gal-7 (130), Gal-4 (245)	AAS99318
	AvBD5	Gal-9 (130), Gal-5 (245)	AAS99320
	AvBD6	Gal-4 (130), Gal-6 (245)	AAS99315
	AvBD7	Gal-5 (130), Gal-7 (245)	AAS99316
	AvBD8	Gal-12 (88), Gal-8 (245)	AAU07922
	AvBD9	Gal-6 (130), Gal-9 (245)	AAS99317
	AvBD10	Gal-8 (130), Gal-10 (245)	AAS99319
	AvBD11	Gal-11 (245)	AAT45551
	AvBD12	Gal-10 (130), Gal-12 (245)	AAS99321
	AvBD13	Gal-13 (245), Gal-11 (88)	AAT48937
	AvBD14	Gal-14 (131)	AM402954
	Cathelicidin-1	(130), Fowlicidin-1 (243)	AAS99323
	CMAP27	(232), Fowlicidin-2 (243)	AAX20012
	Fowlicidin-3	(243)	AAZ42401
Turkey	AvBD1	THP-1 (48)	AAC36053
	AvBD2	THP-2 (48)	AAC36054
	AvBD3	GPV-1 (264)	AAG09213
Mallard duck	AvBD2	Duck β-def. (131)	AAV52799
	AvBD7	Duck β-def.-6-like (131)	ABN50328
King pigeon	AvBD4	King pigeon β-def. (131)	ABI20694
Ostrich	AvBD1	Osp-2 (212)	P85114
	AvBD2	Osp-1 (253)	P85113
	AvBD4	Ostrich gallinacin-4 (131)	ABK40533
	AvBD7	Osp-3 (212)	P85115
	AvBD8	Osp-4 (212)	P85116
King penguin	AvBD103a	Sphe-1 (218)	P83429
	AvBD103b	Sphe-2 (218)	P83430

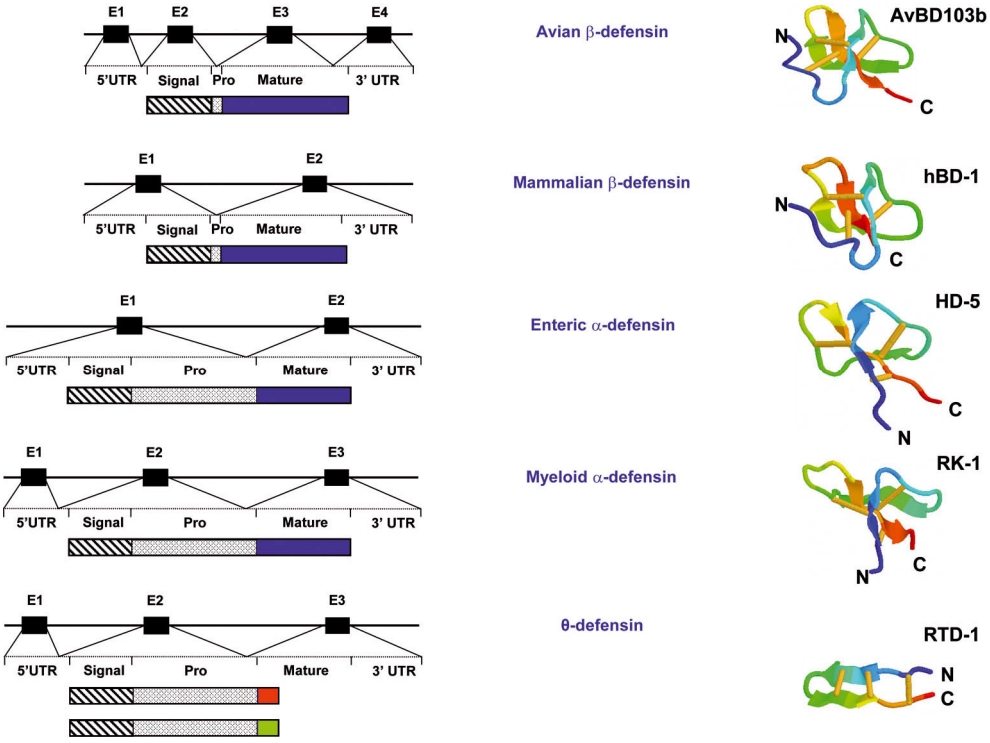

Fig. 1. Genomic organization of avian and mammalian defensins and corresponding mature peptide structures. Differential transcription and subsequent translation of exons (E1 to 4) into the 5' and 3' untranslated regions (UTR) and peptide encoding regions for: signal peptide (diagonal striped bars), propiece (dotted bars) and mature peptide (solid blue bars; red and green bars depict homo- or heterodimers based formation of a single cyclic θ-defensin peptide). Despite differences in genomic organization, 3D-structures of mature avian β-defensins and mammalian α- and β-defensins are very similar: AvBD103b, king penguin avian β-defensin 103b (Sphenicin-2); hBD-1, human β-defensin-1; HD-5, human α-defensin-5; RK-1, rabbit kidney defensin-1; RTD-1, rhesus theta defensin-1.

Single-nucleotide polymorphisms (SNPs) are commonly found in β-defensin genes, and although present only at a low frequency in the coding region, the occurrence of SNPs may seriously affect an individual's predisposition to disease (17), by altering the efficiency of transcription and translation or by generating an altered protein sequence with diminished biological functions (100). SNP analysis of the chicken β-defensin cluster revealed a much higher SNP rate (13.2 SNPs/kb) for the 3.25 kb region containing the AvBD2, -3, -4, -5 and -7 genes (84), than the rate across

the whole chicken genome (5 SNPs/kb) as was previously reported (239). However, all 43 identified SNPs were intronic, with the exception of a nonsynonymous SNP found in the AvBD5 gene that resulted in an amino acid substitution of proline to threonine. In addition to the variability in gene nucleotide sequence, some defensin genes may be completely absent in some individuals. Studies on interpopulation variability of the human α-defensin gene DEFA3 between African, Caucasian, Chinese/Japanese and Spanish subjects, revealed that 10 to 37% of the subjects lacked this gene (7). In contrast to other innate immune genes, several, but not all β-defensin genes show a high degree of polymorphism in gene copy number (90, 124, 125). It has been suggested that a defective β-defensin induction could be due to a low β-defensin copy number (54), which is supported by the observation that a lower hBD2 copy number in the human β-defensin locus predisposes to Crohn's disease (54). Few studies have addressed the disease predisposition of birds in relation to innate immune gene polymorphisms. A recent study addressed the association of avian β-defensin gene polymorphisms in 1 week-old F_1 chickens with their phenotypic immune response to *S. enteriditis* challenge (84). In this study, AvBD2 and -5 gene polymorphisms were moderately associated with caecal and spleen bacterial loads at 1 week post-challenge. The significant associations observed between polymorphisms of the AvBD3 and AvBD7 genes and *S. enteriditis* vaccine antibody response at 21 days, suggest that avian β-defensin genes may facilitate the transition from an innate immune response to an adaptive immune response in newly hatched birds (84). Thus, polymorphisms in avian innate immune genes, encoding signalling pathway components as well as innate immune effectors, can be directly associated with disease resistance. Hence, the allelic selection of genes involved in host immunity by selective breeding may confer an increased innate immune system (71). However, it remains to be elucidated to which extent gene polymorphisms (SNPs) and variations in β-defensin copy number play a role in the resistance of birds to *Salmonella* infections.

2.1.2. *Structural features*.

Deduced primary amino acid sequences for avian mature defensins indicate that, like mammalian β-defensins (167), their peptides consist of 36 aa or more residues with the consensus sequence motif: x_n-**C**-x_{2-4}-G-x_{1-2}-**C**-x_{3-5}-**C**-x_{9-10}-**C**-x_{5-6}-**CC**-x_n. NMR spectroscopy analysis of synthetic king penguin AvBD103b in aqueous solution revealed a 3-dimensional structure most similar to mammalian β-defensins (114) (Fig. 1). The overall fold consisted of a three-stranded β-sheet and an α-helical N-terminus in the structure and contains a hydrophobic patch (Phe^{19}-Pro^{20}-Ile^{22}-Val^{37}-Trp^{38}), shown by comparative structure analysis to be well but not strictly conserved in other

avian defensins. The 10 Arg residues and lack of Glu or Asp residues renders king penguin AvBD103b highly cationic (10+). Some avian β-defensin genes, e.g. AvBD3, -11 and -13, contain a large postpiece, although the AvBD13 nucleotide sequence reported by Higgs *et al.* lacks this postpiece suggesting that there might be strain-specific splice variants or isoform of the AvBD13 gene (88). Comparative analysis of the nucleotide sequences of chicken and turkey AvBD3 (91% identical on a nucleotide level), the latter of which lacks a postpiece, revealed that a two-base insertion just before the chicken AvBD3 original stop codon, causes a frame shift, and an additional 15 bp insertion was responsible for the generation of an anionic postpiece in chicken AvBD3 (264). The AvBD11 postpiece contains a defensin-like motif: x_9-**C**-x_4-G-x-**C**-x_6-**C**-x_7-**C**-x_6-**CC**-x_3, which might be a consequence of gene duplication. Interestingly, of the newly identified human β-defensins mapped to chromosome 6 and 20 (hBD-18 to 21, hBD-23, hBD-25 to 29 and hBD-31), those clustered on chromosome 20 all contained similar long C-terminal tails (192). These C-terminal tails differ substantially in amino acid composition and do not exhibit homology with other sequences (167). It has been suggested that the accumulated negative charges present in some of these large postpieces could function similarly to the anionic charges present in the α-defensin propiece (180). The net anionic charges in the propiece of α-defensins are thought to balance out the cationic net charge of the mature peptide, which might be important for folding and prevention of intracellular binding to membranes (126, 137, 228, 242). The biological function of these large postpieces and their putative role during folding and intracellular trafficking remains to be elucidated.

2.1.3. *Biosynthesis and tissue-specific processing.*

Although mechanisms ruling the synthesis, storage and activation of avian β-defensins are unknown, they can be expected to parallel that of mammalian defensins. Avian β-defensins have been isolated from heterophils of chickens, turkeys and ostrich (47, 83, 212) or were found to be constitutively or inducibly expressed by epithelial cells (264).

Myeloid β-defensins are synthesized in the bone marrow, where in the Golgi apparatus and maturing granules of promyelocytes the prodefensins are processed by as yet unknown proteases and stored into specific granules as mature peptides (65, 227). The abundant mRNA levels of AvBD4 to -7 found in chicken bone marrow, and the absence of significant AvBD4 to -7 expression in leukocyte extracts (245), suggest that, similar to mammalian defensins (251), myeloid avian β-defensin mRNA synthesis is largely absent in mature leukocytes. After biosynthesis and intracellular trafficking through the Golgi apparatus, the ~19 aa signal peptide, that functions to anchor the prodefensin peptide in the endoplasmic reticulum (ER) membrane, is rapidly

proteolytically cleaved to generate a prodefensin peptide with little or no microbicidal activity (186). The large (~40 aa) α-defensin propiece has demonstrated to be essential for subcellular trafficking and sorting of pro-α-defensins into specific secretory granules of polymorphonuclear leukocytes (neutrophils) (126). The fact that β-defensins possess only a relative small propiece suggests that the subcellular trafficking of pro-β-defensins is very different. Non-myeloid biosynthesis of β-defensin has been described to some detail for mammalian epithelial cells (32, 161). In birds, non-myeloid β-defensin expression has been described for epithelial cells (250, 264), but data describing their biosynthesis in these cells and tissues has not yet been reported. Paneth cells are specialized secretory cells located at the base of the crypts of Lieberkühn in some species (170). Paneth cells or related cells have not been identified in avian species (170).

The proteolytic processing of β-defensin proforms is host and tissue-specific due to the local repertoire of proteolytic enzymes and inhibitors and may result in multiple forms with different properties. For instance, epididymis-specific β-defensin-like peptides (HE2, ESP13.2, Bin1b, E-2, EP2, HE2), secreted by epididymal epithelial cells into epididymal fluid and ejaculate are processed by furin-like convertases, major processing enzymes of the secretory pathway located in the trans-Golgi network (76, 233). Human β-defensin 1 produced by oral keratinocytes is present in one major form (47 aa) and several minor forms (40-44 aa) (34). Multiple truncated forms of hBD-1 occur in kidney and plasma (89), the latter of which could be explained by prodefensin cleavage by a chymotrypsin-like enzyme (266).

It remains to be examined if birds possess Paneth-like cells and contribute to intestinal innate immunity. Furthermore, details of β-defensin intracellular trafficking, storage and activation in myeloid and non-myeloid cells in general, and for avian defensins in particular, are lacking.

2.1.4. *Tissue-specific gene expression*.

Beta-defensin-like sequences have been described for several domestic and wild bird species, of which the repertoire and putative functions have been most extensively investigated in domestic chicken.

Chicken β-defensins AvBD1 and -2, originally isolated from peripheral leukocytes (83), and AvBD4 to -7, are all strongly expressed in bone marrow, whereas moderate to weak mRNA expression was found for AvBD4 to -7 in bone marrow and heterophils (Table 2). Together with the reported isolation of multiple β-defensins from turkey and ostrich heterophils (48, 212, 253), this shows that avian heterophils, like the neutrophils of some mammalian species (191, 201), contain multiple β-defensins.

Table 2. Tissue-specific chicken β-defensin gene expression.

Tissues	AvBD1	AvBD2	AvBD3	AvBD4	AvBD5	AvBD6	AvBD7	AvBD8	AvBD9	AvBD10	AvBD11	AvBD12	AvBD13
Tongue	-	-	s	-	m/s	-	-	-	-	-	-	-	-/m
Esophagus	-	-	m	-	-	-	-	-	w/s	-	-	-	-
Crop	-	-	-	-	-	-	-	-	-/s	-	-	-	-
Proventriculus	-	-	-	-	-	-	-	-	-/m	-	-	-	-/m
Gizzard	-	-	-	-	-	-	-	-	-/~	-	-	-	-
Small intestine	-/w	-/m	-	-/~	-	-/~	-/~	-	-/w	-	-	-	-/s
Large intestine	-/w	-/m	-/~	-/~	-	-/m	-/~	-	-/~	-	-	w	-
Caeca				-	-	-	-	-	-	-	-	-	-
Colon				-	-	-	-	-	-	-	-	-	w
Cloaca	w	-/w	-	-/~	-	-	-	-	-	-/w	-	-	-
Pancreas	w	-/w	-	-	-	~	-		~	-		-	m
Liver	-	-/w	-	-/w	-	-/w	-	m	m/s	m/s	-	-	m/s
Gall bladder	w	w	-	~	-	w	~	m	s	s		-	s
Trachea	-	-/w	-/s	-/w	~/w	-/~	-/~	-	w/s	-	-	-	~/m
Lung	m/s	m/s	-	-/w	~	-/m	-/~	-	-/w	-/m	-	-	m
Air sacs	-	-	m					-					m
Kidneys	-	-	-/w	-/~	-	-/w	-	-	m/s	m/s	s	w	m
Testis	s	s	-	-/s	-/~	-/s	-/s	-	-/m	m/s	-	w	-
Vas deferens				-	-	-	-	-	m	~	-	-	-
Ovary	-	-	w	-	-	-	-	-	-/m	m	-	~	~
Oviduct	-	-	-	-	-	-	-	-	-	m	s	s	w
Infundibulum	m	m	m	-	-	-	-	-	-	m	-	-	-
Uterus	-/w	-/w	~	-	-	-	-	-	-	w	w	s	-
Vagina	m	w	m	-	-	-	-	-	-	-	-	-	~
Egg yolk	-	-	-	-	-	-	-	-	-	m	-	-	-
Skin	-	-	-/m	-	-	-	-	-	-/m	-/w	m	-	-
Thymus				-	-	-	-	-	-/w	-	+	-	-
Spleen	-	-/w	-	-	-	-	-	-	-/w	-	-	-	-/s
Bursa	-/m	-/m	s	-/w	-/w	-/m	-/~	-	w/s	~/w	-	m	~/m
Heart				-	-	-	-	-	-	-	-	w	-
Skeletal muscle				-	-	-	-	-	-/m	-	-	-	-
Brain	w	w	~	-/~	~/w	-/~	-/~	-	w/m	-/~	-	-	-
Bone marrow	s	s	w	m/s	w/s	s	s	-	w	-	-	-	-
Leukocytes	s	s		-	-	w	w	-	-	-	-	-	-
Ref.	**(83, 130, 159, 264)**	**(83, 130, 159, 264)**	**(130, 159, 264)**	**(130, 245)**	**(130, 245)**	**(130, 245)**	**(130, 245)**	**(88, 245)**	**(130, 231, 245)**	**(130, 245)**	**(245)**	**(130, 245)**	**(88, 245)**

Expression levels: (s)trong, (m)oderate, (w)eak, ~ trace, - not detected.

In the respiratory tract, high β-defensin expression is observed for AvBD3 (264) and AvBD9 (231) in trachea. In lung tissue strong expression is found for AvBD1 and -2 (130, 264). Most other known β-defensins are weakly or moderately expressed in these tissues (Table 2). Air sac membranes have not been extensively examined for β-defensin presence, but AvBD3 and -13 expression has been detected (88, 264).

Chicken skin was shown to express moderate levels of AvBD3, -9 and -11, while moderate AvBD9 mRNA expression was observed for uropygial gland tissue (231, 245, 264), a large sebaceous skin gland that regularly secretes its contents onto skin and plumage (97). Another avian β-defensin, designated AvBD14, has been recently deposited in the Genbank database (AM402954) and has been observed to be predominantly expressed in chicken skin (Dr. Pete Kaiser, personal communication). The expression levels of β-defensins in chicken skin agrees with the reported basal expression of hBD1, -2, -3 and -4 in keratinocytes of normal human skin, of which the latter three were strongly increased during induced keratinocyte differentation (80). Marked hBD1 and hBD2 expression has also been found in human philosebaceous units, both of which were upregulated in acne vulgaris lesions (28). hBD2 is a major constituent of psoriatic skin, a chronic non-infectious disease in which surprisingly few cutaneous infections occur (81), whereas hBD2 and other AMPs are deficient in atopic dermatitis, in which bacterial and viral skin infections are a recurrent problem (160). Thus, considering their presence in chicken skin and uropygial gland, avian β-defensins may contribute to skin innate immune defense in birds.

With the exception of AvBD11, weak to strong mRNA expression of chicken β-defensins is found throughout the digestive tract. In the proximal digestive tract, strong expression is observed for AvBD3 and -5 (130) in tongue and AvBD9 (231) in esophagus and crop tissue. The crop is an extension of the esophagus in which food can be stored for up to 24 hours and is well developed in gallinaceous birds. As chickens practice coprophagy to recover vitamins, amino acids and other nutrients produced by their hindgut bacteria (140), an adequate local innate immune system is required. The high expression levels of AvBD9 in adult chicken crop tissue and its variable expression in juvenile broilers indicate an important role of AvBD9 in crop tissue defense (231).

In the glandular and muscular stomach, β-defensins are practically absent, apart from the moderate AvDB9 (231) and weak AvBD13 (88) expression levels found in the proventriculus. In the intestinal tract of newly-hatched chickens, AvBD1 and AvBD2 mRNA levels were found to decrease during the first week and increase during the second week post-hatch (11). Likewise, developmental expression studies of chicken AvBD4 mRNA using 1, 4, 17 and 38 day-old animals, showed AvBD4 expression to be maximal within the first week post hatch and declined thereafter

(138). In the chicken small and large intestine of older animals, low to medium mRNA levels are found for β-defensins that have been found to be expressed in heterophils and/or bone marrow only, suggesting it to originate from resident myeloid cells. Considerable AvBD13 mRNA expression was found in small intestinal tissue, liver and gall bladder (88). Similarly, moderate to high mRNA expression in liver (and gall bladder) was found for AvBD8, -9, and -10 (88, 130, 245), which may reflect an important role of avian β-defensins in the liver during systemic infections. In contrast, no β-defensin expression was detected in caecal tissue, whereas low levels of AvBD13 mRNA were found in colon (245) and only weak to moderate mRNA expression levels were found for AvBD1, -2 and AvBD10 in the cloaca (130). The cloaca and colon are a point of entry for potential microorganisms as in birds, anti-peristalsis of the lower intestine, the so-called intestinal reflux, is capable of transporting faeces back into the intestine and past the ileocaecal junction (40). The caecal pouches are the main fermentation sites of poorly digestible substrates and are emptied only once every 8 hours on average. Moreover, in birds, the cloaca is also the collecting point of the urogenital tract. Thus, an efficient local immune barrier can be expected to be present at this site to prevent or limit pathogen invasion in the intestinal and urogenital tracts via this infection route. Ohashi *et al.* (159) examined AvBD1, -2 and -3 mRNA expression in the hen reproductive tract by semi-quantitative RT-PCR and showed that the highest levels occurred in infundibulum for all three gallinacin genes and in vagina for AvBD1 and AvBD3. Localization of expression sites in vaginal tissue using in situ hybridisation identified AvBD1, -2 and -3 in basal cells of the surface epithelium in the mucosal folds. The onset of egg-laying activity at approximately 18 weeks of age and absence of significant AvBD1 to -3 expression in the oviduct reported in 3 month-old hens by Zhao *et al.* (264) suggest that expression levels of these gallinacins in the oviduct may be developmentally affected by estrogen levels. AvBD1, -2 and -3 mRNA levels were significantly higher in the vaginal mucosa of older birds, i.e. 180 day-old vs. 720 day-old hens (250). The decreased avian β-defensin levels found in the regressed oviducts of feed-withdrawal-induced non-laying birds further supports the idea of fluctuating avian β-defensin expression levels as a function of egg-laying activity regulated by gonadal steroid hormone levels. Stimulation of cultured chicken vaginal cells with *S. enteriditis* or LPS increased levels of AvBD1 to -3 within 24 hours. The importance of β-defensins in the protection of the mammalian male and female reproductive tracts is well established. Abundant expression of multiple β-defensin genes in the male (163, 165, 184, 185, 246) and female (3, 173, 229) reproductive tracts has been reported. The male reproductive system is largely devoid of an adaptive immune system and is therefore depending on an effective innate immune system to prevent infection that may affect temporary or permanent fertility (165). A recent report indicates that human β-defensin-2 expression contributes, in

cooperation with resident flora, to protection against vaginal infection (230). The high mRNA levels of multiple avian β-defensins in kidney and throughout the male and female reproductive tracts (Table 2) suggest a similar role for avian β-defensins in the protection of the avian urogenital tract.

The contrast in β-defensin expression levels between bursa of Fabricius, spleen and thymus, bursa >> spleen > thymus, can be explained by their localization. The bursa is located near the cloaca and therefore continuously exposed to microorganisms. It is therefore likely that the local moderate to high expression of several avian β-defensins in this organ, aids to its protection against pathogenic microorganisms.

In summary, the cloacal region harbors the site of B-cell generation, collects urine, and at the same time forms an important junction of the intestinal and reproductive tract. Therefore, it would be very interesting to investigate the role of β-defensins in the local innate immune defense of this region.

Apart from studies involving the king penguin (*Aptenodytes patagonicus*) β-defensins, little is known about the repertoire and functions of avian β-defensins in wild birds. Although β-defensin sequences related to known chicken β-defensins have been found for king pigeon (*Columba livia,* AvBD4) and mallard duck (*Anas platyrhynchos*, AvBD2, -9) and have been deposited in the Genbank database (Table 1), no related functional data have been published. Recently, AvBD4 related β-defensin sequences have been found in gastrointestinal tissues of the blue tit (*Parus caeruleus*), herring gull (*Larus argentus*) and wood pigeon (*Columba palumbus*) (138). Reverse transcriptase PCR analyses detected expression of AvBD4-related sequences in all three non-domesticated species, with highest expression levels in wood pigeon gizzard, and additional low expression in small intestine and liver. For Herring gull, low levels were found in small intestine, gizzard and liver, whereas in the blue tit, low AvBD4-like expression was observed in small intestinal tissue, but not in gizzard.

Whereas β-defensin genes may be constitutively expressed in some tissues, their expression can be upregulated in other tissues in response to microbial infection or by proinflammatory stimulants. In mammals, β-defensins have shown to be expressed by peripheral blood cells, dendritic cells, keratinocytes, and the epithelial cells lining the respiratory, gastrointestinal and urogenital tracts (8, 39, 158) and to be induced or upregulated by cytokines IL-1α (156), IL-1β (206, 224), TNF-α (79, 224), IFN-γ (39), transforming growth factor 1 (209), insulin-like growth factor 1 (209), LPS (49, 224) bacteria (49, 79, 156), yeast (169) and other stimulants such as phorbol myristate acetate (PMA) (112), isoleucine (53) and 1,25-dihydroxyvitamin D_3 (235).

Induction also seems to be the case for the avian β-defensins, as seen for AvBD3, which was significantly upregulated in tracheal tissue of *Haemophilus paragallinarum*-challenged animals, but not in other tissues (264). The presence of

transcription factor binding sites known to be involved in mammalian β-defensin regulation in the chicken AvBD9 promoter region and the observation of highly variable AvBD9 levels in crop tissue of 13 day-old chicken broilers, indicate a possible tissue-specific upregulation of the AvBD9 gene (231). Small intestinal AvBD4, -5 or -6 mRNA levels were not upregulated in response to an oral challenge with *Salmonella* serovars (138).

Because tissue-specific β-defensin expression and upregulation might be breed-dependent, Sadeyen *et al.* (182) investigated the relationship between this and gene expression of innate immune response factors in *S. enteriditis* carrier state in two inbred chicken lines differing in resistance to caecal colonization by *S. enteriditis*. In 6 week-old broilers, as compared to the 15I line (susceptible phenotype) which was less susceptible to caecal *Salmonella* carriage state, basal expression levels of AvBD1 and AvBD2 were reproducibly higher in the 6_1 line (resistant phenotype) and were not upregulated during the course of infection. However, in adult birds of the same inbred lines increased resistance to caecal *Salmonella* carrier state was found for animals of the 6_1 line (181), which also displayed 10-fold higher levels of AvBD1 and -2 in caecal tonsil tissue, which strongly correlated with TLR-4 expression levels. These findings suggest that elevated AvBD1 and -2 levels were not responsible for the increased resistance against *Salmonella* carrier state.

In male king penguins (*Aptenodytes patagonicus*), gastric β-defensin expression can be upregulated when fasting during the breeding season (218). King penguins only feed at sea, which can entail a 400-500 km journey, and while on land they must live off their reserves. After egg-laying, females return to sea to forage and egg-incubation is taken over by the male. Usually, females come back in time to feed the chick at hatching, but their mates cope with a delayed return of their partner by fasting, thus preserving food in their stomach for 2 - 3 weeks, enabling them to feed the newborn chick for about 10 days (68). Analysis of the stomach contents of male penguins identified two β-defensins, AvBD103a and AvBD103b and other yet unidentified antimicrobial substances (218). AvBD103a and -103b concentrations in the stomach contents were compared between food conserving and normally digesting birds during the egg-incubation period. AvBD103b was detected in the stomach of all birds, whereas AvBD103a was detected in only three samples of one conserving bird (218). Stomach β-defensin concentrations were markedly higher in food conserving birds than in digesting birds, i.e. increasing 13-fold from the onset (74 nM) to the end of the fast period (943 nM). Defensin levels in digesting birds remained invariably low (24 nM). By comparison, in mammals defensin concentrations of more than 3 mM have been found in the granules of mammalian leukocytes (64, 65), whereas the concentration of cryptdins (released by Paneth cells) in the crypt lumen was estimated

to be ~2.4 mM (5). Approximately 4.5 to 23 μM of porcine β-defensin-1 was found in pig dorsal tongue scrapings (205).

Comparison of the published data from different research groups (Table 2) show that avian β-defensin expression levels are highly variable. Besides differences in used breeds, animal age and immune status, tissue-specific gene expression levels may even vary considerably between individual animals.

2.1.5. *Antimicrobial activity*.

The few avian β-defensins that have been studied for their antimicrobial activities display a wide range of microbicidal or microbistatic activities against Gram-negative and Gram-positive bacteria, and fungi (Table 3). It should be noted that the MIC values mentioned in this section, are highly dependent on the type of assay, incubation medium and incubation time used.

Evans *et al.* (47) demonstrated bactericidal and fungicidal activity of chicken and turkey heterophil AvBD1, at peptide concentrations of 0.4 -3.4 μM and 0.4 – 1.8 μM, respectively, against avian pathogens. However, these peptides were not able to kill *P. multocida* or neutralize Infectious Bronchitis Virus, an enveloped coronavirus of chickens. A (20 aa) fragment of turkey AvBD2 inhibited the growth of *S. aureus*, but not of *E. coli* (48). Synthetic chicken AvBD9 peptide showed strong microbicidal activity against the Gram-negative bacterium *C. jejuni* (3.7 μM), Gram-positive bacteria, *C. perfringens*, *S. aureus* (1.9 – 3.7 μM) and the yeasts *C. albicans* and *S. cerevisiae* (1.9 μM), but was less potent against *E. coli* (7.5 μM) and not bactericidal against *S. typhimurium* (>30 μM) (231). In contrast, synthetic chicken AvBD13 peptide was only bactericidal at high peptide concentrations against *L. monocytogenes* (114 μM), *S. typhimurium* wild-type (114 μM) and a *S. typhimurium Pho P* mutant (57 μM), whereas inhibition of *E. coli*, *S. aureus* and *S. pyogenes* at peptide concentrations ≤57 μM was negligible or absent (88).

In radial diffusion assays, ostrich heterophil β-defensins, AvBD1, -2, and -7, efficiently inhibited the growth of *E. coli* O157:H7 and methicillin-resistant *S. aureus* strain 1056 (MRSA) with MICs ranging from 0.2 to 0.6 μM (212). Ostrich AvBD8 was less potent against these bacterial strains (MIC, 2.4 μM), whereas only Ostrich AvBD1 was fungicidal against *C. albicans*.

Analysis of the stomach contents of male king penguins revealed numerous antimicrobial activities, including the avian β-defensin peptides AvBD103a and AvBD103b, which are identical with the exception of an Arg residue instead of a His residue at position 14 for AvBD103b.

Table 3. Antimicrobial activity of avian β-defensins and cathelicidins.

	Microorganisms	cAvBD1 [47, 48, 83]	cAvBD2 [48, 83]	cAvBD9 [231]	cAvBD13 [88]	Cathelicidin-1 ‡,[15, 243]	CMAP27 ‡	Fowlicidin-2 [243]	Fowlicidin-3 [15, 243]	tAvBD1 [47, 48]	tAvBD2 fragment [48]	oAvBD1 [212]	oAvBD2 [212]	oAvBD7 [212]	oAvBD8 [212]	pAvBD103b [218]
G (-)	*Escherichia coli*	√	√	√	×	√	√	√	√	√	×	√	√	√	√	√
	Salmonella enteriditis	√				√	√		√	√						
	Salmonella typhimurium	√		√	×	√	√	√	√	√						>
	Pasteurella multocida	×								×						
	Campylobacter jejuni	√		√						√						
	Bordetella avium	√								√						
	Klebsiella pneumonia					√		√	√							>
	Pseudomonas aeruginosa			×		√	√	√								>
	Enterobacter cloaca															×
	Alcaligenes faecalis															×
	Vibrio metshnikovii															>
	Vibrio anguillarum															>
G (+)	*Listeria monocytogenes*	√	√		×	√		√	√							√
	Staphylococcus aureus	√		√	×	√	√	√	√		√	√	√	√	√	√
	Staphylococcus haemolyticus															√
	Staphylococcus saprophyticus															>
	Streptococcus pyogenes			√	×											
	Streptomyces pactum						√									
	Streptomyces fradiae						√									
	Clostridium perfringens			√		√	√									
	Kocuria rhizophilae						√									√
	Bacillus subtilis															√
	Bacillus cereus					√	√									√
	Bacillus megaterium															√
	Bacillus licheniformis						√									
	Nocardia asteroides															√
	Aerococcus viridans															√
M	*Mycoplasma gallisepticum*	√								√						
F	*Candida albicans*	√	×	√		√	√			√		√	×	×	×	>
	Candida tropicalis															>
	Candida glabrara															>
	Saccharomyces cerevisiae			√		√	√									
	Neurospora crassa															>
	Aspergillus fumigatus															>
	Chrysosporium keratinophilum						√									
	Scopulariopsis brevicaulis						×									
V	Infectious Bronchitis Virus	×								×						

‡See Chapters 5 and 6 of this thesis. Chicken (cAvBD), turkey (tAvBD), ostrich (oAvBD) and penguin (pAvbD) avian β-defensins. Microbicidal activity (√), microbistatic (>) activity or no growth inhibition (×) at peptide concentrations below 10 μM. G (-), Gram-negative bacteria; G (+), Gram-positive bacteria; M, mycoplasma; F, fungi; V, enveloped virus.

Synthetic penguin AvBD103b peptide displayed potent bacterical activity against Gram-positive bacteria (*K. rhizophilae*, *Bacillus* spp., *Staphylococcus* spp., *N. asteroides and A. viridans*), with the exception of *S. saprophyticus*, at peptide concentrations less than 4 μM (218). Mainly bacteriostatic activity was observed for AvBD103b against Gram-negative bacteria, although it displayed bactericidal activity against an *E. coli* strain. In contrast to its impotence against *Candida glabrata* (>100 μM) and *Candida albicans* (50 - 100 μM), the yeast *Candida tropicalis* and filamentous fungi *Neurospora crassa* and *Aspergillis fumigatus* were efficiently inhibited (3 – 6 μM) by AvBD103b.

2.2. CATHELICIDINS

Cathelicidins comprise a second major group of antimicrobial peptides and have been found in mammals (254), fish (24, 226) and birds (130, 232, 243). The name cathelicidin is derived from the similarity of the cathelicidin large middle domain to cathelin, a cathepsin L inhibitor originally isolated from porcine leukocytes (175). Based on structural characteristics of their C-terminal mature antimicrobial peptides, at least three subfamilies can be distinguished (9, 254): ***i.*** α-helical peptides, linear peptides of 23-37 aa that adopt an amphipathic helical structure when in contact with environments mimicking biological membranes; ***ii.*** β-hairpin peptides, short cyclic peptides (12-18 aa) formed by one or two intramolecular disulfide bridges; ***iii.*** peptides containing an unusual proportion of one or two amino acids, such as Tryptophan-(indolicidin, 13 aa) or Proline/Arginine-rich peptides. The α-helical cathelicidin peptides are the most widely spread of the 3 groups and found in all investigated mammalian species (254). Mammalian cathelicidins possess potent antimicrobial activity against various bacteria, fungi and enveloped viruses and, like defensins, deploy multiple other functions. For instance, they can attract neutrophils, monocytes and T cells (248), contribute to wound healing (204), modulate LPS-induced inflammatory responses via LPS-binding (197), induce histamine release from mast cells (154), or induce angiogenesis (121).

Little is known about the cathelicidin repertoire in avian species. In general, it is known to vary considerably between species, with humans (hCAP18/LL-37) (58), rhesus monkey (263), mice (62), rat (216) and guinea pig (148) possessing only one gene (58, 62), whereas 8 or more cathelicidin genes have been found in pig, sheep and cattle (70). Up to date, three avian cathelicidin-like peptides have been described for chicken, cathelicidin-1 (130), chicken antimicrobial peptide 27 (CMAP27) (232) and fowlicidin-3 (243), the first two are also referred to as fowlicidin-1 and -2, respectively.

2.2.1 *Genomic organization*.

Cathelicidins are synthesised as inactive prepropeptides, consisting of a short signal peptide, a large cathelin-like propiece and a mature peptide. All three chicken cathelicidin genes are encoded by four exons, similar to those described for mammalian cathelicidins (94, 196, 262), the first exon encoding the 5' UTR, signal peptide sequence and part of the cathelin domain, the second and third exon the major part of the cathelin domain and the fourth exon encoding the last few amino acid residues of the cathelin domain, the mature peptide sequence and 3'UTR (Fig. 2).

The three chicken cathelicidin genes are clustered on chromosome 2 within a 7.7 kb region at less than 3.5 Mb from the proximal end (243). The cathelicidin-1 and CMAP27 genes are uniformly oriented, separated by a 2.4 kb gap, adjacent to a 736 kb gap that separates the reversed oriented fowlicidin-3 reversed from the CMAP27 gene (243). Comparative analysis showed that mammalian, avian and fish cathelicidins are grouped in distinctly separated clusters (232, 243). In addition, chicken cathelicidins show more sequence similarity to neutrophilic granule protein (NGP)-like cathelicidin, such as rabbit P15 and mouse, rat, pig and bovine NGP, than to other cathelicidins (243) and are suggested to share a common ancestor. Like NGPs in mammals, chicken cathelicidin genes are located in close proximity to Kelch-like 18, a conserved gene in human, rodents and dog, whereas in mammals possessing NGP genes other cathelicidins are located more than 500 kb away from the Kelch-like 18 and NGP genes. In human, chimpanzee and dog genomes, NGPs appear to be absent. Therefore, it is thought that after the divergence of birds and mammals, duplication of NGP genes resulted in generation of other mammalian cathelicidin genes and that in mammalian species this NGP lineage was lost. Alternatively, two different primordial genes could have been present in the ancestors of mammalian and avian species, of which the non-NGP line was lost in avian species.

2.2.2. *Structural features*.

Mammalian cathelicidins consist of a ~30 aa signal peptide sequence followed by a cathelin-like domain of 98-114 aa containing four conserved cysteines and an antimicrobial C-terminal domain ranging from 12 to 100 aa residues (254, 255). The predicted signal peptide sequence of chicken cathelicidins is much shorter (~17 aa), whereas the size of their cathelin-like domain (105-110 aa) is within the same range and contains the characteristic four conserved cysteines. In accordance with reported neutrophil elastase cleavage sites in horse (207), pig (164) and cattle procathelicidins

(195), putative elastase-specific cleavage sites were detected between the cathelin-like and C-terminal domains for all three chicken cathelicidins. Based on the presence of these putative elastase cleavage site, mature peptides of cathelicidin-1 and fowlicidin-3 were predicted to consist of 26 and 29 aa residues, respectively (243). The CMAP27 precursor protein sequence contains two putative elastase cleavage sites, SSDPV↓L and LVQRG↓R, resulting in a putative 33 aa (designated fowlicidin-2) (243) or 27 aa mature peptide (designated CMAP27) (232), respectively.

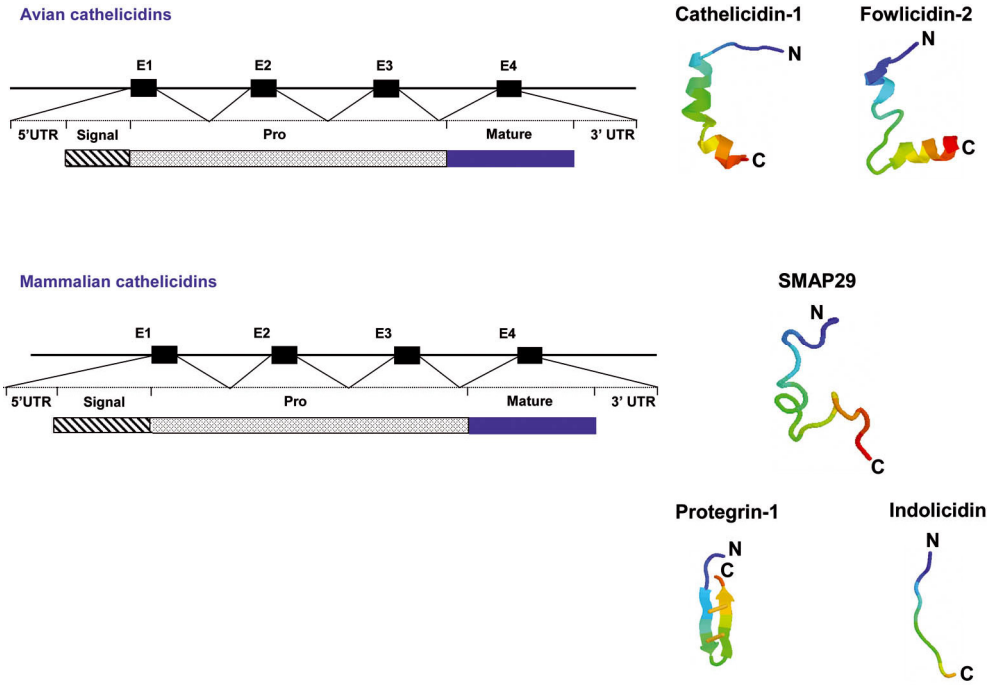

Fig. 2. Genomic organization of avian and mammalian cathelicidins and representive mature peptide structures. Transcription and subsequent translation of exons (E1 to 4) into the 5' and 3' untranslated regions (UTR) and peptide encoding regions for: signal peptide (diagonal striped bars), propiece (dotted bars) and mature peptide (solid blue bars). 3D-structures of mature avian and mammalian cathelicidin peptides. Alpha-helical peptides: chicken cathelicidin-1, chicken fowlicidin-2 (alternatively deduced mature peptide of CMAP27 gene) and SMAP29, sheep myeloid antimicrobial peptide 29. Cyclic peptide: Protegrin-1 (pig). Trp-enriched peptide: Indolicidin (cattle).

The secondary and tertiary structures of chicken cathelicidin-1 and fowlicidin-3 were studied by circular dichroism and NMR spectroscopy. In aqueous solutions, both peptides were largely unstructured, but adopted a typical α-helical conformation in the presence of the structure–promoting agents SDS and trifluoroethanol (15, 244). NMR spectroscopy showed that the putative 26 aa cathelicidin-1 and 27 aa fowlicidin-3 mature peptides were composed of a flexible unstructured N-terminal region and two short α-helical segments around a Gly residue (at position 16 for and 17, respectively) that acts as a hinge between the α-helical segments (15, 244) (Fig. 2). This hinged double helical structure is known to be adopted by other α-helical cathelicidin peptides, such as mouse cathelicidin-related antimicrobial peptide (CRAMP) (252). Figure 2 shows that the tertiary structure determined for fowlicidin-2 is similar to that of cathelicidin-1, but with a larger coiled region in between both helical segments. As predicted, the presence of a Pro residue at position 14 instead of a Gly residue produces a rigid kink in the centre. The limited flexibility of the "hinged region" in this peptide structure will possibly affect its biologic properties.

2.2.3. *Biosynthesis and tissue-specific processing*

Up to date, the synthesis, storage and activation of avian cathelicidins have not been studied. However, based on the data described in Chapters 5 and 6 of this thesis, the underlying mechanisms seem to parallel those reported for mammalian cathelicidins.

In peripheral chicken blood cells, CMAP27 was found to be abundantly expressed by heterophilic granulocytes, but not in other granulocytes, lymphocytes, monocytes or thrombocytes (Chapter 5). Likewise, chicken cathelicidin-1 mRNA was found to be highly expressed in bone marrow (130), whereas the tissue distribution of fowlicidin-3 has not been reported. Neutrophil cathelicidin synthesis occurs early in neutrophil development at the maturation stage when specific or large granules are assembled and switched off during differentiation (50, 256). In mammalian neutrophils, cathelicidins are synthesized as inactive propeptides, stored in the specific or large granules of neutrophils and, if triggered, extracellularly released via exocytosis together with the serine protease-rich contents of azurophilic granules, resulting in a rapid cleavage of inactive proforms into mature 'active' peptides (256). Likewise, Western blot analysis of total leukocyte and heterophil extract showed that CMAP27 is synthesized and stored as a propeptide (Chapter 5). Mature peptide formation occurred in the supernatant containing granule contents and not in the pellet fraction, indicating that proCMAP27 is not membrane bound.

Although little is known about avian heterophil exocytosis, this process has been well studied in human neutrophils and to some extent in other mammals (51, 52,

139, 208, 251, 256). Phorbol esters are potent stimulators of human neutrophil specific and gelatinase granule exocytosis, and to a lesser extent of azurophilic granules (52, 105). On the other hand, fMLP, a powerful neutrophil chemotactic factor, stimulates the release of gelatinase granule, but mobilizes hardly any specific or azurophilic granules (52, 105, 106). Using different agonists of exocytosis, we demonstrated that chicken heterophil stimulation with LPS, PMA and fMLP resulted in the release of proCMAP27 into the environment (LPS>>PMA>fMLP). Furthermore, LPS, and to a lesser extent PMA, but not fMLP, resulted in subsequent processing of proCMAP27 into mature peptide. On SDS-PAGE, mature peptide co-migrated with synthetic CMAP27 peptide, which has a molecular weight of 3,205 Da, indicating that native mature peptide has a mass similar to that of sCMAP27. LPS-stimulation of mammalian neutrophils was shown to induce a significant release of azurophilic granule markers (110), as well as markers of specific granules (45) in which procathelicidins are stored (208). These findings indicate that, like mammalian cathelicidins, CMAP27 is stored as proform in heterophil specific granules, physically separated from its activating proteases, and activated only when simultaneously released into the environment.

Upon stimulation via cell surface receptors, neutrophils activate their oxygen-dependent and -independent antimicrobial activities by releasing both azurophilic and specific granules into the phagosome or in the exterior of the cell (50). The simultaneous release of serine proteases and cathelicidin proforms results in a rapid proteolytic cleavage at the junction between the cathelin-like and C-terminal domains, setting free the antimicrobial C-terminal peptide. The involved proteases differ between species, for instance, while processing of bovine and porcine neutrophil cathelicidins is elastase-mediated (164, 195), released human CAP18 is cleaved by proteinase-3 (210). Although the involved proteases in avian cathelicidin processing still have to be identified, elastase cleavage sites have been identified in all three chicken cathelicidin sequences (232, 243).

The processing of epithelial-derived cathelicidins is tissue-specific and performed by locally expressed proteases. In human eccrine sweat gland and ductal epithelial cells, hCAP18 is constitutively expressed in abundance and transported via sweat onto the skin surface (144). Released proforms (hCAP18) as well as mature LL-37 are further processed to smaller peptides (RK-31, KS-30, KR-20) by the serine proteases kallikrein 5 (stratum corneum tryptic enzyme or SCTE) and kallikrein 7 (stratum corneum chymotryptic enzym or SCCE) (142, 247), which greatly affects the biological properties of these peptides. Contrary to LL-37, processed forms RK-31 and KS-30 exhibit enhanced inhibition of *S. aureus*, but whereas LL-37 is able to augment inflammatory responses by stimulation of the keratinocyte IL-8 production, this activity is lost in processed peptides (142). Epididymal epithelium-derived hCAP18, however, present in seminal plasma at high concentrations in its unprocessed form

(134), is processed by prostate-derived aspartic protease gastricsin (pepsin C) at the acidic pH environment corresponding to the vaginal pH (211), generating a 38 aa residue peptide (ALL-38).

2.2.4. *Tissue-specific gene expression*

In all other studies, the localization and tissue- and cell-specific expression levels of avian cathelicidins (and defensins) are based on determination of mRNA levels. The results described in Chapters 5 and 6, however, provide the first and only evidence up to date, of protein based localization and actual protein concentrations involved in avian AMP (CMAP27) expression.

Both chicken cathelicidin-1 and CMAP27 mRNA are highly expressed in bone marrow (130, 232) and CMAP27 was shown to be abundantly present in heterophilic granulocytes (Chapter 5). The tissue distribution of fowlicidin-3 has not yet been reported, but based on the similarities in expression patterns found for cathelicidin-1 and CMAP27 a predominant presence in myeloid and lymphoid cells and tissues is anticipated.

In the respiratory tract, low levels of cathelicidin-1 and CMAP27 mRNA were detected in trachea with additional low expression of CMAP27 mRNA expression in lung tissue (130, 232). CMAP27 expression was not detected in normal chicken epidermis, nor was it detected in skin appendages, such as comb or wattles. However, skin injury was shown to result in a massive infiltration of CMAP27-positive heterophils at the site of injury as well as local induction of CMAP27 expression in keratinocytes near the site of re-epithelialization (Chapter 6). Epidermal CMAP27 expression was restricted to the wound edge, in line with reported induced LL-37 expression by human skin keratinocytes upon injury (38). Similarly, in murine and human skin, cathelicidin expression is low or absent in normal skin (38, 57), but induced in mouse and human keratinocytes upon skin injury or inflammation (38, 58, 85, 189). In addition, mammalian cathelicidins can be locally released by various other cell types, including neutrophils (31, 164), mast cells (33) and via eccrine sweat glands (144). Interestingly, a surprisingly high CMAP27 mRNA expression was observed in uropygial (preen) gland tissue (232), a single large sebaceous gland located at the base of the tail in most birds that secretes a waxy substance which is distributed onto the plumage during preening activity. Dot blot analysis and Western blot analysis of proteinaceous extracts, prepared from gland wax, showed that low amounts of CMAP precursor were present in uropygial gland wax, conservatively estimated at >0.25 nmol/g secretion. In comparison, human cathelicidin hCAP18/LL-37 concentrations in sweat secretions were shown to be considerably variable between individuals (~1 µM

on average), of which ~10% is processed into mature peptide, at least 20-fold lower then necessary for antimicrobial activity (143). In human skin, cathelicidin precursor hCAP18 is processed by the serine proteases stratum corneum tryptic enzyme (SCTE, kallikrein 5) and stratum corneum chymotryptic enzyme (SCCE, kallikrein 7) into LL-37 and smaller mature peptides (247). Thus, it is possible that proCMAP27 undergoes similar proteolytic processing when deposited onto skin. In addition, the previously reported expression of moderate β-defensin mRNA levels in uropygial gland tissue extracts (231) suggests a possible synergistic action between cathelicidin and defensin peptides (147) when simultaneously released onto the skin surface.

Localization of CMAP27 by immunohistochemistry showed that its expression in the uropygial gland was restricted to the most basal cell layers in the secretory area of the proximal tubular regions, secondary cavities and lower part of the primary cavities, whereas in the thinner upper parts of the primary cavities immunoreactivity was seen throughout the secretory and transitional area (Chapter 6). A similar pattern has been reported for the expression of β-defensin-1 and -2 in the human philosebaceous units (28), where marked defensin expression was found in basal cells covering sites containing stem cell populations and suprabasal defensin expression in regions highly exposed to microbial invasion and skin microflora. These observations, together with the absence of anti-CMAP27 immunoreactivity in the distal tubule regions, supports a role of CMAP27 in the protection of the germinative layer in the gland regions most exposed to microorganisms.

In the digestive tract, low CMAP27 expression was detected in the proventriculus, probably linked to the proximity of the esophageal tonsil (232). Chicken cathelicidin-1 was moderately expressed in gizzard, small and large intestine (130). Low cathelicidin-1 expression was found in liver and gall bladder (130), whereas high CMAP27 mRNA levels were found in liver (232). Highest expression of CMAP27 in the digestive tract was found in cecal tonsil tissue, whereas low expression was found throughout the intestinal tract.

Cathelicidin-1 and CMAP27 were strongly expressed in testis, kidney and bursa of Fabricius (130, 232). Moderate CMAP27 expression was observed in spleen and thymus (232) and low cathelicidin-1 expression was found in cloaca (130). As was suggested for β-defensins, the role of cathelicidins in the innate immune defense of the cloacal region should be further investigated.

In addition to their constitutive expression, tissue-specific upregulation of cathelicidin expression in response to microbial infection or by proinflammatory stimulants may occur (57, 174, 241). In mammals, upregulation of cathelicidin expression is highly species and tissue-specific (189, 194).

Although *S. enteriditis*-challenge of 4 day-old chicken broilers resulted in increased infiltration of CMAP27 positive heterophils in the jejunum villi lamina

propria and, to a lesser extent, crypt regions of infected animals, no induction of CMAP27 peptide expression was detected in crop, jejunum or liver tissue within 8 hrs and 48 hrs post infection. Interestingly, CMAP27-positive liver-specific natural killler (Pit) cells were also identified in *Salmonella*-infected liver sections. Besides their strong cytotoxic activity towards tumor cells and virus-infected cells (129), NK cells participate in the resistance against bacterial pathogens, which has been shown to involve the secretion of soluble factors (177). In addition, activated bovine NK cells display cytotoxic activity against mycobacterium-infected macrophages (46). Thus, chicken liver-specific NK cells may aid in clearance of *Salmonella*-infected macrophages in the liver.

Because of the variable CMAP27 expression observed in immunohistochemical, Dot blot and Western blot analyses of uropygial gland tissue and secretion, the CMAP27 promoter region was examined for transcription factor binding sites known to be involved in regulation of cathelicidin expression. Indeed, several putative binding sites for transcription factors known to be involved in the regulation of cathelicidin expression in mammalian cells were present: NFκβ, AP-1, NF-IL6 (c/EBPβ), RAR and VDR/RXR (Chapter 6) . Human CAP18/LL-37 has been demonstrated to be regulated in human epidermal keratinocytes and monocytes by $1,25(OH)_2VitD_3$ via a vitamin D_3 responsive element (VDR) in the cathelicidin promoter (188, 235). In contrast, CRAMP induction in murine keratinocytes is not regulated by $1,25(OH)_2Vit\ D_3$ (187). Although significant similarity exists between the mouse CRAMP and human and primate CAMP promoter regions, the conserved region of the human and chimpanzee CAMP promoter in which a vitamin D response element (VDRE) is located, is lacking in the mouse CRAMP promoter region (74). It is evident that in animals with their skin covered with a dense complexion of hairs or feathers, conversion of vitamin precursor by UV-B light is more complicated, which perhaps explains why VDREs are absent in the murine, rat and dog CAMP promoter regions (74).

Interestingly, in fowl, the uropygial gland is also the main site of synthesis and storage of the vitamin D_3 precursor 7-dehydrocholesterol (225), which is converted by UVB light to Vitamine D_3 (20). Both Vitamin D_3 and 7-dehydrocholesterol are present in uropygial gland secretion and although only low amounts are found in the back epidermis, much more is found in the unfeathered skin of the legs and feet, which is considered to be the main site of Vitamin D_3 photosynthesis (225). In mammalian keratinocytes, Vitamin D_3 is subsequently converted to 1, 25 dihydroxyvitamin D_3 by two hydroxylation steps mediated by a 25-hydroxylase and 25(OH)-1α-hydroxylase, the latter of which is expressed *in vitro* by numerous cells, including skin keratinocytes (60). The presence of multiple VDREs in the chicken CMAP27 promoter regions therefore, makes it temping to speculate about a possible VDR-dependent induction of

CMAP27 in keratinocytes upon skin injury. Although different organs are involved and mechanisms are unknown, these findings suggest that, parallel to mammalian skin-related immune defense, CMAP27 is probably not directly responsible for maintenance of skin homeostasis, but may be substantially induced by resident and infiltrating cells upon injury and during skin inflammation.

Sebum production is continuous and known to be controlled by hormones, retinoids and growth factors (265). The CMAP27 promoter region contained putative binding sites associated with transcription regulation of sebum production. Multiple putative binding sites were found for c/EBPβ (NF-IL6), PPAR/RXR, SREBP, AR and RAR (Chapter 6). Androgen receptors (AR) are localized in the chicken comb and bottom half of the uropygial gland tubules, but not in the proximal part of tubules, secondary or primary cavities (203). SREBP-1, an important regulator of cholesterol and fatty acid biosynthesis, is highly expressed in the chicken uropygial gland and in human sebocytes (4, 82). Likewise, high PPARα expression was found in the chicken uropygial gland (36). These are important observations since CCAAT enhancer binding proteins (c/EBPs) and peroxisome proliferator-activated receptors (PPARs) are crucial for terminal differentiation of adipocytes (82) and are known to be highly expressed in human sebocytes (26). These findings implicate that under non-pathogenic conditions, a tissue-specific coordination of CMAP27 biosynthesis and sebum production must exist which is most likely governed by key regulators of lipogenesis.

2.2.5. *Antimicrobial and cytotoxic activity*.

Table 3 shows the antimicrobial activity of the three chicken cathelicidins against bacteria (Gram-positive, Gram-negative), yeasts and filamentous fungi. In colony-counting assays, synthetic CMAP27 peptide displayed potent bactericidal activity against all tested strains, including a multidrug-resistant *S. typhimurium* DT104 strain at concentrations ranging from 2.5 to 10 μM (Chapter 5). *Salmonella* strains isolated from chicken feces (*S. typhimurium* 13563 and *S. enteriditis* 13367) did not show enhanced resistance towards cathelicidin-mediated killing. Compared to synthetic cathelicidin-1 peptide, synthetic CMAP27 peptide displayed slightly more potent bactericidal and fungicidal activity towards most strains. In broth microdilution assays, bactericidal activities observed for fowlicidin-3 were similar to those for cathelicidin-1 with MICs in the range of 1-2 μM, including the multidrug-resistant strains *S. aureus* ATCC 43300 (MRSA), *S. aureus* BAA-39 (MRSA) and *S. typhimurium* DT104 (15, 243). In conclusion, the potent microbicidal activity of mature CMAP27 peptide against *Salmonella* isolates and its abundant expression in chicken heterophilic

granulocytes at the site of infection in *Salmonella*-challenged animals, indicate that cathelicidins play an important role in paratyphoid and other bacterial infections.

Particularly ground-foraging birds, such as the domesticated chickens, are constantly exposed to soil-derived microorganisms. Feather (mainly β-) keratin is a poor substrate for most microorganisms and is highly resistant to proteolytic enzymes (122, 213, 238), yet common soil bacteria and fungi have the capacity to degrade feathers *in vitro* (44, 119, 155, 219, 239). Keratinolytic bacteria are wide-spread in the plumage of wild birds and mostly belong to *Bacillus* species, predominantly *B. licheniformis* (21). Keratinolytic fungi have been isolated from bird plumage (92, 93, 171, 172) or old nests (92). In domestic fowl, the breast skin surface was shown to be predominantly colonized by *Staphylococcus epidermidis* and to a lesser extent *Kocuria rhizophilae* and *Streptomyces* spp. (10), whereas multiple keratinolytic fungal species were found, among which *Chrysosporium keratinophilum* and *Scopulariopsis brevicaulis* exhibited the strongest keratinolytic activity (41, 101, 102). It was demonstrated that the vast majority of the tested feather-degrading bacterial and fungal strains were inhibited in the presence of sCMAP27 (Table 3, Chapter 6). sCMAP27 displayed potent microbicidal activity against keratinolytic bacteria and the keratinolytic fungus *C. keratinophilum* (1.2 – 5 μM), and to a lesser extent to opportunistic skin pathogens *P. aeruginosa* and *S. aureus* (10 – 20 μM), while germination and growth of *S. brevicaulis* conidia was not greatly affected (>40 μM). Interestingly, it is now known that in many filamentous fungi, the spores, fungal walls and the surface of fruiting bodies are covered by considerable amounts of hydrophobins, a multigene family of small (~6 – 10 kDa) amphipathic proteins, and that certain hydrophobins have been implicated in fungal pathogenesis (123, 240). Furthermore, under conditions of nutrient deprivation, such as in colony-counting assays, hydrophobin expression has been observed to be upregulated (198). Within a species, different hydrophobins seem to fulfill specific roles, meaning that only some types of hydrophobins are predominantly found in spore walls as well as secreted at high concentrations into the environment (123). Moreover, the hydrophobin pattern in mycelium as well as spore walls is highly variable at the species and sub-species level (150). Thus, although only two species were investigated, it is postulated that the amphipathic properties of cell wall and secreted hydrophobins, could render some fungi less susceptible to AMP-mediated killing and thereby contribute to their pathogenicity.

Bacterial growth inhibition by fowlicidin-2 and cathelicidin-1 was practically unaffected in the presence of physiological salt concentrations up to 150 mM (243). In the presence of 100 mM NaCl, cathelicidin-1-mediated inhibition decreased 2-fold for *Salmonella* spp., *K. pneumoniae*, *L. monocytogenes* and MRSA strain BAA-39, but not MRSA strain 43300 (243). Similarly, fowlicidin-2 mediated growth inhibition declined

2-fold for *E. coli* O157:H7, *S. typhimurium* DT104, *K. pneumoniae* and all (MRSA) *S. aureus* strains, but showed a 2-fold increased growth inhibition in the presence of salt against *P. aeruginosa (243)*. Synthetic CMAP27 and cathelicidin-1 peptides exhibited similar cytotoxic activity towards chicken erythrocytes; at 5 μM peptide, at which most strains were completely inhibited, 26-28% of the chicken RBCs were lysed (Chapter 5). At 10-20 μM, 50% of Madin-Darby canine kidney (MDCK) cells were killed by cathelicidin-1 and fowlicidin-2 in the absence of 10% FBS, with fowlicidin-2 being the least toxic. Similar results were observed with RAW264.7 macrophage cells (243). Compared to cathelicidin-1, fowlicidin-3 displayed slightly more potent bactericidal activity, and was 4-6 fold less cytotoxic towards eukaryotic cells.

To examine the effect of helicity and N- and C-terminal cationic regions on antibacterial and cytotoxic activities, the relation between structure and activity in cathelicidin-1 analogs were studied against *E. coli* ATCC 25922, *S. t*yphimurium ATCC 14028, *L. monocytogenes* ATCC 19115 and *S. aureus* ATCC 25923. The N- and C-terminal residues proved to be dispensable for cathelicidin-1 antibacterial activity as most of the antibacterial activity was retained in cathelicidin-1 analogs lacking the seven N-terminal residues or three C-terminal Lys residues (244). All or part of the bactericidal activity of cathelicidin-1 appears to be originating from the central hydrophobic α-helical segment (Lys_8 – Ile_{23}) (244). The omission of the C-terminal helical region after the Gly_{16} residue resulted in a less than 2-fold reduction of MIC value against Gram-negative bacteria, but in a 7 to 18-fold reduction in MIC towards Gram-positive bacteria, indicating its importance in antibacterial potency against Gram-positive bacteria, but not against Gram-negative bacteria (244). Flexibility minimization at the hinge region between the two helices, by substituting the Gly_{16} residue by a helix-stabilizing Leu residue, reduced antibacterial activity (244). A cathelicidin-1 analog with its Pro, Thr, Ile, Gly, Asn residues in the central hydrophobic region substituted by three Lys and two Leu residues ($K_7L_{12}K_{14}L_{16}K_{18}$), displayed reduced antibacterial activity towards *L. monocytogenes* and *E. coli* despite its augmented amphipathicity and cationicity, yet retained all or part of its antibacterial activity against *S. aureus* and *S. typhimurium* (244). In the case of cathelicidin-1 analogs, removal of 7 N-terminal aa residues, 3 C-terminal aa residues, the C-terminal helical segment, minimization of hinge region flexibility, or augmented amphipathicity/cationicity did not alter its kinetics of killing (244).

Reducing cationicity by deletion of N- and C- terminal located cationic aa residues did not greatly affect peptide cytotoxicity towards human erythrocytes and MDCK cells (244). Compared to the parent cathelicidin-1 peptide, the analogue lacking the three C-terminal Lys residues was only 4-fold less cytotoxic towards these eukaryotic cells, whereas lytic activity similar to the parent peptide was observed when the first four N-terminal aa residues were absent (244). In stark contrast, deletion of the

first seven N-terminal aa residues resulted in greatly reduced cytotoxicity, with only 20% hemolysis of human erythrocytes occurring at peptide analog concentrations of 360 μM and 50% lysis of MDCK cells at 159 μM (244). Omission of the entire C-terminal helical segment (Gly_{16}-Ile_{23}) resulted in an almost complete loss of cytotoxicity towards both cell types (244). Cytotoxicity was not altered by a Gly/Leu substitution at the hinge region (244). Compared to the parent peptide the cathelicidin-1 ($K_7L_{12}K_{14}L_{16}K_{18}$) analog showed a 6-fold increase against human red blood cells, whereas lytic activity towards MDCK cells was only slightly enhanced (244). These findings suggest a stronger binding affinity of this amphipathic helix to erythrocyte membranes than to epithelial membranes, probably due to differences in their membrane lipid composition (244). Fowlicidin-2 and cathelicidin-1 showed killing kinetics similar to SMAP-29, achieving within 30 min maximal killing of *E. coli* ATCC25922 cells at peptide concentrations of 0.16 and 0.10 μM, respectively (243). Incubation of *E. coli* cells with both peptides combined, did not show obvious synergistic effects (243). Permeabilization studies were performed using *E. coli* ML-35p cells incubated with different cathelicidin-1 and fowlicidin-3 peptide concentrations and monitoring the temporal release of cytosolic β-galactosidase in the presence of the chromogenic substrate o-nitrophenyl-β-galactopyranoside (116). Almost immediately after addition of 0.5, 1 or 2 μM peptide to bacterial cells, maximal membrane permeabilization was achieved at 30 to 40 min for both peptides, in accordance with killing kinetics observed for cathelicidin-1 in an earlier study, suggesting that disruption of the bacterial membrane is their primary mode of action (15, 243). In accordance with its salt-independent bactericidal activity in colony-counting assays, membrane permeabilization of ML-35p cells by fowlicidin-3 was slightly delayed in the presence of 100 mM NaCl (15).

3. PAMP-MEDIATED SIGNALLING PATHWAYS

Pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides, lipoteichoic acids, peptidoglycans, flagellin, unmethylated (CpG motifs) DNA and double stranded RNA, are recognized by pattern recognizing receptors (PRRs), including Toll-like receptor (TLRs), interleukin-1 receptor (IL-1R), protease-activated receptor (PAR) and intracellular receptors, such as nucleotide-binding oligomerization domain 2/caspase recruitment domain 15 (NOD2/CARD15) (59).

TLRs are type 1 transmembrane receptors and considered as key sensors of PAMPs, as they are expressed on immune system sentinel cells, such as macrophages and dendritic cells (157). Humans and mice possess at least 11 TLRs: ***i.*** nucleic acid-associated receptors TLR3 (dsRNA), TLR7/8 (ssRNA) and TLR9 (CpG DNA), ***ii.***

protein-associated receptors TLR5 (flagellin) and TLR11 (profilin) and, ***iii.*** lipid-associated receptors TLR2/TLR1 and TLR2/TLR6 (lipopeptide, lipoprotein, peptidoglycan from Gram-positive bacteria and lipopolysaccharide) (157). Up to date, with the use of bioinformatics analysis several chicken orthologs of these TLRs have been identified: cTLR1/6/10 (also referred to as cTLR16) (103, 132), cTLR2 types 1 and 2 (16), cTLR3 (132), cTLR4 (120), cTLR5 (96), cTLR7 (168), cTLR15 (87) and cTLR21 (176). No chicken ortholog for TLR9 has been discovered (176) and TLR15 may represent an avian-specific TLR which has been lost in other taxa or gained in the chicken (87). As in mammalian genomes, the chicken TLR7 locus lies in tandem with a TLR8 locus, but shows a gap in the draft TLR8 locus where its TIR domain should be located, which could imply a pseudogene (176). However, the avian TLR classification is based on sequence similarity only. Thus, corresponding avian ligand-TLR interactions do not necessarily match those observed for mammalian TLRs. Like its mammalian ortholog, cTLR3 specifically responds to polyI:C, while cTLR7 does not respond to a mammalian-specific TLR7 ligand (193). In HEK293 cells, chicken TLR2 type 2 responds to both LPS (if cotransfected with human MD-2 or CD14) and bacterial lipoproteins, whereas in humans these ligands are recognized by two separate receptors, TLR4 and TLR2, respectively (61). Chicken TLR2 type 1 responds to lipoproteins, but not to LPS (61). Furthermore, the ligand specificity of some TLRs, such as human TLR2, are known to be determined through formation of functional TLR heterodimers (223). The observation that both diacylated (hTLR2/6) and triacylated (hTLR2/1) peptides stimulate potent NFκβ activation through cTLR16/cTLR2 type 2 heterodimer complexes, but not through cTLR16/cTLR2 type 1 heterodimer complexes (103), emphasizes the specificity of TLR interactions with other receptors and ligands. Albeit at different amounts, chicken heterophils (110), macrophages, B cells, CD4+ T cells, CD8+ T cells, αβ T cells, γδ T cells were found to express most of these TLRs (96).

Based on sequence homology Lynn *et al.* (132) identified many avian homologues of TLR pathway components by *in silico* analysis, including MyD88, MAL, IRAK-4, Tollip, TRAF6, TAK, TAB1, TAB2, IKKα, IKKβ, IκBα, NF-κB1 and NF-κB2. A chicken MD-2 like sequence is predicted from the annotated genomic sequence by GNOMON based on mRNA and EST evidence (Genbank, XP418301). Although CD14 has not yet been identified in chicken, experimental evidence (35, 109) suggests the existence of a CD14-like protein in chicken. These findings suggest that avian TLR signalling largely resembles that of mammals. Figure 3 shows the two major signalling pathways that are activated by TLRs in mammals, one leads to activation of the transcription factor NF-κB, the other to activation of the mitogen-activated protein (MAP) kinases p38, Jun amino-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) (59, 157). TLRs are embedded in the

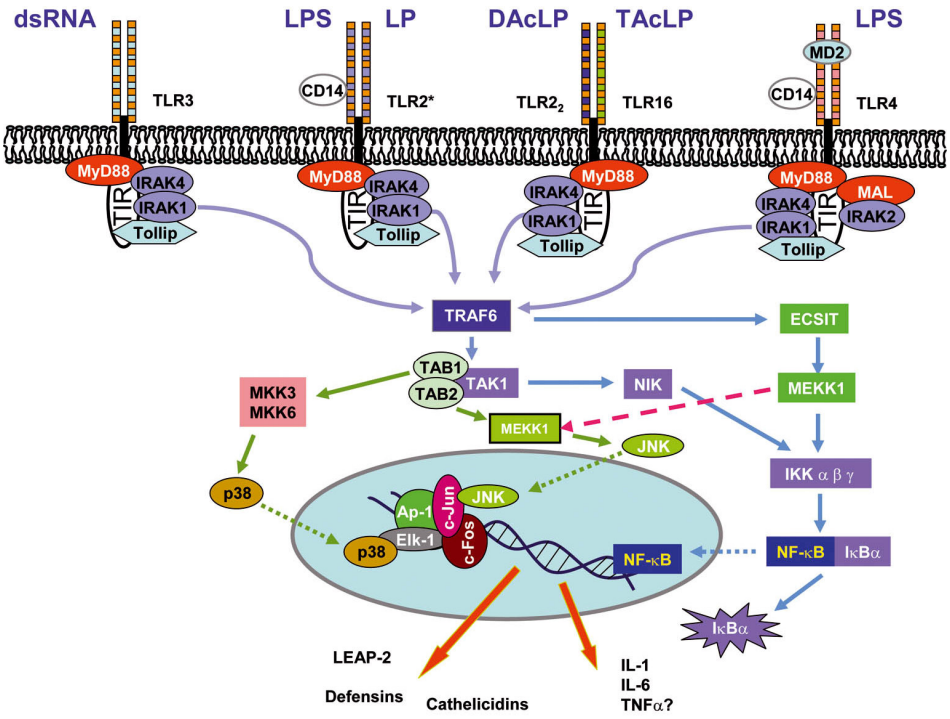

Fig. 3. Chicken Toll-like receptor signalling pathways. Adapted from Lynn *et al.* (132). * cTLR2 type 2 responds to both lipoproteins and LPS, cTLR2 type 1 responds to lipoproteins only.

cytoplasmic membrane with an extracellular variable leucine-rich repeat (LRR) domain and a conserved intracellular Toll/IL-1R (TIR) domain with homology to the mammalian interleukin 1 receptor (IL-1R) (179). Interaction of an extracellular LLR domain with its specific ligand initiates a signalling cascade which results in the recruitment of different adaptor proteins (MyD88, IRAK1, 4, TRAF6) to the receptor complex (30, 136) that after rapid autophosphorylation dissociates from the receptor complex (30) and can associate with downstream adaptor proteins, such as TAK and ECSIT. Toll-interacting protein (Tollip) negatively regulates TLR signalling by association with the TIR domains of TLR2 and TLR4 and inhibiting TLR-mediated signalling by preventing autophosphorylation of IRAK and therefore its dissociation (261). Downstream of TRAF6, NF-κB activation occurs when interactions between NF-κB and its inhibitory proteins (IκBs) are broken by IκB degradation by the IκB kinase kinase complex (IKK-α, -β and -γ) (111), thereby unmasking the nuclear

localization domain of NF-κB. After translocation to the nucleus, NF-κB binds to specific transcription factor binding sites in the promoter region of multiple genes involved in innate immune response, inducing the expression of proinflammatory cytokines (IL-1, IL-6, TNFα), chemokines and antimicrobial peptides (defensins, cathelicidins, LEAP-2) (59, 132). IKK complex activation itself is believed to be mediated by phosphorylation via kinases belonging to the MAPKKK (mitogen-activated protein kinase kinase kinase) family. Considering this aspect, NF-κB-inducing kinase (NIK) and mitogen-activated protein/ERK kinase kinase 1 (MEKK-1) are important because these kinases have demonstrated phosphorylation of IKKs resulting in NF-κB activation (111). ECSIT (Evolutionary Conserved Signalling Intermediate in Toll pathways) interacts with TRAF6 and binds to MEKK-1, which in turn can activate both NF-κB and AP-1 (activator protein 1) (111). Alternatively, simultaneous NF-κB and AP-1 activation may be achieved by TRAF6 signalling through TAK/TAB (transforming growth factor β-activated protein kinase/TAK-1 binding protein) via NIK to the IKK complex and interaction of TAK/TAB to JNK (111). In addition, an MAPKK-independent pathway has been suggested by Ge *et al.* (69) in which p38α is activated by autophosphorylation of TAB1 via MKK3 and MKK6 (128) and leads to AP-1 activation.

4. MECHANISMS OF ACTION

Mature β-defensin peptides have a 3-dimensional amphipathic structure, i.e. they possess spatially opposite domains of clustered hydrophobic and cationic aa side chains. Three intramolecular disulfide bridges restrict conformational changes of these peptides and are well-conserved in this family. For most mature defensins, disulfide bridges and their connectivity appear not to be important for direct antimicrobial activity, but may play a prominent role in other functions, such as chemotaxis. Klüver *et al.* (107) demonstrated that hBD-3 analogues with different connectivities displayed similar antimicrobial and cytotoxic activities. Mandal *et al.* (135) observed antibacterial activity for single, two and three disulfide bridge containing peptides, which were all able to adopt β-hairpin or β-sheet configurations in the presence of micelles and lipid vesicles. Krishnakumari *et al.* (113) reported that cyclic as well as linear peptide analogues corresponding to the C-terminal part of bovine β-defensin-2 were bactericidal. Disulfide bridges may function to protect against proteolysis (200), a feature which is necessary when these peptides are released in the protease-rich environment of the digestive tract. On the other hand, amino acid composition and positioning are highly variable and appear to determine the extent to which individual β-defensins specifically target certain types of microorganisms (221). Their ability to

inhibit growth and/or kill microorganisms differs considerably and is likely achieved via multiple mechanisms. Figure 4 shows a hypothetical "carpet-wormhole model" of action for defensins (63). Cationic peptides are able to electrostatically interact with negatively charged membrane components, such as lipopolysaccharides (LPS), lipoteichoic acid (LTA) and anionic phospholipids and subsequently pass the membrane via the "self-promoted uptake pathway" (77).

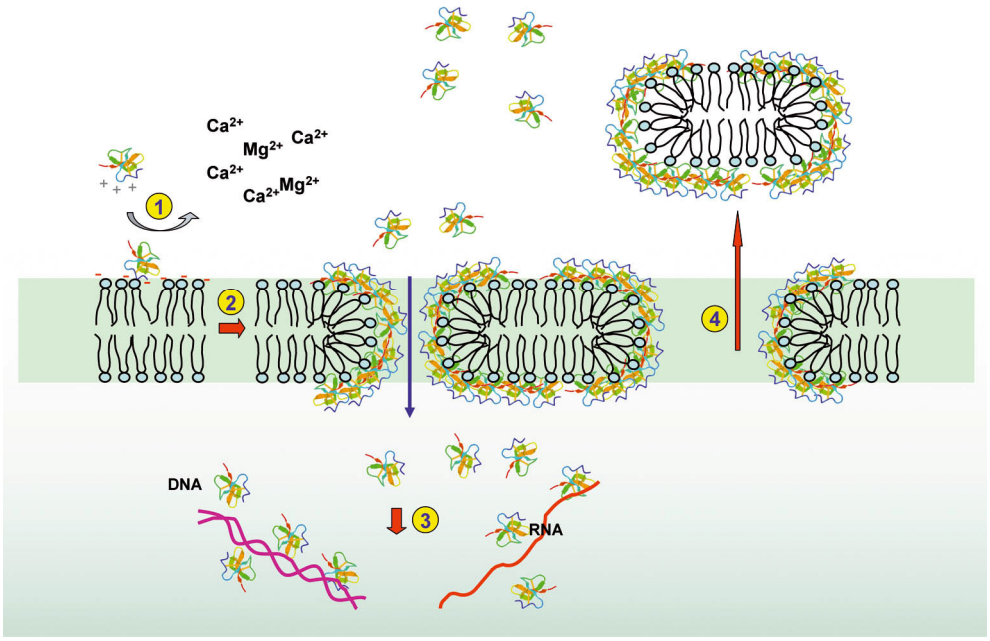

Fig. 4. Mechanisms of action of cathelicidins and defensins according the "carpet/wormhole model". Displacement of membrane-stabilizing ions during initial electrostatic interaction with outer membrane components (1) is followed by accumulation of peptides parallel to the membrane and formation of transient pores or in detergent-like membrane disruption (2). Additionally, intracellular interactions with DNA, RNA or proteins may disable protein synthesis and function (3). Alternatively, aggregate channels may be formed (4).

Due to their higher affinity for divalent cation binding places in the outer membrane, cationic peptides can competitively displace Ca^{2+} and Mg^{2+} ions, important for microbial membrane stability, and subsequently by their larger size, perturb the

membrane structure (77, 78). Driven by the large electric potential of the membrane, the perturbing peptide migrates through the membrane and aggregates into multimeric peptide clusters with their hydrophilic sides facing inwards, resulting in stable or transient pore formation (63). Although the above described mechanisms of permeabilization, involving carpet formation and pore formation, are supported by ultrastructural studies, the above described model and alternative models are primarily based on work done with artificial membranes. Kinetics of β-defensin-mediated killing and permeabilization suggest that although membrane permeabilization is necessary, it is not their primary target, because growth inhibition can be achieved without the need for complete lysis (231). At lower peptide concentrations, pore-formation results in loss of potassium and other small molecules and leads to membrane depolarization (115). Without a membrane proton-motive force, active transportation of substances across the membrane and rotation of bacterial flagella will stop leading to starvation, a loss of motility and, eventually, lysis. Once transported to intracellular sites, cationic peptides can interfere with DNA, RNA and protein synthesis by binding to DNA and RNA molecules (115, 215).

In addition, some defensins are able to bind to membrane glycoproteins and may be potentially important for anti-viral activity (236). Alternatively, the displacement of divalent cations in the cell wall by cationic peptides can promote autolysis by activating the autolytic cell wall enzymes (muramidases) in bacterial membranes. The main regulators of autolysins in Gram-positive bacteria are (lipo)teichoic acids (72). In dividing Gram-positive cells, activated autolysins remodel the bacterial peptidoglycan layer by their muramidase activity (108). Teichoic and teichuronic acids in the peptidoglycan layer of the Gram-positive cell wall bind autolysins non-competitively and in that way inhibit muramidase activity. Cationic peptides bind to teichoic acids with a higher affinity and can thereby displace and activate autolysins leading to uncontrolled degradation of the muramidase layer and often spontaneous lysis of the cytoplasmic membrane (13). In fact, activation of autolysins in the cell wall of the Gram-positive bacteria has been observed in the presence of poly-L-lysine (14). In Gram-negative bacteria, the regulation of autolysins is not fully understood (91).

The mechanisms underlying the direct antimicrobial actions of α-helical cationic peptides are similar to those described for defensins. After initial electrostatic interaction with negative cell membrane components, α-helical cationic peptides rapidly permeabilize the outer leaflet of Gram-negative bacteria (259). According the "carpet/wormhole model", peptides would then accumulate parallel to the cytoplasmic membrane and are thought to form transient pores or disrupt the membrane in a detergent-like way (Fig. 4) (162). Alternatively, formation of so-called aggregate channels (78), membrane regions with increased permeability, have been proposed.

The selectivity towards prokaryotic and eukaryotic membranes is differentially influenced by the propensity of helix formation and the properties of the polar and hydrophobic peptide domains (259). For deeper insertion in the membrane lipid bilayer, an optimal spatial arrangement of aliphatic side chains is permitted by a helical conformation, stabilized by the strong interactions of these side chains with membrane lipids (259). Based on their behaviour in bulk solutions, α-helical cationic peptides can be divided into two categories, aggregating and non-aggregating peptides. Potent salt- and medium-insensitive *in vitro* antimicrobial activity is exhibited by canonical α-helical peptides, such as frog magainins, insect cecropins (162) and cathelicidins BMAP27, BMAP28 and SMAP-29 (70), which are considered non-aggregating. Alpha-helical peptides that are prone to aggregation at relative low concentrations of salt and/or medium, include human LL-37 (98) and some of its orthologs. Most α-helical cationic peptides have a free random coil monomeric structure in bulk solution and only adopt a helical conformation if in contact with a membrane-like environment. Circular dichroism studies of LL-37 like peptides indicated that these peptides favor a structured formation in bulk solution. According to the proposed model of Zelezetsky *et al.* (260) "aggregating"-type peptides possess features that tilt the equilibrium under physiological conditions (salt, medium) towards an aggregated state rather than a random coil state. Certain anions, such as SO_4^{2-}, HCO_3^- and to a lesser extent Cl^-, can promote helix formation by salting out apolar groups resulting from the effects of salts on water surface tension, the so-called Hofmeister effect (98). At high salt concentrations (>1 M) proteins display reduced solubility, but at lower salt concentrations this salting out effect promotes a shift in the equilibrium of α-helical cationic peptides towards an oligomeric state (98). Peptides in the aggregated state are thought to interact differently with biological membranes, affecting both antimicrobial and cytolytic activity. Medium components strongly interact with the "sticky" peptide aggregates and sequester binding sites, thus reducing microbicidal and cytotoxic activity.

The antimicrobial activity of many defensins is diminished in the presence of salts, such as sodium chloride concentration at physiological concentrations (~150 mM or 300 mOsm) or by various divalent cations or plasma proteins (67, 73, 206, 237). Most likely, mono- or divalent cations inhibit by simple charge competition of the initial interaction between the cation peptide and its anionic targets (117).

Chicken AvBD9-mediated growth inhibition of *E. coli* and *S. typhimurium* was not affected in the presence of 20 mM sodium chloride, whereas growth inhibition of *C. perfringens* and *S. aureus* declined to ~ 50% (231). Synthetic AvBD9 proved to be relatively salt-insensitive, as 24 to 49 % growth inhibition was retained for most strains in the presence of 150 mM sodium chloride. Living in a salt water habitat, it was not surprising that the antibacterial activity of synthetic king penguin AvBD103b

against *E. coli* and *S. aureus* was not affected by sodium chloride concentrations up to 160 mM (348 mOsm), which is close to the osmolarity measured in penguin stomach contents (324 mOsm) (114). This indicates that these peptides can retain their microbicidal activity *in vivo* in stomach contents and contribute to protection against food degrading microorganims. At 480 mM NaCl the efficacy against *S. aureus* decreased 16-fold and growth inhibition of *E. coli* decreased 2- and 4-fold in the presence of 1 and 50 mM $MgCl_2$, respectively (114). Some β-defensins have the propensity to form oligomeric structures. The capacity to create stable dimers in solution has been suggested as a possible reason for the high and salt-independent antibacterial activity of hBD-3 (190). The solution structure of AvBD103b revealed a global β-defensin fold consisting of a three-stranded β-sheet and an N-terminal α-helix, common features of mammalian β-defensin, and was shown to be monomeric by NMR spectroscopy although the authors did not rule out the formation of a symmetrical dimer (114). Although oligomerization may enhance their antimicrobial activity (22, 190), in the case of AvBD103b and hBD-3, high cationicity (10+ and 11+, respectively) and overall hydrophobicity (107) appears to be more important. Despite the demonstrated inactivation of some AMPs at physiological salt concentrations *in vitro*, AMP gene products correlate well with increased antimicrobial resistance in animal model experiments (183). A possible explanation for this phenomenon was postulated by Dorschner and coworkers (37), who suggested that bicarbonate, which is ubiquitously present in blood, sweat, and the mucosal surfaces of the respiratory, urogenital, and gastrointestinal tracts, could enhance microbial susceptibility to AMP-mediated killing. Dorschner *et al.* observed potent LL-37-mediated killing of *S. aureus* when grown in bicarbonate containing MEM medium containing 10% serum, but not against cultures grown in TSB medium. They demonstrated that bacteria grown in the presence of bicarbonate responded with an altered gene expression profile that made them more susceptible to cationic peptide-mediated killing. Transmission electron microscopy of *S. aureus* treated with $NaHCO_3$ showed thinning of the cell wall. DNA microarray analysis of *E. coli* cultured in the presence or absence of $NaHCO_3$ identified decreased expression of several genes, including >30 flagellar genes. Quantitative RT-PCR showed a 5-fold decrease in *E. coli fliA* gene expression in the presence of bicarbonate, which encodes a sigma factor that regulates many flagellar genes and can influence bacterial virulence and AMP sensitivity (86). *S. aureus* grown in bicarbonate-containing media showed a 10-fold decrease in sigma factor *sigB*, a gene contributing to virulence and involved in a broad range of stress responses, including control of cell wall thickness and integrity (99). These findings support the *in vivo* direct antimicrobial activity of AMPs.

5. CONCLUSIONS

The biological functions of avian cathelicidins and defensins are still largely unknown, but are expected to reflect those described for their mammalian counterparts. Many questions remain to be answered. The relationship between copy number, gene polymorphisms and AMP gene repertoire with disease resistance need to be sorted out. The apparent lack of antimicrobial activity of chicken AvBD13 and the occurrence of elongated defensin-like genes, due to an additional large post-piece, in some human and avian defensins suggest biological functions other than direct antimicrobial activity for these molecules. Evidence is mounting that heterophils play an important role in avian innate immune defense against bacterial infections, but the repertoire and role of heterophil AMPs in the extracellular and intracellular release of AMPs, their subsequent activation and regulation needs to be further investigated. Similarly, the tissue distribution and scarce data on upregulation of avian AMP expression is almost completely based on regulation on the transcriptional level. Therefore, additional research on translational regulation and post-translational processing and modifications should be performed and coupled to functional studies with respect to tissue-specific expression. Particularly the cloaca and surrounding tissues, where reproductive tract, B cell synthesis and intestinal contents come together, is an interesting subject for further studies. The observed cathelicidin expression in uropygial gland tissue and secretion suggest that in some avian species this gland might have developed an immune function that contribute to the protection of skin and plumage against opportunistic pathogens. As reported for mammals, avian skin injury resulted in infiltration of cathelicidin containing granulocytes and likely also local induction of cathelicidin expression by keratinocytes. Our findings suggest that the tissue-specific expression regulation of CMAP27, and perhaps other AMPs, in the uropygial gland, is most likely linked to sebum production. Although several putative regulatory mechanisms of AMP inducibility have been found in the CMAP27 (Chapter 6) and AvBD9 (231) promoter regions, they will have to be validated in future studies.

6. ABBREVIATIONS

aa, amino acid**; AMP**, antimicrobial peptide; **AP-1**, activator protein 1; **AR**, androgen receptor; **AvBD**, avian beta-defensin; **ATCC**, American Type Culture Collection; **AWAK**, avian wap motif containing, Kunitz domain containing peptide; **CAMP**, cationic antimicrobial peptide; **c/EBPβ**, CCAAT enhancer binding protein beta; **CMAP27**, chicken myeloid antimicrobial peptide 27; **CRAMP**, cathelicidin-related antimicrobial peptide; **DEFA**, alpha-defensin ; **DEFB**, beta-defensin; **DAcLP**, diacetylated lipopeptide; **dsRNA**,

double-stranded ribonucleic acid; **ECSIT**, evolutionary conserved signalling intermediate in Toll pathways; **ER**, endoplasmic reticulum; **ERK**, extracellular signal-regulated kinase; **FBS**, fetal bovine serum; **fMLP**, formyl Met-Leu-Phe peptide; **Gal**, gallinacin; **GPV-1**, gallopavin-1; **hBD**, human beta-defensin; **hCAP18**, human cationic protein 18; **IFN-γ**, interferon gamma; **IGF-1**, insulin growth factor 1; **IκBα**, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; **IKK**, IκB kinase kinase; **IL-1α**, interleukin 1alpha; **IL-1β**, interleukin 1beta; **IL-1R**, interleukin 1 receptor; **IRAK**, interleukin 1 receptor-associated kinase; **JNK**, Jun amino-terminal kinase; **LEAP-2**, liver expressed antimicrobial peptide-2; **LP**, lipoprotein; **LPS**, lipopolysaccharide; **LRR**, leucine-rich repeat domain; **MAL**, MyD88 adaptor-like protein; **MAPKKK**, mitogen-activated protein kinase kinase kinase; **MDCK**, Madin-Darby canine kidney ;**MEKK-1**, mitogen-activated protein/ERK kinase kinase 1; **MKK**, mitogen-activated protein kinase kinase; **MRSA**, methicillin-resistant *Staphylococcus aureus*; **MyD88**, myeloid differentiation primary response gene 88; **NF-IL6**, nuclear factor interleukin 6; **NFκβ**, nuclear factor kappa beta; **NGP**, neutrophilic granule protein; **NIK**, NFκβ-inducing kinase; **NMR**, nuclear magnetic resonance; **NOD2/CARD15**, nucleotide-binding oligomerization domain 2/caspase recruitment domain 15; **PAMP**, pathogen-associated molecular pattern; **PAR**, protease-activated receptor; **PG**, peptidoglycan; **PMA**, phorbol myristate acetate; **PRR**, pattern recognizing receptors; **PPAR**, peroxisome proliferator-activated receptor; **PPAR/RXR**, peroxisome proliferator-activated receptor/retinoid X receptor heterodimer; **p38**, mitogen-activated protein kinase p38; **RAR**, retinoic acid receptor; **RT-PCR**, reverse transcriptase polychain reaction; **SDS-PAGE**, sodium dodecyl sulfate polyacrylamide gel electrophoresis; **SCCE**, stratum corneum chymotrypsin-like enzyme; **SCTE**, stratum corneum trypsin-like enzyme; **SNP**, single nucleotide polymorphism; **SREBP-1**, steroid-responsive element binding protein 1; **ssRNA**, single-stranded ribonucleic acid; **TAB1**, TAK-1 binding protein; **TAK-1**, transforming growth factor beta-activated protein kinase 1; **TGF-1**, transforming growth factor 1; **THP**, turkey heterophil peptide; **TIR**, Toll/IL-1R domain; **TLR**, Toll-like receptor; **TNF-α**, tumor necrosis factor alpha; **Tollip**, Toll-interacting protein; **TRAF6**, TNF receptor-associated factor 6; **TAcLP**, triacetylated lipopeptide; **UVB**, ultraviolet B; **VDR**, vitamin D receptor; **VDRE**, vitamin D response element; **VDR/RXR**, vitamin D receptor/retinoid X receptor heterodimer; **1,25(OH)$_2$VitD3**, 1,25 dihydroxyvitamin D3.

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Chapter 3

The β-defensin gallinacin-6 is expressed in the chicken digestive tract and has antimicrobial activity against food-borne pathogens

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ABSTRACT

Food-borne pathogens are responsible for most cases of food poisoning in developed countries and are often associated with poultry products, including chicken. Little is known about the role of β-defensins in the chicken digestive tract and their efficacy. In this study, the expression of chicken β-defensin gallinacin-6 (Gal-6) and its antimicrobial activity against food-borne pathogens were investigated. RT-PCR analysis showed high expression of Gal-6 mRNA in esophagus and crop, moderate expression in glandular stomach, and low expression throughout the intestinal tract. Putative transcription factor binding sites for nuclear factor kappa beta, activator protein-1 and nuclear factor interleukin-6 were found in the Gal-6 gene upstream region which suggests a possible inducible nature of the Gal-6 gene. In colony counting assays, strong bactericidal and fungicidal activity was observed, including bactericidal activity against food-borne pathogens *Campylobacter jejuni, Salmonella typhimurium, Clostridium perfringens*, and *Escherichia coli*. Treatment with 16 μg/ml synthetic Gal-6, resulted in a 3 LOG unit reduction in *Clostridium perfringens* survival within 60 min, indicating fast killing kinetics. Transmission electron microscopy examination of synthetic Gal-6 treated *Clostridium perfringens* cells showed dose-dependent changes in morphology after 30 min, including intracellular granulation, cytoplasm retraction, irregular septum formation in dividing cells and cell lysis. The high expression in the proximal digestive tract and broad antimicrobial activity suggest that chicken β-defensin gallinacin-6 plays an important role in chicken innate host defense.

INTRODUCTION

Since the 1950's, sub-therapeutic doses of antibiotics were added to feed in order to promote growth in chicken broilers (45). Increasing concern about the development of antibiotic resistance and prevalence of its transmission to human pathogens has led to a European ban on the use of antibiotics in animal feeds to promote growth. Hence, alternative methods to suppress microbial outgrowth in poultry are needed. An alternative strategy could be to stimulate the expression of endogenous antimicrobial proteins at mucosal surfaces of the chicken digestive tract by dietary modulation.

Currently, several chicken antimicrobial peptides, belonging to the cathelicidin, liver expressed antimicrobial peptide (LEAP) and β-defensin families have been discovered (24, 39, 41, 46), but little is known about their role in the chicken digestive tract.

Chicken cathelicidin 1 is expressed at moderate levels in gizzard, small intestine and large intestine (24), whereas low levels of chicken myeloid antimicrobial peptide 27 (CMAP27) were found throughout the intestinal tract (41). High levels of chicken LEAP-2 expression were observed in small intestine and liver (24) and upregulated in these tissues when challenged with *Salmonella enteriditis* (39). Apart from gallinacin-11 (Gal-11) which is highly expressed in small intestine, liver, gall bladder and spleen, and Gallinacin-13 (Gal-13) which is found in colon, no significant β-defensin levels have been detected in the digestive tract (18, 24, 46).

Little is known about the antimicrobial properties of antimicrobial peptides in the chicken digestive tract. Recombinant chicken LEAP-2 was effective at microgram amounts against *Salmonella typhimurium* SL1344, but not against *Salmonella typhimurium* C5 and *Salmonella enteriditis* (39). Chicken myeloid β-defensins, gallinacin-1, -1α and -2, isolated from chicken heterophils, showed activity against gram-positive, gram-negative bacteria and yeast (10). Although *Salmonella typhimurium* and *Listeria monocytogenes* were inhibited by synthetic Gal-11, complete killing was only achieved at 500μg/ml (18) suggesting a different role in the chicken gut.

Here we report the expression of β-defensin gallinacin-6 in the chicken digestive tract. The antimicrobial properties of synthetic and recombinant gallinacin-6 peptides were tested against gram-positive and gram-negative bacteria and yeasts using colony counting and broth microdilution assays. Additionally, kill-curve studies and transmission electron microscopy were used to investigate the killing mechanism(s) involved.

MATERIALS AND METHODS

Tissue distribution of gallinacin-6. Three healthy 6 week-old Ross 308 broiler chickens were sacrificed and 23 tissue samples were taken within 30 min for RNA isolation. Samples were rinsed with cold, sterile saline, frozen immediately in liquid nitrogen and stored at -80°C. Total RNA was extracted using Trizol® (Invitrogen, Carlsbad, CA) according to the manufacturers protocol, diluted to 2 µg RNA/µl, pooled per tissue and DNAse I treated (Invitrogen) before first strand cDNA synthesis. To investigate the variability of expression in the upper part of the digestive tract, crop tissue samples were also collected from 13 day-old (n=9) and 85 week-old (n=5) Ross 308 chickens and 21 day-old Hybro chicken broilers (n=3) and individually analyzed.

The tentative consensus sequence for chicken gallinacin-6 was retrieved from The Institute for Genomic Research (TIGR) chicken Expressed Sequence Tag (EST) database (http://www.tigr.org/tdb/tgi/gggi, TC82510). Primer sequences were designed corresponding to the regions flanking the Gal-6 start and stop codon (Gal-6 forward and reverse primers) (Table 1). The β-actin forward and reverse primers were used to assess the quality and quantity of the chicken mRNA samples. All PCR reactions were performed with Faststart DNA taq polymerase (Roche Diagnostics Gmbh, Mannheim, Germany) as follows: after an initial denaturing step of 5 min at 95°C, forty cycles of 30s at 95°C, 30s at 53°C and 45s at 72°C for Gal-6; and thirty-three cycles of 30s at 95°C, 30s at 61°C and 45s at 72°C for β-actin, followed by a final elongation step at 72°C for 7 min.

Promoter analysis. The published Gal-6 gene cDNA sequence (National Center for Biotechnology Information (Genbank), http://www.ncbi.nlm.nih.gov, AY621324) containing a 4480 bp 5' flanking sequence was investigated for the presence of putative transcription factor binding sites using the JASPAR (http://jaspar.cgb.ki.se/cgi-bin/jaspar_db.pl) and MatInspector software (http://www.genomatix.de). A putative transcription start site (TSS) was predicted using the neural netwerk promoter prediction software (Berkeley Drosophila Genome Project, http://www.fruitfly.org/seq_tools/promoter.html).

Prediction of mature Gallinacin-6 peptide sequence. A protein Basic Local Alignment Search Tool (pBLAST) search (http://www.ncbi.nlm.nih.gov/BLAST/) using the Gal-6 precursor amino acid sequence was performed to investigate similarities with other β-defensins. The prepropeptide amino acid sequence of chicken gallinacin-6 was aligned with other avian and mammalian β-defensins using Clustal X (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). Signal peptide sequences were predicted using SignalP software (http://www.cbs.dtu.dk/services/SignalP/, version 3.0).

Table 1. Vector construction, recombinant peptide and RT-PCR primers for Gallinacin-6

Primer type and name	Primer sequence
RT-PCR primers	
Gal-6 (forward)	5'-TCCACAGCAGAGGACAATC-3'
Gal-6 (reverse)	5'-AACTGCGTGGTCAGTGAGG-3'
β-actin (forward)	5'-ACCCTGTCCTGCTTACTGAGG-3'
β-actin (reverse)	5'-TCCCAATGGTGATCACCTGCC-3'
Protein expression vector construction primers	
oligo-1 (forward)	5'-GTTTAAACCCGGGCCGCCACCATGGGATCCCCTCCTTCACTCGGACACACACC-3'
oligo-1 (reverse)	5'-GAGATCTGCCCGGGCTATGCGGCCGCGCTAGCTATTTTGATGAAATGAAGAGT TTCGCCAAGGCTTTC-3'
oligo-2 (forward)	5'-GTTTAAACCAAGGCCCAACTCCCCGAACCACTC-3'
oligo-2 (reverse)	5'-CTGGATCAGAATTCAAGCATGCCCGCGGG-3'
oligo-3 (forward)	5'-CGGAGATCTAAGCTTGGCTGTGGAATGTGTGTCAGTTAG-3'
oligo-3 (reverse)	5'-GTTTAAACGAGCTTGGATCTGTAACGGCGCAG-3'
Recombinant Gal-6 primers	
rGal-6 (forward)	5'-GGATCCACCTTAGCATGCAGGCAG-3'
rGal-6 (reverse)	5'-GCGGCCGCTTAGGAGCTAGGTGCCCAT-3'.

The mature Gal-6 peptide sequence was deduced from comparison of the propiece sequences and cleavage sites of known mature β-defensins. Precursor and known mature peptide sequences were retrieved from the Genbank or EMBL Nucleotide Sequence Submission (EMBL; http://www.ebi.ac.uk) database. GenBank/EMBL accession numbers are as follows: mgBD-1, AAG09213; Gal-3 , Q9DG58; Sphe-1, P83429; Gal-8, AAS99319; Gal-6, AAT48933; ptBD-1, AAK61462; hBD-1, AAC51728; hcBD-1, AAK61464; pBD-2, AAR90346; mBD-37, CAD33899; SBD-2, AAB61996; chBD-1, O97946; bbEBD, AAP57565; LAP, AAB33727; TAP, AAB61757; btEBD, AAC48804; and BNBD-1, AAB25864. The Ostricacin-1 (Osp-1) amino acid sequence was retrieved from the original citation (48).

Peptide synthesis. A 41 amino acid residues peptide corresponding to the deduced mature Gal-6 peptide was synthesized by Genosphere Biotechnologies (Paris, France) using Fmoc solid-phase synthesis on a Symphony synthesizer (Protein Technology Inc., Tucson, AZ). Disulfide bridge formation was accomplished by air-oxidation method as described by Hidaka et al. (17). Synthesized Gal-6 was dissolved in 100 mM Tris/HCl (pH 8.0) in the presence of reduced and oxidized glutathione (2 mM GSH, 1 mM GSSG) and incubated under a N_2 atmosphere at room temperature for 3 days. The folded Gal-6 peptide was purified by Reversed Phase HPLC on a Zorbax C8 column (Agilent Technologies, Palo Alto, CA) eluted with 20 min linear gradient of 0-100% acetonitrile in 0.1 % (w/v) trifluoroacetic acid. Finally, RP-HPLC purified folded peptide was dissolved in milli Q and characterized by mass spectrometry, amino acid analysis and electrophoresis on Tris-tricine PAGE gels.

Protein expression vector construction. Plasmids pTT3 (8), pSGHV(0) (21) and pNUT-VWFcas (40) were used as templates for the construction of the pTT3-SRα-hGH-his8-TEV secreted protein expression vector. The PCR fragment-1 containing the BamH I – Nhe I – Not I multiple cloning site was generated using oligo-1 forward and reverse primers (Table 1) and plasmid pNUT-VWFcas as a template. PCR fragment-2 containing the human growth hormone gene (hGH), a His8-tag and a Tobacco Etch Virus (TEV) protease cleavage site, was generated using oligo-2 forward and reverse primers and plasmid pSGHV0 as template. PCR fragment-3 containing the SRα promoter, was generated using oligo-3 forward and reverse primers and plasmid pSGHV0 as template. The empty expression plasmid was constructed as follows. Pme I – Bgl II digested PCR fragment-1 was ligated into Pme I – BamH I digested pTT3, yielding pTT3a. PmeI – BamH I digested PCR fragment-2 was ligated into Pme I – BamH I digested pTT3a, yielding pTT3b. Bgl II (blunted) – Pme I digested PCR fragment-3 was ligated into Sal I (blunted) – Pme I digested pTT3b, yielding pTT3-SRα-hGH-his8-TEV.

Production of recombinant Gal-6. PreproGal-6 cDNA was produced from a chicken liver mRNA as described above. The sequence coding for the putative mature

peptide was amplified using the rGal-6 forward and reverse primers (Table 1). PCR reactions were performed using the cycling protocol: 5 min 94ºC, 40 cycles 94/58/72ºC for 30/30/45 s. The PCR oligo's introduced a BamH1 site immediately for the codon coding for the N-terminal threonine residue of the mature peptide, and a NotI restriction site after the stop codon. The amplified construct was cloned in the pGEM-T-Easy vector (Promega Co., Madison, WI), and subsequently sequenced in both directions to confirm that it contained no errors. The construct was digested using BamH1 and NotI and ligated into digested pTT3-SRα-hGH-his8-TEV expression vector. HEK293-EBNA cells (ATCC CRL10852) were grown in 90% FreeStyle (Invitrogen) and 10% Ca^{2+}-free Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), containing 5% FCS (Invitrogen), 1% pluronic (Sigma-Aldrich, St. Louis, CA), 10 mM HEPES, 4 mM L-glutamine, 200 U/l penicillin G, 0.1 mg/l streptomycin and 50 µg/ml geneticin. Cells were maintained in exponential growth using Erlenmeyer flasks at 120 rpm on an orbital shaker mounted in a Reach-In CO_2 incubator (Clean Air Techniek, Woerden, The Netherlands). HEK293-EBNA cells were transfected using DNA-PEI (Polysciences, Warrington, PA) according to Durocher *et al*. (8). Briefly, 24 hours before transfection, cells were seeded at 2.5 x 10^5/ml in medium without FCS by dilution. The next day DNA-PEI complexes were formed by a 10 minutes incubation of DNA at 20 µg/ml with PEI at 40 µg/ml in Optimem (Invitrogen), 25 µl of this mixture was used for each ml of cell culture to be transfected. Small scale transfections (4 ml) were performed in 6 well plates, large scale transfections were performed in a Bioreactor (New Brunswick Scientific).

Purification of recombinant Gal-6. The supernatant of transfected HEK293-EBNA cells was collected after 6 days and concentrated using a hollow fiber column (molecular mass cut-off, 10,000 Da, Amersham Biosciences, Uppsala, Sweden). Purification of the fusion protein hGH-His8-TEV-rGAL6 was performed by affinity chromatography (Histrap^{TM} HP, 1 ml, Amersham Biosciences). The supernatant was applied to the column which was subsequently washed 3 times with buffer A (25 mM Tris, 300 mM NaCl, pH 8.2) containing 0, 20 and 50 mM imidazole, respectively. Bound protein was eluted with 250 mM imidazole in buffer A, collected and dialyzed overnight (molecular mass cut-off, 1,000 Da; Spectrum Laboratories Inc., Rancho Domigues, CA) against 25 mM Tris-HCl, 150 mM NaCl, pH 8.2. After dialysis, the fusion protein was cleaved by TEV-proteolysis for 2 h at 30 ºC (fusion-protein : TEV = 10:1 mol:mol) and re-applied to the affinity column. The cleaved mature rGal-6 eluted at 50 mM imidazole in buffer A and was dialysed against 25 mM ammoniumformate. The purified protein was lyophilized and resuspended in 10 mM sodium phosphate buffer pH 7.0 for MALDI-TOF analysis and amino acid analysis.

Anti-His immunostaining of purified recombinant Gal-6. Protein samples were analyzed by one-dimensional Tricine-SDS-PAGE (32) and protein bands were visualized by silver staining (Bio-Rad Laboratories, Richmond, CA). For immunoblot analysis, the proteins were transferred electrophoretically from the gels onto nitrocellulose membrane. Immunostaining was performed using mouse monoclonal anti-His6 (Roche) as primary antibodies and goat anti-mouse IgG containing a peroxidase conjugate as the second antibody. Visualization was achieved using 3,3'-Diaminobenzidine tetrahydrochloride (Sigma-Aldrich).

Characterization of recombinant Gal-6. Protein concentration and amino acid composition was determined by quantitative amino acid analysis on a HP 1090 Aminoquant, using a two step pre-column derivatization with o-phthalaldehyde-3-mercaptopropionic acid for primary and 9-fluorenylmethylchloroformate for secondary amino acids (33). N-terminal sequence analysis was performed using an Applied Biosystems-Perkin Elmer sequencer Model 476A. Matrix assisted laser desorption/ionization-time of flight mass (MALDI-TOF MS) analysis was performed by Eurosequence b.v. (Groningen, The Netherlands) on a Voyager-DE™ PRO (Applied Biosystems, Foster city, CA) in a positive linear mode, using alpha-cyano-4-hydrocinnamic acid as matrix (16).

Quantitative determination of sulfhydryl groups in recombinant Gal-6. Free thiol groups in rGal-6 were determined using the standard Ellman's Test (9) with small modifications. Briefly, a stock solution containing 100 mM Tris, pH 8.0; 0.1 mM 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB) and 2.5 mM sodium acetate was prepared. To 90 µl of this stock solution, 10 µl (256 ug/ml) rGal-6 was added and the mixture was incubated at room temperature for 5 minutes. After incubation, the absorbance at 412 nm was determined as a measure of free thiols. Calibration was performed using L-cysteine solutions in the range of 0-100 µM.

Antimicrobial activity. *Bacillus cereus* ATCC 9193, *Campylobacter jejuni* ATCC 33291, *Candida albicans* ATCC 10231, *Clostridium perfringens* ATCC 12915, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Saccharomyces cerevisiae* CBS2978, *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 29213, and *Streptococcus pyogenes* ATCC19615 were used for antimicrobial assays. *C. albicans* at 37°C and *S. cerevisiae* at 25°C were grown overnight in Yeast Maltose Broth (YMB; Oxoid Limited, Hampshire, United Kingdom). *C. jejuni* was grown overnight (37 °C, 5% CO_2) to stationary phase in Heart infusion broth (HIB; Biotrading Benelux b.v., Mijdrecht, The Netherlands). *S. pyogenes* (37 °C, 5% CO_2) and *C. perfringens* (37 °C, anaerobe) were grown overnight to stationary phase in Trypticase Soy Broth (TSB; Oxoid Limited). All other bacteria were maintained in TSB medium at 37 °C. Bacteria and *C. albicans* were cultured to mid-logarithmic phase by transferring 100 µl of stationary phase suspension into TSB

or YMB medium followed by incubation and shaking for 4 hours at 37 °C. Hundred µl of *S. cerevisiae* stationary phase culture was transferred into YMB medium, incubated and shaken for 10 hours at 25 °C. Mid-logarithmic phase cultures were centrifuged for 10 min at 4 °C at 900 × *g*, and the bacterial and yeast pellets were diluted in minimal medium, (TSB or YMB medium diluted 1000-fold in distilled water for bacterial and yeast pellets, respectively). Initial concentrations of bacteria and yeasts were determined by measuring the optical density at 620 nm. To determine cell viability, 100 µl of 10-fold serial dilutions in peptone physiological salt solution were transferred on to Trypticase Soy Agar (TSA; Oxoid Limited) or Yeast Maltose Agar (YMA; Oxoid Limited) plates and colonies were counted after 24 hours of incubation. *C. jejuni* viability was determined by transferring the serial dilutions on to Saponin agar plates (SA; Oxoid Limited) and were counted after overnight incubation at 37 ºC and 5% CO_2. Final dilutions were prepared in minimal TSB or minimal YMB media to reach a cell density of ~2.5×10^6 cfu/ml for sGal-6 assays and ~2.5×10^5 cfu/ml for rGal-6 assays.

The antimicrobial activity of sGal-6 was determined using colony counting assays. Twenty-five µl of bacterial or yeast culture were mixed with 25 µl of 0 to 256 µg/ml sGal-6 in polypropylene microtiter plates and preincubated for 3 hours at conditions suited to the investigated strain. After a 3 hour incubation period, 200 µl of minimal medium was added, further diluted 10 to 1000-fold in minimal medium and transferred onto TSA, SA or YMA plates and colonies were counted after 24 hours incubation.

To investigate the effect of increased nutrient concentration and thereby higher metabolic activity of the bacteria on sGal-6 antimicrobial activity, additional colony count assays were performed in 100-fold diluted TSB minimal medium. Minimal medium containing 100-fold diluted TSB was buffered with 10 mM sodium phosphate (pH 6.5) to prevent acidification resulting from increased bacterial metabolism. Bacterial pellets were washed in phosphate buffered 100-fold diluted TSB minimal medium instead of 1000-fold diluted TSB in water and antibacterial activity of sGal-6 was determined as described above.

The influence of ionic strength on sGal-6 microbicidal activity was studied in 100-fold diluted TSB medium containing 10 mM sodium phosphate (pH 6.5) supplemented with 20 or 150 mM NaCl. Bacterial pellets, washed in phosphate buffered 100-fold diluted TSB minimal medium, were subdivided for further dilution in 0, 20 or 150 mM NaCl in the same medium. The antibacterial activity of sGal-6 was assessed as described above.

Additionally, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the sGal-6 and rGal-6 peptides against bacterial

and yeast strains were assessed using broth microdilution assays. Bacterial and yeast suspensions were exposed to sGal-6 and rGal-6 concentrations ranging from 0 to 256 µg/ml in 50 µl of minimal medium as described for the colony counting assays. After 3 hours of preincubation, 180 µl of TSB, HIB or YMB medium was added to each well and incubated for 24 hours. The optical density of the resuspended well contents was measured at 595 nm in a 96-wells plate reader (Bio-Rad Laboratories). Growth inhibition was defined as the lowest concentration of peptide that reduced growth by more than 90%. Minimal bactericidal concentrations (MBC) and minimal fungicidal concentrations (MFC) were evaluated by plating the contents of wells without visible growth onto TSA, SA or YMA plates and incubating for 24 to 72 hours and defined as the lowest concentration of peptide that prevented any residual colony formation.

Kill-curve studies. Thirty microliters of 32 µg/ml rGal-6, sGal-6 and 128 µg/ml sGal-6 (final concentrations: 16 and 64 µg/ml) were mixed with 30 µl of 2.5×10^5 cfu/ml (rGal-6) or 2.5×10^6 cfu/ml (sGal-6) *C. perfringens* mid-logarithmic phase culture in minimal TSB medium and anaerobically incubated at 37 °C. At various time points, a 50 µl bacterial suspension aliquot was taken, diluted 50-fold in TSB medium of which 100 µl was plated on TSA medium. The number of colony forming units was counted after overnight incubation at 37 °C. As a negative control, the bacterial supension was incubated with 30 µl of minimal TSB medium.

Transmission electron microscopy. Mid-logarithmic phase *C. perfringens* cells (2×10^8 cfu/ml) were treated with sGal-6 (final concentration: 0, 1.56 , 6.25, 12.5 and 25 µg/ml) for 30 min at 37°C under anaerobic conditions. After treatment, bacterial pellets were prefixed in Karnovsky's reagent (2% paraformaldehyde, 2.5% glutaraldehyde, 0.25 mM $CaCl_2$, 0.5 mM $MgCl_2$ in 80 mM sodium cacodylate buffer, pH 7.4) and postfixed in 2% osmiumtetroxide buffered in 0.1 M cacodylate (pH 7.4) for 2 hours. The samples were blockstained with 2% uranyl acetate for 1 hour and subsequently dehydrated in acetone (50, 70, 80, 96 and 100 %). The cells were immersed in acetone/durcupan resin (1:1) overnight, immersed in pure durcupan resin (Fluka, Buchs, Switzerland) for 2 hours and embedded in durcupan resin at 60°C. Ultrathin sections (thickness, 50nm), stained with lead citrate (30) were examined in a CM10 electron microscope (Philips, Amsterdam, The Netherlands).

RESULTS

Gal-6 localization in digestive tract tissues. In 6 week old Ross 308 chicken broilers the highest Gal-6 mRNA expression was found in trachea, esophagus, crop, bursa, kidney, liver and muscle (Fig. 1A). Moderate Gal-6 expression was observed in uropygial gland, brain, proventriculus, ovary, testis, and skin, whereas low expression

levels were observed in lung, thymus, spleen and throughout the intestinal tract. Gal-6 mRNA was not detected in bone marrow. Additional investigation of Gal-6 mRNA expression levels in the crop tissue of chickens of different ages and breeds (Fig. 1B) revealed that it was strongly and equally expressed in adult Ross 308 chickens and 3 week old Hybro chicken broilers. Yet, in crop tissues from 2 week old Ross 308 chicken broilers, levels of Gal-6 mRNA were low or not detectable in seven of the nine animals investigated.

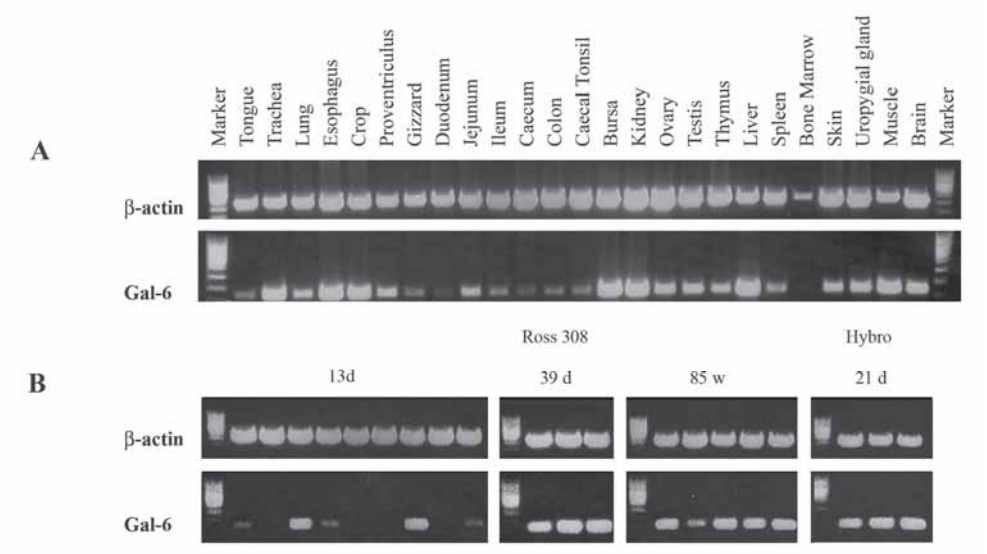

Fig. 1. Tissue gene-expression of chicken gallinacin-6. (A) cDNA fragments were amplified from the pooled total RNA of three animals per tissue (1 µg) with one-step RT-PCR (40 cycles). cDNA fragments of ß-actin were amplified (33 cycles) with the same RNA samples as a control. (B) cDNA fragments were amplified from total RNA samples (1 µg) obtained from the crop tissue of individual animals of different breeds (Ross 308, Hybro) and age with one-step RT-PCR (40 cycles), cDNA fragments of ß-actin were amplified (33 cycles) with the same RNA samples as a control.

Variability in β-defensin expression levels between animals of the same age and breed could indicate a tissue-specific upregulation and/or inducibility. Therefore the genomic region upstream of the Gal-6 gene was searched for the presence of transcription factor binding sites associated with β-defensin regulation. A putative transcription start site (TSS) was predicted 70 bp upstream of the first nucleotide of the ATG start codon, preceded by a TATA box (-29 bp) and a CAAT box at –111 bp (Fig. 2). Further analysis of the Gal-6 upstream region using the JASPAR and Matinspector

programs indicated the presence of several putative transcription factor binding sites: nuclear factor kappa beta (NF-κB; –506 bp), activator protein-1 (AP-1; -1011 bp, -2700 bp, -2872 bp) and nuclear factor interleukin-6 (NF-IL6; -1108 bp, -1949 bp, -2630 bp, -2777 bp).

Fig. 2. Nucleotide sequence of the Gal-6 gene promoter region deduced from the published cDNA sequence (AY621324). Nucleotides are numbered relative to the predicted transcription start site (TSS, arrow), the partial exon 1 is shown in uppercase and the putative 5'UTR in italics. Putative transcription factor binding sites are in boldface and underlined: TATA; CCAAT box; NF-kB, nuclear factor kappa beta: AP-1, activator protein 1; NF-IL6, nuclear factor interleukin 6.

Clustal-W alignment of Gal-6 with avian and mammalian β-defensins. An NCBI database pBLAST search using the Gal-6 precursor sequence, showed more similarity between Gal-6 and mammalian β-defensins, than compared to other avian β-defensins (Fig. 3).

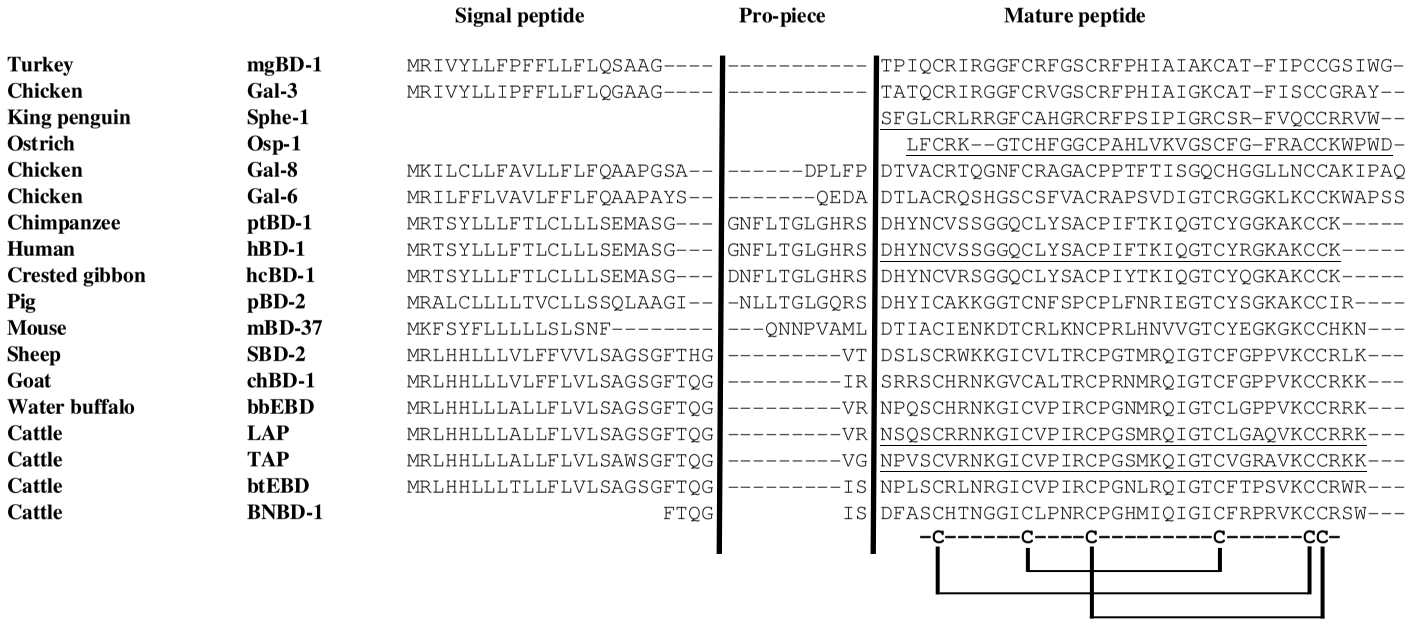

Fig. 3. Alignment of gallinacin-6 prepropeptide sequence with other avian and mammalian β-defensins. Mature peptide sequences are underlined.

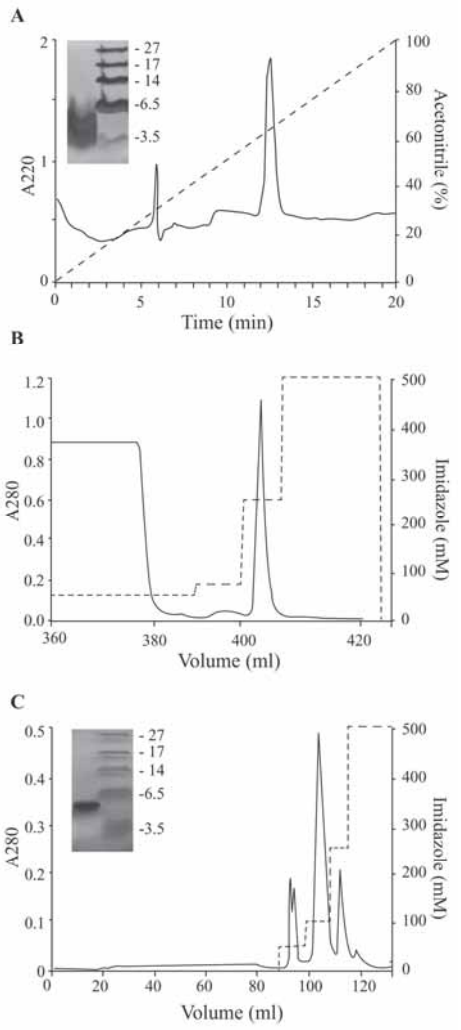

Fig. 4. Purification of synthetic and recombinant Gal-6. (A) Synthetic peptide was loaded on a C8 RP-HPLC column and eluted with a 20 min linear 0-100% gradient of acetonitrile in 0.1% TFA at a 1 ml/min flow rate. Peaks were detected at 220 nm. Purity of synthetic Gal-6 was verified on Tricine-SDS-PAGE gel. Maldi-Tof analysis showed a monoisotopic mass of 4282 ± 4 Da, in accordance with the calculated mass. (B) Fusion protein hGH-His8-TEV-rGal6 was purified from cell supernatant by affinity chromatography. Bound fusion protein was eluted from the HisTrap™ nickel column with 250 mM Imidazole in buffer A (25 mM Tris, 300 mM NaCl, pH 8.2). (C) Dialyzed fusion protein was proteolytically cleaved by TEV protease for 2 hours at 30ºC and re-applied to the nickel affinity column. The cleaved mature recombinant gallinacin-6 was eluted from the column at 50 mM imidazole in buffer A. Purity of rGal-6 was verified by Tricine-SDS-PAGE. The molecular mass of 4313 ± 4 Da, determined by Maldi-Tof analysis, was in accordance with the calculated mass.

Gal-6 was most similar to human β-defensin-1 (43% identity; 51% conserved), primate BD1-like defensins (43-45% identity; 54-58% conserved), bovine neutrophil β-defensins (33-44% identity; 54-61% conserved) and sheep β-defensin-2 (41% identity; 52% conserved). However, with the exception of gallinacin-8 (40% identity; 45% conserved), much less similarity was observed between Gal-6 and other avian β-defensins. A comparison of cleavage sites between pro-piece and antimicrobial peptide domains of β-defensins with known cleavage sites was used to predict a mature Gal-6 peptide sequence. A putative cleavage site was found between Ala^{26} and Asp^{27} (QEDA↓D) which resembled proteolytic cleavage sites in human β-defensin-1 and its primate orthologs (hBD-1, ptBD-1, hcBD-1; GHRS↓D), and bovine neutrophil β-defensin-1 (BNBD-1, QGIS↓D).

Production of synthetic gallinacin-6. Synthetic mature Gal-6 (sGal-6), synthesized by Fmoc solid-phase chemistry, was slowly refolded in buffer by air-oxidation to obtain the peptide in its thermodynamically most stable form. Final purification of folded peptide by reversed phase chromatography resulted in elution of a single peak at 12.46 min retention time (Fig. 4A). Lyophilized sGal-6 was dissolved in water and characterized by SDS-PAGE, amino acid analysis and mass spectrometry. SDS-PAGE analysis of purified sGal-6 showed a single band migrating at approximately 4.2 kDa (Fig. 4A). Amino acid analysis of the purified mature product was consistent with the expected sGal-6 composition and did not detect significant impurities. The molecular mass of 4282 ± 4 Da [M+] determined by MALDI-TOF analysis was in accordance with the calculated molecular mass of 4285 Da for synthetic Gal-6.

Production and characterization of recombinant gallinacin-6. Recombinant mature Gal-6 was expressed in HEK293-EBNA cells as a fusion protein with human growth hormone and subsequently purified by affinity chromatography (Fig. 4B and 4C) as described above. SDS-PAGE (Fig. 4C) and amino acid analysis of the purified mature product did not detect any impurities in the rGal-6 sample. The total yield was typically 125 μg/l culture of HEK293-EBNA cells. Further characterization of rGal-6 using N-terminal amino acid sequencing showed complete homology of the first 37 amino acids with the expected product. This corresponds to the mature sequence (as indicated in Table 2) with the Asp residue substituted by a Glycine and Serine residue at the N-terminus. These two amino acids are part of the TEV cleavage site and remain part of the Gal-6 N-terminus after TEV treatment. MALDI-TOF analysis of the purified rGal-6 peptide resulted in two observed peaks corresponding to the MH+ and MH2+ ions of a peptide with a molecular weight of 4313 ± 4 Da, which corresponds well with the calculated mass of 4314 Da. Quantitative analysis of sulfhydryl groups revealed that no cysteine residues were available for complexing with the Ellman's

reagent, indicating, in combination with the MALDI-TOF results, that all cysteines were involved in intramolecular disulfide linkages.

Table 2. Synthetic and recombinant gallinacin-6 peptides

Peptide	Amino acid sequence	Length	Charge
Gal-6 Propeptide	QEDADTLACRQSHGSCSFVAC-RAPSVDIGTCRGGKLKCCKWAPSS	45	+2
sGal-6	DTLACRQSHGSCSFVAC-RAPSVDIGTCRGGKLKCCKWAPSS	41	+4
rGal-6	GSTLACRQSHGSCSFVAC-RAPSVDIGTCRGGKLKCCKWAPSS	42	+5

Fig. 5. Concentration- and time-dependent inhibition of the growth of bacteria and yeasts by synthetic and recombinant gallinacin-6. The data are means ± standard deviation (n=3). (A) In colony counting assays, *E. coli*, *S. typhimurium*, *C. jejuni*, *C. perfringens*, *S. aureus*, *C. albicans* and *S. cerevisiae* (~2.5 x 10^6 cfu/ml) were incubated for 3 hours with various concentrations of synthetic Gal-6. *S. cerevisiae* cells were counted after 24 hours incubation on agar media at 25 ºC. All other strains were counted after 24 hours incubation on agar media at 37 ºC. (B) Additional colony counting assays were performed with *E. coli* (●), *S. typhimurium* (○), *S. aureus* (▽) and *C. perfringens* (▼) in 1% TSB containing 10 mM phosphate buffer (pH 6.5) as described above. (C) Inhibition of *E. coli*, *S. typhimurium*, *S. aureus* and *C. perfringens* in 1% TSB containing 10 mM phosphate buffer, pH 6.5 (white bars) supplemented with 20 mM (grey bars) or 150 mM (black bars) NaCl. (D) The killing kinetics of 16 µg/ml sGal-6 (▼), 64 µg/ml sGal-6 (▽) and 16 µg/ml rGal-6 (○) were determined against logarithmic phase *C. perfringens* cells in minimal medium (~2.5 x 10^6 cfu/ml for sGal-6, ~2.5 x 10^5 cfu/ml for rGal-6) in kill-curve studies. During a 150 min incubation time at 37 ºC under anaerobic conditions, aliquots were removed at various times and incubated overnight at 37ºC on TSA plates and surviving colonies counted. Bacteria resuspended in minimal medium in the absence of antimicrobial peptide and subjected to the same experimental conditions served as a control (●). All kill-curve studies were performed in duplicate.

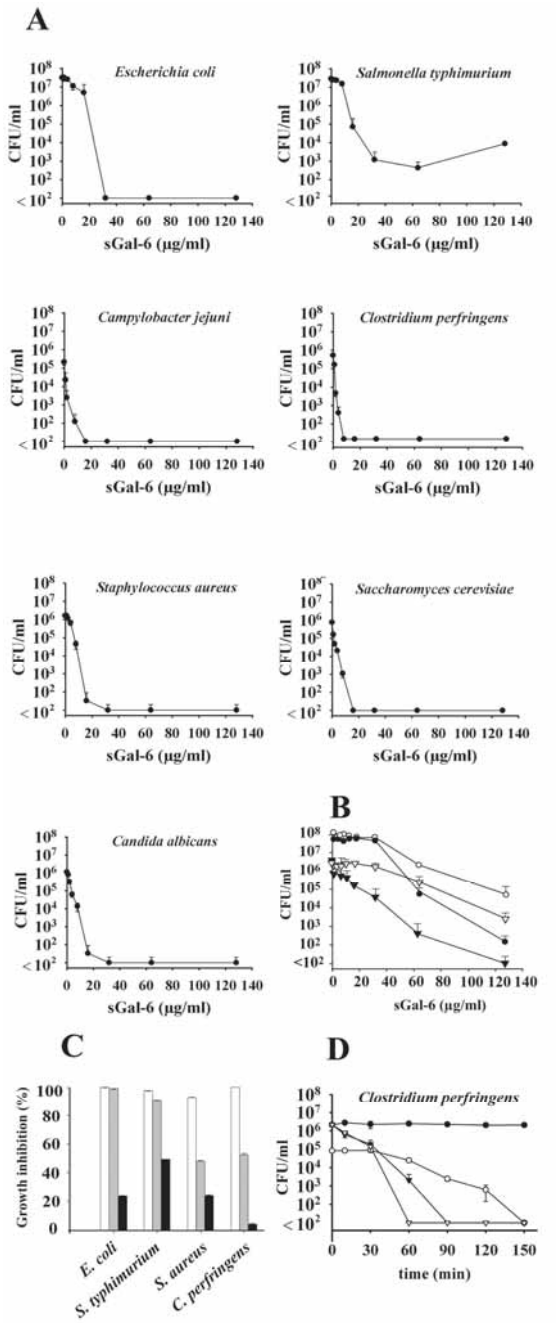
A
Escherichia coli
Salmonella typhimurium
Campylobacter jejuni
Clostridium perfringens
Staphylococcus aureus
Saccharomyces cerevisiae
Candida albicans
CFU/ml
sGal-6 (µg/ml)
B
C
Growth inhibition (%)
E. coli
S. typhimurium
S. aureus
C. perfringens
D
Clostridium perfringens
time (min)

Table 3. Antimicrobial activity of synthetic gallinacin-6 by broth microdilution method. Determination of minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC)

Microorganisms	sGal-6[a]		rGal-6[a]	
	MIC µg/ml	MBC µg/ml	MIC µg/ml	MBC µg/ml
Gram-negative				
Campylobacter jejuni	64	>128	64	128
Salmonella typhimurium	>128	>128	>128	>128
Escherichia coli	64	64	>128	>128
Pseudomonas aeruginosa	>128	>128	>128	>128
Gram-positive				
Clostridium perfringens	8	8	8	8
Streptococcus pyogenes	64	64	n.d.[b]	n.d.
Staphylococcus aureus	>128	>128	>128	>128
Bacillus cereus	>128	>128	<128	>128
Fungi				
Candida albicans	8	>128	32	>128
Saccharomyces cervisiae	8	>128	32	>128

[a] Final concentrations of 0 to 128 µg/ml peptide were incubated with 10^6 cfu/ml (sGal-6) or 10^5 cfu/ml (rGal-6) of each bacterial or fungal strain. All assays were performed in triplicate.
[b] Not determined.

Antimicrobial activity of synthetic and recombinant peptide. The dose-dependent survival of synthetic Gal-6 treated bacteria and yeasts were tested in colony counting assays (Fig. 5A). A fast decline in surviving cells was observed for *C. jejuni*, *C. perfringens*, *S. aureus*, *C. albicans* and *S. cerevisiae*, survival of these strains was reduced at 16 µg/ml to below the detection level of 100 cells/ml. A slower decline was observed in the assays performed with *E. coli* and *S. typhimurium*; even at 128 µg/ml sGal-6, *S. typhimurium* cells were not completely eradicated. Additionally, broth microdilution assays were performed to compare synthetic and recombinant Gal-6 antimicrobial spectra. Both peptides were active against gram-negative bacteria, gram-positive bacteria and yeasts (Table 3). Synthetic Gal-6 inhibited *C. jejuni* (MIC = 64 µg/ml), *E. coli* (MIC/MBC = 64 µg/ml), *C. perfringens* (MIC/MBC = 8 µg/ml) and *S. pyogenes* (MIC/MBC = 64 µg/ml), no inhibition was observed within these assays for *S. typhimurium*, *P. aeruginosa*, *S. aureus* and *B. cereus*. The yeasts *C. albicans* and *S. cerevisiae* were inhibited at low concentrations of sGal-6 (MIC = 8 µg/ml); prolonged

incubation at the highest concentration tested (128 μg/ml) showed this inhibition to be fungistatic of nature in this assay. Since recombinant peptide yield was relatively low, broth microdilution assays were performed with 10 μl of 10^5 cfu/ml in stead of the 25 μl of 10^6 cfu/ml used for synthetic peptide testing. The antimicrobial spectrum observed for recombinant gallinacin-6 was similar to that of synthetic Gal-6, although less potent. Recombinant Gal-6 was active against *C. jejuni* (MIC = 64 μg/ml; MBC = 128 μg/ml), *C. perfringens* (MIC = 8 μg/ml), *C. albicans* (MIC = 32 μg/ml) and *S. cerevisiae* (MIC = 32 μg/ml). As seen for sGal-6, the recombinant peptide showed bacterical and fungistatic activity in broth microdilution assays. pH measurements indicated that, in contrast to the colony count assays performed in 1000-fold diluted TSB minimal medium, bacterial cultures in 100-fold diluted TSB minimal media significantly decreased the pH of the assay medium during the 3 hr incubation period if the medium was not buffered. In colony count assays, sGal-6 inhibition of *E. coli* in 10 mM sodium phosphate buffer was investigated at pH 5.5, 6.0, 6.5 and 7.0 (data not shown). *E.coli* inhibition curves were similar at all pH values, indicating that sGal-6 killing was pH-independent in this range (data not shown). Exposure to sGal-6 in the presence of 10 mM sodium phosphate and 1% TSB significantly increased bacterial survival (Fig. 5B). Despite the 3 LOG unit decline in survival for *E. coli* and a 4 LOG unit decline in survival for *C. perfringens* observed at 64 μg/ml, at 128 μg/ml only *C. perfringens* survival was reduced to below the detection limit under these conditions. The presence of low salt (20 mM NaCl) did not affect sGal-6 mediated growth inhibition for *E. coli* and *S. typhimurium*, whereas growth inhibition of *S. aureus* and *C. perfringens* decreased to ~50% (Fig. 5C). In the presence of high salt (150 mM NaCl), growth inhibition was reduced to 49% for *S. typhimurium*, 24% for *E. coli* and *S. aureus* and 4% for *C. perfringens*.

Killing kinetics of synthetic and recombinant peptide. Kill-curve studies were performed with synthetic and recombinant Gal-6 peptide against a logarithmic-phase *C. perfingens* culture. A 3 LOG units decrease in viable *C. perfringens* cells was observed after 60 min when treated with 16 μg/ml sGal-6 (Fig. 5D). Treatment with 64 μg/ml sGal-6 decreased *C. perfringens* survival within 60 min to below the detection limit of 100 cell/ml. However, treatment with recombinant Gal-6 resulted only in a 0.5 LOG unit decline in surviving cells after 60 min of treatment and needed up to 150 min incubation time to reduce colony counts to practically zero. No effect on *C. perfringens* growth was detected in the presence of minimal TSB medium (negative control). Thus, again sGal-6 killing of *C. perfringens* proved to be more potent than rGal-6.

Effects of sGal-6 treatment on the ultrastructure of C. perfringens. To elucidate the nature of the killing mechanism(s) used by Gal-6, *C. perfringens* cells were treated for 30 min with 1.56 to 25 μg/ml sGal-6 and were investigated by transmission electron microscopy. Compared to the control treatment (Fig. 6A), sGal-6 treatment resulted in dose-dependent morphological changes: at 1.56 μg/ml sGal-6,

granulation of intracellular material (Fig. 6B) and irregular septa in dividing cells were observed (Fig. 6C). At 6.25 µg/ml similar observations were made for an increasing fraction of cells (Fig. 6D). Cells treated with 12.5 µg/ml sGal-6 showed other characteristics, such as retracting cytoplasm and membrane leakage (Fig. 6E), whereas lysis at the septa of dividing cells (Fig. 6F) became more prominent. At 25 µg/ml the majority of cells developed a ghost-like appearance combined with detachment of the cytoplasmic membrane from its peptidoglycan layer and concomitant membrane fragmentation (Fig. 6G) and resulted more often in complete lysis (Fig. 6H). Control (minimal TSB medium only) treated bacteria showed none of these effects.

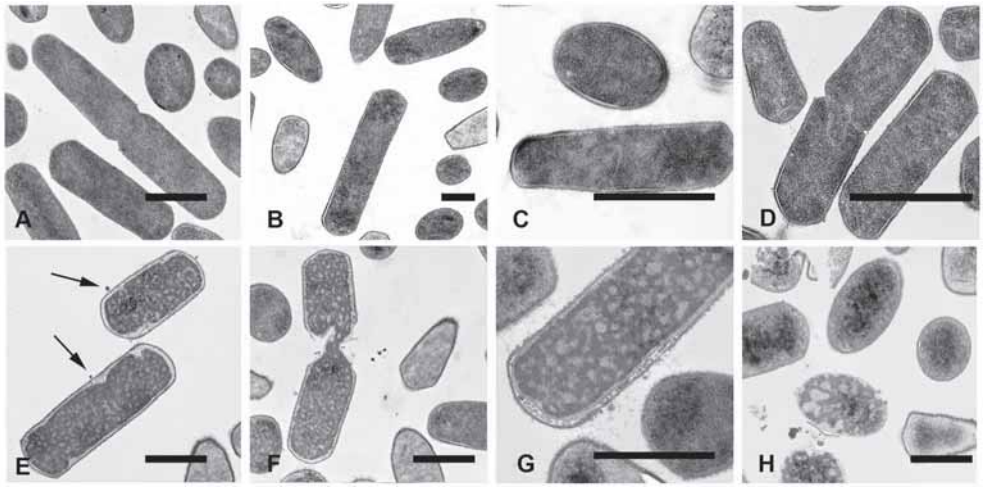

Fig. 6. Transmission electron microscopy of *C. perfringens* cells incubated with synthetic Gal-6. (A) Bacteria incubated in minimal medium for 30 min, were undamaged. In contrast, bacteria incubated for 30 min with increasing concentrations of sGal-6 showed dose-dependent changes in ultrastructure. Granulation of intracellular material was already observed at 1.56 µg/ml (B). Irregular septum formation in dividing cells was observed at 1.56 µg/ml (C) and 6.25 µg/ml (D). At 12.5 and 25 µg/ml, cells showed retracting cytoplasm (E), lysis at the septa of dividing cells (F), cytoplasmic membrane degradation (G), and complete cell lysis (H).

DISCUSSION

The predominant Gal-6 expression found in urogenital tract, liver, and bursa corroborated earlier reports (24, 46). However, in contradiction to our findings these studies showed no significant β-defensin expression in the proximal part of the

digestive tract. We found high expression levels of β-defensin gallinacin-6 in esophagus, crop and to a lesser extent in the proventriculus (glandular stomach). These differences could be due to variability in β-defensin expression between individual animals, choice of breed, animal age and experimental procedures. The similarly high levels of Gal-6 mRNA found in the crop tissues obtained from Hybro and Ross 308 broilers, showed that the high Gal-6 expression in crop tissue is not breed-dependent. However, the highly variable Gal-6 mRNA levels found in crop tissues of 13 day-old Ross 308 animals does suggest that Gal-6 may be developmentally regulated.

To investigate the possibility of a gallinacin-6 upregulation or inducibility in the upper digestive tract, the genomic sequence flanking the Gal-6 gene was searched for the presence of transcription binding sites. Several β-defensins have demonstrated to be upregulated by transcription factors involved in inflammation. Indeed, putative transcription binding sites were found for NF-κB, NF-IL6 and AP-1 in the 5' region flanking the Gal-6 gene. Inducible β-defensins, such as human β-defensin-2 (hBD-2) (2) and bovine tracheal antimicrobial peptide (TAP) (7), have been shown to be upregulated through the NF-κB pathway by a variety of inflammatory mediators. Transcription factors NF-κB and NF-IL6 have been demonstrated to synergistically participate in activation of numerous innate immune responses (3, 27) and are both markedly conserved in the promoter regions of TAP, hBD-2 and insect defensins attacin and diptericin (7). Additionally, activator protein-1 (AP-1) which plays a role in both basal and inducible transcription of numerous genes (12), may even be required in cooperation with NF-κB to achieve full gene expression, as demonstrated for monocyte chemoattracting protein-1 (MCP-1), tissue factor (TF) and hBD-2 genes (25, 26, 42, 44). Our findings suggest that Gal-6 expression might be induced or upregulated in the chicken digestive tract via NF-κB and AP-1 pathways.

The fact that high β-defensin mRNA levels were found in crop tissue, was not unexpected. Chickens practice coprophagy to recover vitamins, amino acids and other nutrients produced by their hindgut bacteria (28). The crop, an extention of the oesophagus, is especially well developed in chickens and other gallinaceous birds and serves to temporarily store food when the stomach is full and may hold its contents for up to 24 hours before it passes into the glandular stomach. Hence it requires an adequate local innate immune system.

To investigate the antimicrobial properties of Gal-6, synthetic and recombinant Gal-6 peptides were produced (Table 2) and tested against gram-positive bacteria, gram-negative bacteria and yeasts using colony counting assays (Fig. 5A) and broth microdilution assays (Table 3). In colony counting assays, synthetic Gal-6 peptide showed strong bactericidal and fungicidal activity against all investigated strains, including food-borne pathogens *Campylobacter jejuni*, *Salmonella typhimurium* and

Clostridium perfringens (fig. 5A). However, the magnitude of activity seemed dependent on the assay used, since sGal-6 showed higher activity and a broader antimicrobial spectrum in the colony counting assay compared to the broth microdilution assay (Table 3). The differences between both types of assays can be explained in part by the greater stringency of the broth microdilution assay, i.e. after the killing reaction has been terminated by dilution, surviving microorganisms form single colonies on agar plates, but can fully outgrow in liquid media. Additionally, the conditions for resuscitation of sub-lethally damaged cells seem to be more favourable in the broth microdilution assay compared to direct plating on agar media (4, 43). Colony count assays performed with minimal media containing 1:100 instead of 1:1000 diluted TSB showed only a marginal increase in bacterial growth for the investigated species and a markedly increased bacterial survival (Fig. 5B). It is well described that the microbicidal activity of cationic peptides is influenced by environmental factors (i.e. temperature, pH, ionic strength, cationicity) and microbial growth phase (20, 35, 38). Logarithmic growth phase organisms have been observed to be more susceptible to cationic peptide-mediated killing than organisms in a stationary growth phase, but exceptions have been noted (15, 20), indicating that this phenomenon is target species-specific. A 10 mM sodium phosphate buffer at neutral pH is commonly used to study the antimicrobial properties of cationic peptides. Yet, several reports suggest that the presence of phosphate buffer could interfere with cationic peptide-mediated inhibition (11, 34), although these effects were attributed to ionic strength.

However, in our experiments, addition of 20 mM NaCl did not affect the bactericidal activity of sGal-6 and even in the presence of 150 mM NaCl, sGal-6 still had bactericidal activity against most bacteria (Fig. 5C). Hence, the increased bacterial survival in these experiments can neither be explained by ionic strength differences nor by an increased nutrient availability.

The production of recombinant Gal-6 involved proteolytical cleavage of the hGH-His8-TEV-rGal-6 fusion protein by TEV protease and resulted in a 42 amino acid peptide in which the Asp^{1} residue was substituted by a Gly-Ser N-terminus (Table 2). Despite the higher positive charge of the recombinant peptide, thought to be associated with increased antimicrobial activity (6), broth microdilution assays showed that rGal-6 was far less potent than sGal-6 (Table 3). Antcheva et al. (1) made a similar observation for a variant of hBD2 lacking its N-terminal aspartic acid (Asp^{4}) residue. Compared to hBD-2, (-D)hBD-2 demonstrated to be less structured and had a markedly lower antimicrobial activity. Circular dichroism spectra of (–D)hBD-2 peptide showed little α-helical content in aqueous solution and in the presence of SDS micelles, whereas the hBD-2 spectrum was compatible with the presence of an α-helical stretch as observed in the crystal structure (19). The slower permeabilization and killing

kinetics observed for (-D)hBD-2 towards *E. coli* ML-35 suggest that the structure of the N-terminal stretch plays a role in mediating interaction with the bacterial membrane.

To elucidate the mechanism(s) involved in gallinacin-6 mediated killing of bacteria, the killing kinetics and morphology of food-borne pathogen *C. perfringens* cells were examined after treatment with synthetic Gal-6. In support of the broth microdilution assays, kill-curve studies of recombinant and synthetic gallinacin-6 showed a much faster killing mechanism and a greater efficacy for synthetic Gal-6. Similar kinetics have been reported for synthetic tick defensin A; treatment with MIC values of defensin A killed *Micrococcus luteus* cells within 60 min (29). A 1.5, 0.5, and 1 LOG unit decrease in survival of *S. aureus 710A* cells within 60 min was observed after treatment with synthetic hBD-2, (MIC), *Hylobates concolor* β-defensin-3 (4 × MIC), and human β-defensin-3 (8 × MIC), respectively, indicating an even slower but effective mechanism (31). Killing and permeabilization kinetics observed for these peptides (29, 31) indicate that cell lysis itself is not the primary mode of β-defensin-mediated bacterial killing. Apparently, a certain level of cell wall permeabilization is required for the β-defensin molecules to reach intracellular targets, possibly affecting DNA replication, RNA and protein synthesis (23).

Examination of sGal-6 treated *C. perfringens* by transmission electron microscopy (TEM) showed dose-dependent morphological effects as seen for other defensins. A 30 min treatment of 10^8 cfu/ml *C. perfringens* cells with sGal-6 concentrations ranging from 1.56 to 25 μg/ml induced dose-dependent changes, such as clumping of intracellular material and irregular septum formation during cell division at lower concentrations. At higher concentrations, most cells showed signs of cytoplasm retraction and detachment of the cytoplasmic membrane from the peptidoglycan layer, sometimes resulting in mesosome-like structures. Prior to complete lysis, often originating at the cell poles or at the septa of dividing cells, many cells developed a ghost-like appearance. Similar observations were described by Lee *et al.* (22), who observed chromatin condensation in *Haemophilus influenza* after 30 min treatment with 10 μg/ml hBD-2. Condensation of plasmid-sized DNA molecules can be induced by low concentrations of chemical agents with a cation valence of +3 or greater (37), including cationic peptides, such as spermine, spermidine, polylysine and protamine (5, 36). DNA condensation has also been observed by treatment with protein synthesis inhibitors, such as rifampicin, chloramphenicol and puromycin (49). *E. coli* cells treated for 30 min with 50 μg/ml HE2β1, an antibacterial peptide with similarity to β-defensins, inhibited DNA, RNA and protein synthesis and caused extensive granulation and cytoplasmic retraction (47). Mesosome-like structures have been observed for bacteria treated with defensins, the artificial peptides Bac2A-NH and

CP11CN, and antibiotics, such as trimethoprim and rifampicin(13, 38). The phenomenon of an antibacterial mechanism specifically aimed against septum formation and resulting in lysis near the septa of dividing cells has been reported for cationic peptides indolicidin and tick defensin A (14, 29). The morphological changes induced by low concentrations of sGal-6 and the lack of massive complete lysis at the highest concentrations tested, show cell lysis not to be the primary mechanism in Gal-6 mediated killing of prokaryotic cells.

In conclusion, to our knowledge this is the first report of a chicken β-defensin highly expressed in the digestive tract, and displaying strong bactericidal activity against food-borne pathogens. The high expression levels found in crop tissue indicate an important role for Gal-6 in chicken innate immunity. The presence of putative transcription factor binding sites involved in β-defensin induction and upregulation makes Gal-6 an excellent target to improve chicken digestive tract health and food safety by dietary modulation.

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Chapter 4

CMAP27, a novel chicken cathelicidin-like antimicrobial protein

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ABSTRACT

Cathelicidins, antimicrobial peptides with broad spectrum activity, have been almost exclusively found in mammals. Here we report the cloning of a novel avian cathelicidin, chicken myeloid antimicrobial peptide 27 (CMAP27) from chicken bone marrow cells. A combined expressed sequence tag (EST) and genomic based search revealed a cathelicidin-like gene located at the terminus of chromosome 2. RT-PCR and 5'RACE techniques resulted in a 154 amino acid prepropeptide, homologous to chicken cathelicidin 1 (51%) and most similar to α-helical myeloid antibacterial peptides (MAPs; 29-33%). A putative elastase cleavage site (LVQRG↓RF) suggests the production of a 27 amino acid antimicrobial peptide, predicted to adopt an α-helical configuration followed by a hydrophobic tail. Comparative analyses between antimicrobial peptide domains showed marked similarity between CMAP27 and MAPs members of the *bovidae* family, but not with the α-helical chicken cathelicidin 1. Strongest expression of CMAP27 mRNA was found in myeloid/lymphoid tissues, testis and uropygial gland. In accordance with the phylogenetic tree analysis, these findings support the theory of a common ancestral cathelicidin gene and suggest an important role for cathelicidins in chicken innate host defense.

INTRODUCTION

The broad antimicrobial spectra and wide-ranging distribution of cathelicidins indicate an important role in innate host defense. Antimicrobial peptides (AMPs) of the cathelicidin-family are encoded in the genome as prepropeptides and are proteolytically cleaved to form biologically active peptides ranging from 12 to 97 amino acids (15). Most cathelicidins are differentially expressed in a variety of tissues, exhibit broad spectrum antimicrobial activity against several Gram-negative and Gram-positive bacteria, fungi, protozoa and enveloped viruses (33). Additionally, they can bind and neutralize endotoxin (4) and induce chemotaxis of neutrophils, T cells and monocytes when separated from their cathelin-like propiece (31). Cathelicidins are well represented in mammals (1, 3, 9, 21, 24, 26, 27) and have been recently found in Atlantic hagfish (29) and chicken (8). However, their repertoire differs considerable among species. Given the extensive numbers of antimicrobial peptides found in some species, it is feasible that in the chicken substantial amounts of cathelicidins are expressed that can contribute to chicken innate host defense. The objective of this study was therefore to search for novel cathelicidin-like antimicrobial peptides and to investigate their tissue distribution.

RESULTS AND DISCUSSION

A translated nucleotide BLAST search (tBLASTn) in the TIGR chicken Expressed Sequence Tag (EST) database (http://www.tigr.org/tdb/tgi/gggi) using the amino acid sequence of chicken cathelicidin 1 precursor (GenBank™, AAS99323) resulted in multiple ESTs originating from a lymphoid tissue library. One tentative EST sequence (TC114957) encoded a precursor that contained a partial cathelin-like domain and a short putative C-terminal antimicrobial peptide domain, but lacked a signal peptide sequence. This EST nucleotide sequence was used in a genomic BLAT search of an annotated chicken genomic sequence database (UCSC Genome Bioinformatics Site; http://genome.ucsc.edu) and revealed a Gnomon-predicted novel cathelicidin-like gene (XP418514), consisting of three exons located at the terminus of chromosome 2. The predicted gene however, deviated much from other cathelicidin genes: 1) it encoded a 109 amino acid precursor, whereas the smallest known cathelicidin, indolicidin, is encoded by a 144 amino acid precursor (19); 2) only tree exons could be recognized in the genomic sequence, whereas other cathelicidin genes consist of four exons separated by three introns (15); 3) although signal peptide sequences are known to be well conserved within species, the signal peptide had no resemblance to chicken cathelicidin 1; 4) the cathelin-like domain showed a high degree of identity with cathelicidin 1, but

was significantly shorter and 5) the C-terminal antimicrobial peptide domain differed significantly from the EST encoded sequence. Furthermore, the genomic sequence positioned directly upstream of the predicted gene appeared not to be identified and could contain the "lacking" signal peptide and cathelin-like domain part.
To confirm the composition of the 3'end, primers were designed based on the putative 3' and 5' regions flanking the start and stop codon predicted by Gnomon (CC2F: 5'-GAGCTATGGGAGCATCTTGG-3'; CC2R: 5'-ACGTGGCCCCATTTATTCAT -3', respectively) and were used to clone and subsequently sequence the predicted gene product. First, total cellular RNA was extracted with Trizol® (Invitrogen, Carlsbad, CA) from bone marrow cells obtained from a 6 weeks old healthy female Ross 308 broiler chicken. DNAse treated RNA was used for cDNA synthesis using M-MLV-RT and dT12-18 primers (Invitrogen, Carlsbad, CA). PCR conditions were: 40 cycles of 95°C for 30s, 55°C for 30 s and 72°C for 45s followed by a final elongation step at 72°C for 7 min. Secondly, 5'RACE (Rapid Amplification of cDNA Ends) was performed to elucidate the upstream transcript sequence, using a BD SMART™ cDNA Amplification kit (BD Biosciences Clontech, Palo Alto, CA). Briefly, first-strand RACE ready cDNA was synthesized using the 5'CDS polyT primer and 1µg of total RNA extracted from bone marrow cells. A PCR reaction was performed with a universal primer mix and the gene specific primer (5'-GACGAGGCGTCTCTGCAGCGTAGAT-3'). Conditions were: 40 cycles of 95°C for 1 min, 68 °C for 1 min and 72°C for 1 min. After electrophoresis, a 570 bp band was cut out, purified with a QIAEX agarose gel extraction kit (Qiagen, Valencia, CA), cloned into a pGEM-T-Easy vector (Promega, Madison, WI) and transfected into *Escherichia coli* HB101 cells. Positive clones were screened with PCR for correct product size and sequenced. Sequencing reactions were performed with a ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA) in both directions using the T7 and Sp6 primer sites and separated on an ABI PRIM 3100 fluorescent DNA sequencer (Applied Biosystems, Foster City, CA).

The final consensus sequence resulted in a fragment of 474 bp, encoding a 154 amino acid precursor (Fig. 1). It contained a classical 17 amino acid N-terminal signal peptide sequence as predicted by SignalP software (version 3.0, http://www.cbs.dtu.dk/services/SignalP/). The remaining propeptide contained a characteristic cathelin-like domain with two conserved cysteine bridges and a signature similar to chicken cathelicidin 1 and mammalian cathelin-like domains. Its C-terminal antimicrobial domain proved to be identical to the Gnomon predicted sequence, not the EST sequence (data not shown).

To predict the size and properties of the C-terminal peptide, the propeptide sequence was compared with known cleavage sites of other cathelicidins (14, 18, 22,

24). Not surprisingly, a putative elastase cleavage site (LVQRG↓RF) encoded by the last exon, was found at position 127 (Gly_{127}-Arg_{128}) of the prepropeptide.

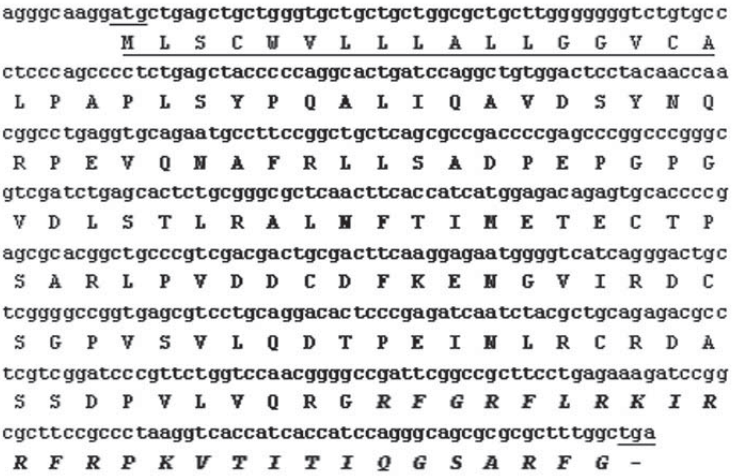

Fig. 1. Nucleotide and amino acid sequences for the CMAP27 precursor. Start and stop codons are underlined. Signal peptide sequence predicted by SignalP version 3.0 software (http://www.cbs.dtu.dk/services/SignalP/) is underlined and the predicted CMAP27 antibacterial peptide is displayed in bold and italics.

In mammals, processing of the intracellularly stored cathelicidin precursors is often mediated by serine proteases such as elastase and proteinase 3 (22). In agreement with our findings the last exon encodes for the C-terminal cationic antimicrobial peptide as well as its cleavage site (15). The elastase-catalyzed cleavage would generate a 27 amino acid cationic peptide with a net charge of +10. However, post-translational carboxyamidation of the glycine residue, is likely to occur, and is thought to provide resistance to proteolytic degradation by C-terminal exopeptidases (28, 32). The peptide would adopt an amphipathic α-helical conformation (Gly_{130}–Phe_{139}), according to the PSIPRED secondary structure method (5), followed by a C-terminal hydrophobic tail, which is sometimes associated with increased antifungal activity (23, 24).

To investigate species similarity, we used the prepropeptide sequence in a NCBI BLAST2 search and aligned it with the resulting sequences (Table 1). As expected, we found that the novel gene was strongly homologous to chicken

cathelicidin 1 (51%) and most similar to α-helical myeloid antibacterial peptides (29-33%), and was therefore designated by us chicken myeloid antimicrobial peptide 27 (CMAP27; Genbank™, AY817057). As expected, signal peptide sequences were extremely well conserved within chicken (94% identity), and highly similar to mammalian species (35-47% identity), but very different from hagfish signal peptide sequences. The cathelin-like domain was less conserved, with 56% intraspecies identity and interspecies homology comparable to that found for signal peptide sequences. The C-terminal antimicrobial peptide sequence, most similar to bovine and caprine peptides (~30%), showed only a weak similarity to equine and fish cathelicidin peptides. Interestingly, CMAP27 showed little similarity to chicken cathelicidin1, which was also predicted by PSIPRED to assume an α-helical configuration.

Table 1. Species similarity (%) between CMAP27 and other chicken, mammalian and fish α-helical cathelicidins at the amino acid level

		Prepro-Peptide	**Signal peptide**	**Cathelin-like domain**	**Antimicrobial peptide**
Avian	Cath1	51.3	94.1	56.0	7.1
Bovine	MAP27	33.1	47.1	32.1	28.6
Caprine	MAP28	31.8	35.3	31.2	32.1
Bovine	MAP28	31.2	47.1	29.4	28.6
Porcine	MAP36	31.2	41.2	31.2	25.0
Ovine	MAP29	29.9	41.2	29.4	25.0
Equine	MAP26	29.9	41.2	31.2	17.9
Fish	HAP29	16.9	23.5	16.5	14.3

In contrast to the C-terminal antimicrobial peptide, the exact function of the cathelin-like domain remains elusive. It has been implicated in cysteine protease inhibition (30) and suggested to be important in intracellular transport of the antimicrobial peptides to the granules (36) or aiding their maturation (35). However, the recent observation of antibacterial properties of the recombinant cathelin-like domain of human CAP18 against LL37-resistant bacterial strains, would suggest it to be directly involved in host defense (34). Its antibacterial mechanism is complementary to that of LL-37 and could act in synergy with each other and/or other antimicrobial substances, such as defensins (10).

The amino acid sequences of cathelicidin precursors from 8 different taxa, including chicken cathelicidin 1 and MAP27, were aligned using the ClustalX program (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). A phylogenetic tree of avian, mammalian and hagfish α-helical cathelicidin prepropeptide sequences was constructed by the neighbor-joining (NJ) method based on the Poisson-corrected estimate of the number of amino acid replacements per site (16) using MEGA version 2.1 software (7).

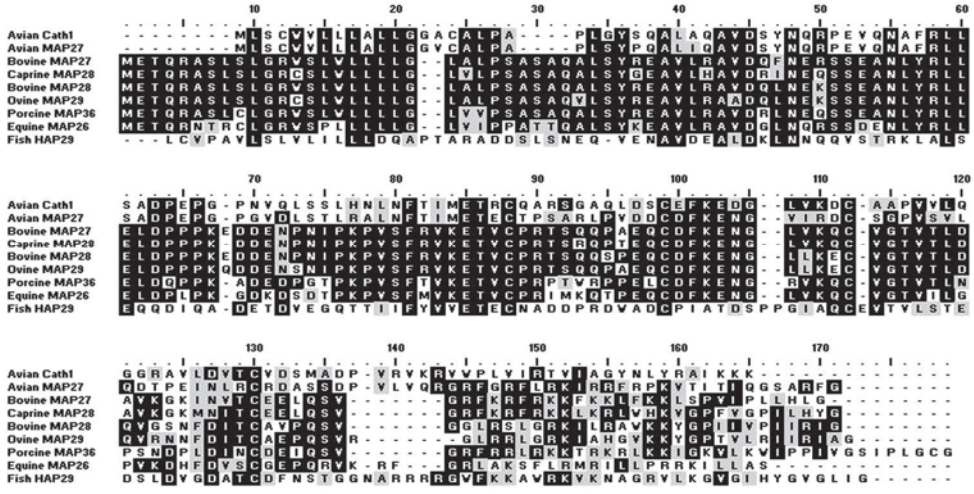

Fig. 2. Alignment of the chicken MAP27 prepropeptide with chicken cathelicidin1 and other α-helical mammalian and hagfish prepropeptide sequences; goat MAP28, sheep SMAP29, Atlantic hagfish HAP29, porcine MAP36, Equine MAP 26, bovine BMAP27 and BMAP28.

The reliability of tree branches in the phylogenetic tree was assessed by bootstrapping, using 1000 bootstrap replicates. In agreement with classical taxonomy based on morphological data, the phylogenetic tree showed a division in three distinct clusters with branch lengths emphasizing their relative evolutionary distances (Fig. 3). The observation that cathelicidins of the α-helical type are widespread, would indicate that initially an α-helical progenitor molecule expanded this gene family by gene duplication and divergence into a variety of homologous genes (35). As observed for β-defensins (20) and dermaseptins (11), the exon encoding the cathelicidin antimicrobial peptide is considered most prone to positive selection. Non-synonymous (amino acid altering) mutations in this exon, with an unbalanced preference for hydrophobic and positively charged amino acids, would therefore result in strongly heterogeneous

antimicrobial peptides. Hence, the presence of such a well conserved antimicrobial peptide signature in avian and mammalian lineages, which diverged ~310 million years ago (6), must reflect the importance of this subgroup in cathelicidin evolution.

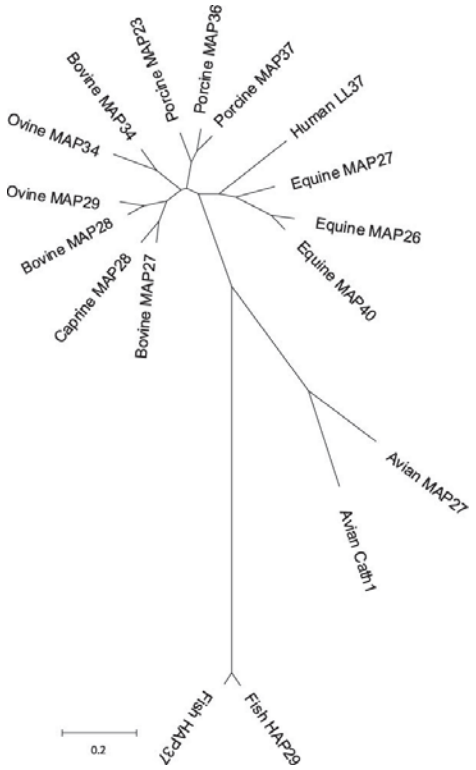

Fig. 3. Phylogenetic tree of prepropeptide sequences of α-helical cathelicidins from 8 taxa, constructed with the neighbor-joining method and tested with 1000 bootstrap replications. Branch lengths are proportional to the numbers of accumulated amino acid substitutions.

In order to determine the tissue distribution of the CMAP27 gene, three healthy 6 weeks old ROSS 308 broiler chickens, two females and one male, were sacrificed and 25 tissue samples were taken within 30 min for RNA isolation. Samples were rinsed with cold, sterile saline, frozen immediately in liquid nitrogen and stored at -80°C. Total RNA was extracted, pooled per tissue and DNAse I treated before cDNA synthesis as described above. Expression levels were investigated with the forward and reverse primers corresponding to the cathelin-like domain (5'-

ACACTGGCGTCGATCTGAG-3', 5'-CGTCTCTGCAGCGTAGATTG-3', respectively). The β-actin primers, forward primer cBactF (5'-ACCCTGTCCTGCTTACTGAGG-3') and cBactR (5'-TCCCAATGGTGATCACCTGCC-3'), corresponding to chicken β-actin, were used to assess the quality and quantity of the chicken mRNA samples. All PCR reactions were performed with Faststart DNA taq polymerase (Roche Diagnostics Gmbh, Mannheim, Germany) as follows: after an initial denaturing step of 5 min at 95°C, 40 cycles of 30s at 95°C, 30s at 55°C and 45s at 72°C were used for CMAP27 and 25 cycles of 30s at 95°C, 30s at 61°C and 45s at 72°C for β-actin, followed by a final elongation step at 72°C for 7 min.

Figure 4 shows that CMAP27 mRNA was highly expressed in myeloid and lymphoid tissues, most strongly in Bursa of Fabricius and bone marrow, whereas strong to moderate expression was observed in testis, spleen, kidney, liver and thymus (Fig. 4). CMAP27 was also highly expressed in the cecal tonsil, lymphoid tissue located at the base of the ceca near the ileal-cecal junction, whereas low expression was found throughout the rest of the intestinal tract. No expression was observed in tissues of the upper digestive tract, the low expression observed in the proventriculus (glandular stomach) is probably due to the presence of the esophageal tonsil located at the border of the esophagus and proventriculus (13) and is with the ceacal tonsil considered to be a part of the gut-associated lymphoid tissue (GALT).

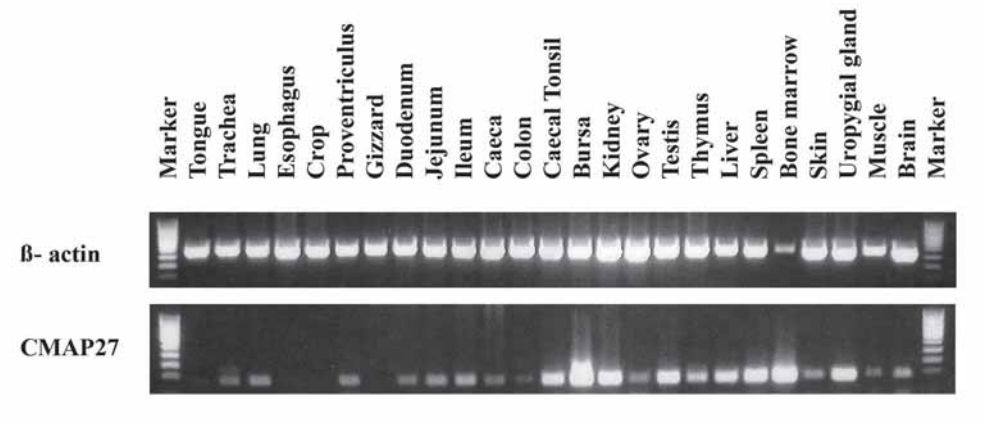

Fig. 4. Expression of CMAP and ß-actin mRNA in the digestive tract, respiratory tract, urogenital tract and lymphoid tissues of 6 weeks old healthy chicken broilers. Expected products were 186 bp (CMAP27) and 453 bp (β-actin).

While CMAP expression was low in skin tissue, a surprisingly high expression level was observed in uropygial (preen) gland tissue. This organ, a sebaceous gland located at the base of the tail of birds is relatively large in galliformes (25). It secretes a holocrine solution of waxes and oils which serves to maintain feather condition and, in birds other than water fowl, is spread over the plumage by dustbathing (17). The secreted preen oil contains alkyl-substituted wax acids and alcohols, which possess antimicrobial properties and may have a protective function (25). Considering the important effector function of cathelicidin-derived antimicrobial peptides in mammalian skin-associated innate immunity (2, 12), an equal role can be expected for excreted avian cathelicidins. Therefore, we postulate that CMAP27 peptides, when excreted with preen oil and distributed over plumage and skin, may contribute to innate host defense against skin pathogens.

ABBREVIATIONS

AMP, antimicrobial peptide; **CMAP27**, chicken myeloid antimicrobial peptide 27; **EST**, Expressed Sequence Tags; **RT-PCR**, reverse transcriptase-polymerase chain reaction; **RACE**, rapid amplification of cDNA ends; **BLAST**, basic local alignment search tool; **BLAT**, BLAST-like alignment tool

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Chapter 5

Localization and function of the chicken cathelicidin CMAP27 in peripheral blood cells and digestive tract tissues

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ABSTRACT

Cathelicidins possess potent antimicrobial and immunomodulatory activities and are part of the first line of host defense. We recently reported the discovery of a chicken myeloid antimicrobial peptide (CMAP27), which is highly expressed in myeloid and lymphoid organs. In this study, we investigated the microbicidal and cytotoxic properties of CMAP27 peptide and its localization in chicken digestive tract tissues and leukocyte subpopulations. Immunohistochemistry demonstrated abundant expression of CMAP27 in peroxidase-negative granulocytes (heterophils), but not in monocytes, thrombocytes or lymphocytes. Immunoblots of total leukocyte extracts with anti-CMAP27 antibody showed that CMAP27 is stored in its proform and is proteolytically cleaved into a mature peptide with an electrophoretic mobility identical to synthetic CMAP27 peptide. Stimulation of heterophils with *Salmonella*-derived LPS and, to a lesser extent, with phorbol ester (PMA), but not with formyl-Met-Leu-Phe (fMLP) induced the extracellular release of mature CMAP27 peptide in a time-dependent way. *S. enteriditis*-challenged 4 day-old Ross 308 broilers showed considerable infiltration of CMAP27-positive heterophils in the jejunum of challenged animals as early as 8 h p.i., whereas only few residential heterophils were detected in the jejunum of control animals. No detectable CMAP27 peptide was observed in the epithelial cell lining of the digestive tract of healthy or infected animals. Synthetic CMAP27 peptide showed potent bactericidal and fungicidal activity against all tested strains, including chicken-specific *Salmonella* isolates. The localization of at least one cathelicidin in heterophilic granulocytes with potent microbicidal activity against avian pathogens indicates a pivotal role for cathelicidins in avian innate immunity.

INTRODUCTION

Cathelicidins have been found in mammals (57), fish (9, 47) and more recently, in birds (33, 49). In general, they consist of a short signal peptide, an approximately 100 amino acid residue cathelin-like domain and a heterogeneous ~12 to 80 amino acid residue antimicrobial C-terminal domain (57). In mammals, cathelicidins have shown to be predominantly expressed by neutrophils (2, 11, 39, 45, 58, 59), but depending on the species also in various other myeloid and non-myeloid cells i.e. monocytes (1), macrophages (3), B cells (1), γδ T cells (1), skin keratinocytes (13, 19) and epithelial cells lining the respiratory, intestinal and urogenital tracts (3, 4, 20, 45). Mammalian cathelicidins have demonstrated to be important for the antimicrobial efficacy of neutrophils, macrophages and mast cells (1, 12, 39, 46, 56). Besides their potent direct antimicrobial activity against a broad spectrum of microorganisms, including bacteria, fungi, protozoa and enveloped viruses (5), some cathelicidin peptides demonstrated multiple immune modulatory activities, such as chemotaxis towards blood cells (54) and the binding and inactivation of endotoxin (53).

In birds, three chicken cathelicidins have been discovered up to date by genome and expressed sequence tag (EST) database searches, e.g. cathelicidin-1 (fowlicidin-1) (33), chicken myeloid antimicrobial peptide 27 (CMAP27)/fowlicidin-2 (49, 52) and fowlicidin-3 (52). Synthetic peptides based on the putative peptide sequences and elastase cleavage sites of these three cathelicidins demonstrated salt-insensitive antibacterial activity with minimal inhibitory concentrations in the range of 0.4 to 5.4 μM (52). The tissue distribution of chicken cathelicidins by reverse transcriptase PCR indicated high levels of cathelicidin-1 mRNA in gizzard, small and large intestine, bursa of Fabricius, testis and bone marrow (33), whereas highest expression of CMAP27 mRNA was observed in myeloid and lymphoid tissues (49). However, nothing is known about avian cathelicidin biosynthesis and processing or their localization at mucosal surfaces and in immune effector cells. Therefore, we investigated the localization of CMAP27 in leukocyte cell populations and digestive tract tissues by immunohistochemistry in healthy and *Salmonella*-challenged broilers, and characterized the antimicrobial properties of synthetic CMAP27 peptide.

MATERIALS AND METHODS

Animal experiments. Ross 308 chicken broilers were kept in wire cages with feed and water provided *ad libitum*, and orally inoculated at 4 days of age with 0.25 ml suspension containing 1×10^4 CFU nalidixin-resistant *S. enterica* serovar Enteriditis PT4 (Animal Sciences Group, Lelystad, The Netherlands). At 8 and 48 hours post

infection, *Salmonella*-infected and control animals were euthanized and dissected. Tissue samples were taken from crop, jejunum and liver of infected and control animals, fixed in 10% neutral buffered formalin solution for 24 hours and processed for paraffin-embedding according routine laboratory procedures for light microscopy (6). Chicken blood was collected from healthy adult animals for total leukocyte and heterophil isolation. All animal experiments were carried out according to protocols approved by the Intervet Animal Welfare Committee.

Peptide synthesis. CMAP27, CysCMAP27 and Cathelicidin-1 peptides were commercially synthesized by solid phase Fmoc chemistry at JPT Peptide Technologies (Berlin, Germany). Synthetic CMAP27 and cathelicidin-1 were produced based on the presence of putative elastase cleavage sites (49), (LVQRG↓RF) at position 127 (Gly_{127} – Arg_{128}) of the CMAP27 prepropeptide and (MADPV↓RV) at position 122 (Val_{122} – Arg_{123}) of the cathelicidin-1 prepropeptide. Because of the high probability of posttranslational carboxyamidation of the C-terminal glycine residue (49), CysCMAP27 and CMAP27 peptides were both synthesized with the C-terminal Gly residue substituted by an amide group. Peptide amino acid sequences were
RFGRFLRKIRRFRPKVTITIQGSARF-$\mathbf{NH_2}$ (CMAP27),
CRFGRFLRKIRRFRPKVTITIQGSARF-$\mathbf{NH_2}$ (CysCMAP27),
RVKRVWPLVIRTVIAGYNLYRAIKKK (cathelicidin-1). Synthetic peptides were purified by reversed phase HPLC and characterized by mass spectrometry, electrophoresis on Tris-tricine PAGE gels and dissolved in distilled water.

Crude leukocyte granule extraction. Total chicken leukocyte granule contents were extracted as described by Anderson and Yu (2). Sodium citrate anti-coagulated chicken blood was mixed with 0.83% ammonium chloride in a 3:1 ratio and left for 25 min on ice. Leukocytes were harvested after 15 min centrifugation at 700 × g (4 °C) and resuspended in ice-cold PBSX buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). After centrifugation (15 min, 700 × g, 4 °C) supernatant (S_1) was collected and stored on ice. Pelleted cells were resuspended in 1 ml of ice-cold PBSX buffer, sonicated and centrifuged for 40 min at 27,000 × g, after which the supernatant (S2) was aspired. Both supernatants and the remaining pellet (P) were extracted overnight in 10% acetic acid at 4 ºC. Extracted peptides were separated from cell debris by centrifugation (20 min, 27,000 × g, 4 °C) and lyophilized.

Isolation of chicken heterophils. EDTA anti-coagulated chicken blood was collected from wing veines and used for the isolation of chicken peripheral heterophils as described by Kogut *et al.* (30) with minor modifications. Whole blood was mixed at a 1.5:1 ratio with 1% methylcellulose (25 centiposes, Sigma Chemical Co., St. Louis, CA) and centrifuged for 30 min at 42 × g. Plasma and buffy coats were mixed with an equal volume of Ca^{2+}- and Mg^{2+}- free Hank's Balanced Salt Solution (HBSS, Sigma).

The leukocyte-enriched solution was layered over a discontinuous Optiprep™ density gradient (Axis Shield, Oslo, Norway) diluted in RPMI 1640 medium (Sigma) to specific gravities of 1.077 over 1.119. After 60 min centrifugation at 250 × g, the 1.077 band containing the mononuclear cells, the 1.077/1.1119 interphase and 1.119 band containing the heterophils were collected, washed twice and resuspended in RPMI 1640 medium. The cell fraction composition was determined by microscopic examination of leukocyte smears on 3-aminoalkyltrietoxysilane (AAS; Sigma) coated glass slides by direct DAB (3,3'diaminobenzidine; Sigma) staining for endogenous peroxidase activity, Giemsa- and diff-quick (Klinipath, Duiven, The Netherlands) staining. Cell viability and cell count of the heterophils fraction were determined by trypan blue exclusion. In brief, 20 µl cell suspension was centrifuged (5 min at 100 × g), and the resulting pellet was washed once in PBS and resuspended in 500 µl PBS. The diluted suspension was gently mixed with an equal volume of a 0.4% trypan blue stain (Invitrogen, Carlsbad, CA) and counted within 3-5 min in a Fuchs-Rosenthal hematocyte counter.

Exocytosis. Freshly isolated heterophils were resuspended in RPMI 1640 medium and diluted to 5×10^6 cells/ml. After 5 min preincubation at 37 ºC, 90 µl aliquots of cell suspension were stimulated with 10 µl agonist at final concentrations of 100 µg/ml phorbol myristate acetate (PMA; Sigma), 100 µg/ml N-formyl-Met-Leu-Phe peptide (fMLP; Sigma) or 100 µg/ml *S. typhimurium* lipopolysaccharide (LPS; Sigma) for 20 or 60 min at 37 ºC. Cell suspension kept on ice for 60 min served as control. After stimulation, cell suspensions were incubated 5 min on ice and centrifuged at 250 × g for 5 min (4 ºC). Cell supernatants were aspirated and stored at -80 ºC. Equivalents of 7×10^5 cells were dissolved in sample buffer and separated on 18% Tris-Tricine-SDS-gels. After electroblotting, nitrocellulose membranes were blocked and immunostained as described.

Generation of antibody. Rabbit polyclonal antibody against synthetic CysCMAP27 peptide was generated at Biogenes (Berlin, Germany). Five mg of synthetic CysCMAP27 was conjugated to limulus polyphemus hemocyanin (LPH) with 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester. Two rabbits were immunized at 7 days, followed by several boosts and bled after 322 days. Forty-five ml of antiserum was collected from one animal and purified by affinity chromatography on a CysCMAP27-coated CNBr-Sepharose column (5 ml, Amersham Biosciences). Forty-five ml of polyclonal antiserum was passed through the column after equilibration with several bed volumes of 50 mM Tris buffer containing 150 mM NaCl (pH 7.5). After substantial washing of the column bead support (15 vol. of 50 mM Tris-HCl, 150 mM NaCl, pH 7.5), 10 bed volumes of 20 mM Tris containing 2 M NaCl were used to elute unspecific bound proteins. The column was then connected to a spectrophotometer (Pharmacia LKB Uvicord SII; 280 nm) equipped with a recorder

(Pharmacia LKB Rec 102) and washed with buffer (50 mM Tris, 150 mM NaCl, pH 7.5) until OD_{280nm} reached the baseline. Monospecific IgG was then eluted from the column with 0.2 M Glycine-HCl buffer containing 250 mM NaCl (pH 2.2) based on peak absorbance at 280 nm and neutralized with 1 M Tris-HCl (pH 7.5). The eluate was centrifuged at 4500 × g for 15 min to remove any remaining debris, and filter sterilized (0.22 µm). To conserve the antibody, 1 µl of 20% Thimerosal per ml was added and aliquots were stored at -80 °C. The monospecific polyclonal antibody, isolated from antiserum, was quantitatively tested by ELISA. Hundred µl per well of 10 µg/ml CysCMAP-BSA conjugate in 0.05 M carbonate buffer (pH 9.5) were agitated overnight. Wells were emptied and blocked by incubation on a shaker with 200 µl/ well Tris buffered saline (TBS) containing 1.0% fetal calf serum (FCS) for 30 min at room temperature. Wells were washed 3 times with 300 µl TBS containing 0.05% Triton X-100 (TXTBS). Antibody dilutions ranging from 1:300 to 1:218,700 were prepared in TXBST containing 1.0% FCS and added in duplicate per well (100 µl/well), using 1.0% FCS in TXBST as a blank, and incubated on a shaker at room temperature. Wells were subsequently washed 4 times with 250 µl TXSBT and incubated with 100 µl anti-rabbit horseradish-labeled IgG (1:10,000 diluted, Biogenes) on a shaker for 1 hour at room temperature. Wells were again washed 4 times with 250 µl TXSBT. Hundred µl of 0.05% H_2O_2 in 50 mM sodium acetate, 0.12 mg/ml tetramethyl benzidine (pH 4.5) was added to each well and the reaction was stopped after 15 min by adding 100 µl of 0.5 M sulfuric acid. The absorption was read in a spectrophotometer at 450 nm. Aliquots were frozen in liquid nitrogen and stored at -80 ºC.

Immunoblotting. Cell extracts were analyzed by semi-quantitative dot blot and one-dimensional Tricine-SDS-PAGE (42). Synthetic CMAP27 peptide was used as a positive control. Samples were 1:1 mixed with sample buffer, 5 min heated at 95 ºC, separated on 18% Tris-Tricine SDS-PAGE gels and electroblotted on Protran® nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Immunostaining was performed using affinity purified rabbit polyclonal anti-CMAP27 antibody. Nitrocellulose membranes were blocked in 3% milk powder containing TTBS buffer (20 mM Tris, 0.5 M NaCl, 0.05 % Tween-20, pH 7.5) overnight at 4 ºC and subsequently incubated at room temperature for 1 hour with polyclonal rabbit anti-CMAP27 antibody (1:1000) diluted in blocking solution. Thereafter, membranes were washed three times for 10 min with TTBS buffer and incubated at room temperature for 1 hour with horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody (1:5000; Nordic, Tilburg, The Netherlands) diluted in blocking solution. Finally, excess secondary antibody was removed by washing 2 × 10 min in TTBS buffer, followed by a single 10 min wash step in TBS buffer (20 mM Tris, 0.5 M NaCl, pH 7.5). Immunoreactive protein bands were detected by enhanced chemiluminescence (ECL) using an Amersham ECL Western Blotting Detection Reagents kit (GE

Healthcare Bio-Sciences, Uppsala, Sweden). Membranes were incubated at room temperature with ECL reagent for 1 min and exposed to CL-XPosure film (Pierce, Rockford, IL).

Immunohistochemistry. Paraffin sections (5 µm) of each sample were cut (American Optical 820 microtome, American Optical corporation, Buffalo, NY), mounted on AAS-coated glass slides and dried overnight at 37 °C. Sections were dewaxed in xylene (2 rinses, 5 min each), rinsed in a 100% alcohol bath and were further rehydrated in alcohol 100%, 96%, 70% and distilled water. Subsequently, slides were Giemsa stained, photographed and kept 2 times 10 min in xylene to remove the cover slides. Sections were further washed in alcohol 100% (2 times, 2 min), 96% (2 min), 70% (2 min) and acidified alcohol (1% v/v HCl in 70% v/v ethanol, 5 min). The slides were thoroughly rinsed in running tap water, followed by a 2 min rinse in distilled water. Slides were dehydrated in alcohol 70%, 96% and 100%, whereafter endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol. The sections were further rehydrated in alcohol 100%, 96%, 70% and distilled water. Next, sections were thoroughly rinsed (3 rinses, 5 min each) in phosphate buffered saline (PBS) containing 0.05% Tween-20. To reduce non-specific background, slides were blocked with 10% normal goat serum (Jackson Immunoresearch, West Grove, PA) for 15 min at room temperature. Blocking serum was removed by tapping the slides, which were then incubated with the primary antibody anti-CMAP27 (1:100) for 60 min at room temperature. After another three washes (5 min each) with PBS/Tween, the sections were incubated for 30 min at room temperature with a biotinylated goat anti-rabbit antibody (Vector Laboratories Inc., Burlingame, CA). After three further 5 min washes in PBS/Tween, sections were flooded for 30 min with ABC reagent (Vector Laboratories Inc.) and subsequently washed with PBS. Finally, sections were incubated with 0.05% DAB in 0.05 M Tris buffer (pH 7.6) containing 0.03% hydrogen peroxide for 10 min. For negative controls primary antibody was omitted and replaced by PBS. Sections were counter stained with Mayer's hematoxylin for 15-30 s, dehydrated and mounted in Pertex (Klinipath). For endogenous peroxidase activity determination in cells and tissue sections, after dewaxing, the sections were rinsed in PBS (3 rinses, 5 min each). Subsequently slides were placed in 0.05% DAB in 0.05 M Tris buffer (pH 7.6) containing 0.03% hydrogen peroxide solution for 10 min and counterstained with Mayer's hematoxylin for 15-30 s.

Bacterial cultures. Reference strains *Bacillus cereus* ATCC 9139, *Candida albicans* ATCC 10231, *Clostridium perfringens* ATCC 13124, *Escherichia coli* ATCC 25922, *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) DT104 and *Staphylococcus aureus* ATCC 29213 were obtained from the American Tissue Culture Collection. *Saccharomyces cerevisiae* CBS 2978 was obtained from the Centraal bureau voor Schimmelcultures (CBS, Utrecht, The Netherlands). The bacterial strains

originally isolated from chicken feces, *S. typhimurium* 13563 and *Salmonella enterica serovar Enteriditis* (*S. enteriditis*) 13367, were a gift from Dr. Jaap P. Wagenaar (Veterinary Medical Diagnostic Centre, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University). *Clostridium perfringens* (37 °C, anaerobe) was grown overnight to stationary phase in Trypticase Soy Broth (TSB; Oxoid Limited). All other bacteria were maintained in TSB medium at 37 °C. The yeasts, *S. cerevisiae* and *C. albicans* were grown overnight to stationary phase in Yeast Maltose Broth medium (YMB; Fluka, Buchs, Switzerland) at 25 °C and 37 °C, respectively.

Antimicrobial activity assays. Colony-counting assays, as previously described (48), were used to evaluate the antimicrobial activity of synthetic peptides CMAP27 and Cathelicidin-1. Mid-log-phase growth bacteria were diluted in minimal TSB medium to reach a cell density of ~2.5×10^6 CFU/ml. Twenty-five µl of bacterial culture were mixed with 25 µl of 0 - 256 µg/ml synthetic peptide in polypropylene microtiter plates and preincubated for 3 hours at conditions suited to the investigated strain. Subsequently, 200 µl of minimal TSB (or YMB) medium was added to the contents of each well and further diluted 10 to 1000-fold in minimal TSB (or YMB) medium. Hundred µl of each well was spreadplated onto Trypticase Soy Agar plates or Yeast Maltose Agar plates and colonies were counted after 24 – 48 hours of incubation.

Hemolysis assay. Peptide-induced lysis of chicken red blood cells was assessed according the method of Klüver *et al.* (28) with minor modifications. Heparinized chicken blood was centrifuged for 10 min at 800 × g (20 °C) to sediment the red blood cells (RBCs). The cell pellet was resuspended in RPMI 1640 medium and stored at 4 °C until needed. The RBCs were washed three times in PBS, centrifuged for 10 min at 800 × g, and diluted 1:200 in PBS. The RBC concentration was determined in a Fuchs-Rosenthal hematocyte meter, using an additionally 100-fold diluted suspension in PBS. In 96-well polypropylene plates, 75 µl of sCMAP27 or cathelicidin-1 serial dilutions (0 to 128 µg/ml) were mixed with an equal volume of the 1:200 diluted chicken RBC suspension and incubated at 37 °C for 1 hour. PBS served as baseline and a 0.2% (v/v) Triton X-100 solution served as a control for complete lysis. Supernatants, collected after 10 min centrifugation at 1,300 × g (20 °C), were transferred into polystyrene 96-wells plates and absorbance was measured at 405 nm. Hemolysis (%) = $(A_{Peptide} - A_{Blank})/(A_{Triton} - A_{Blank}) \times 100$.

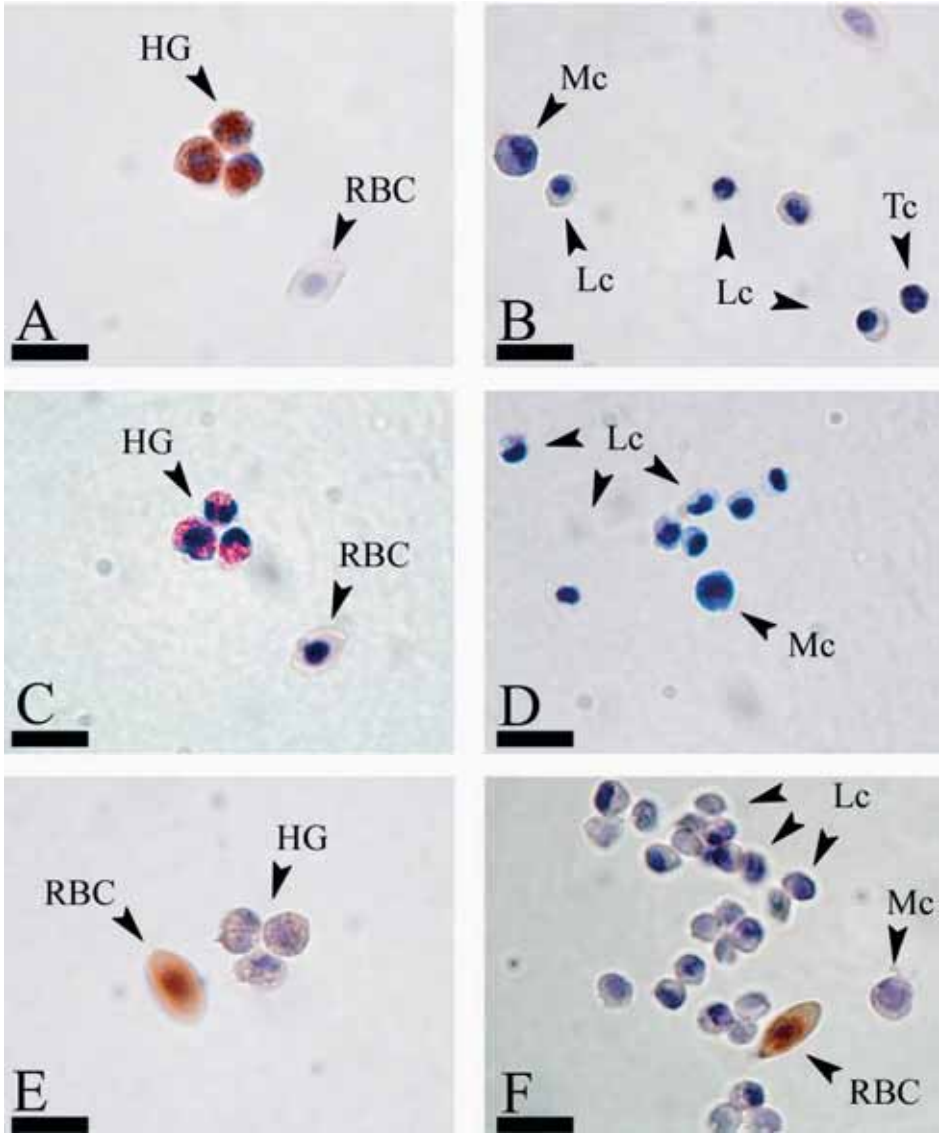


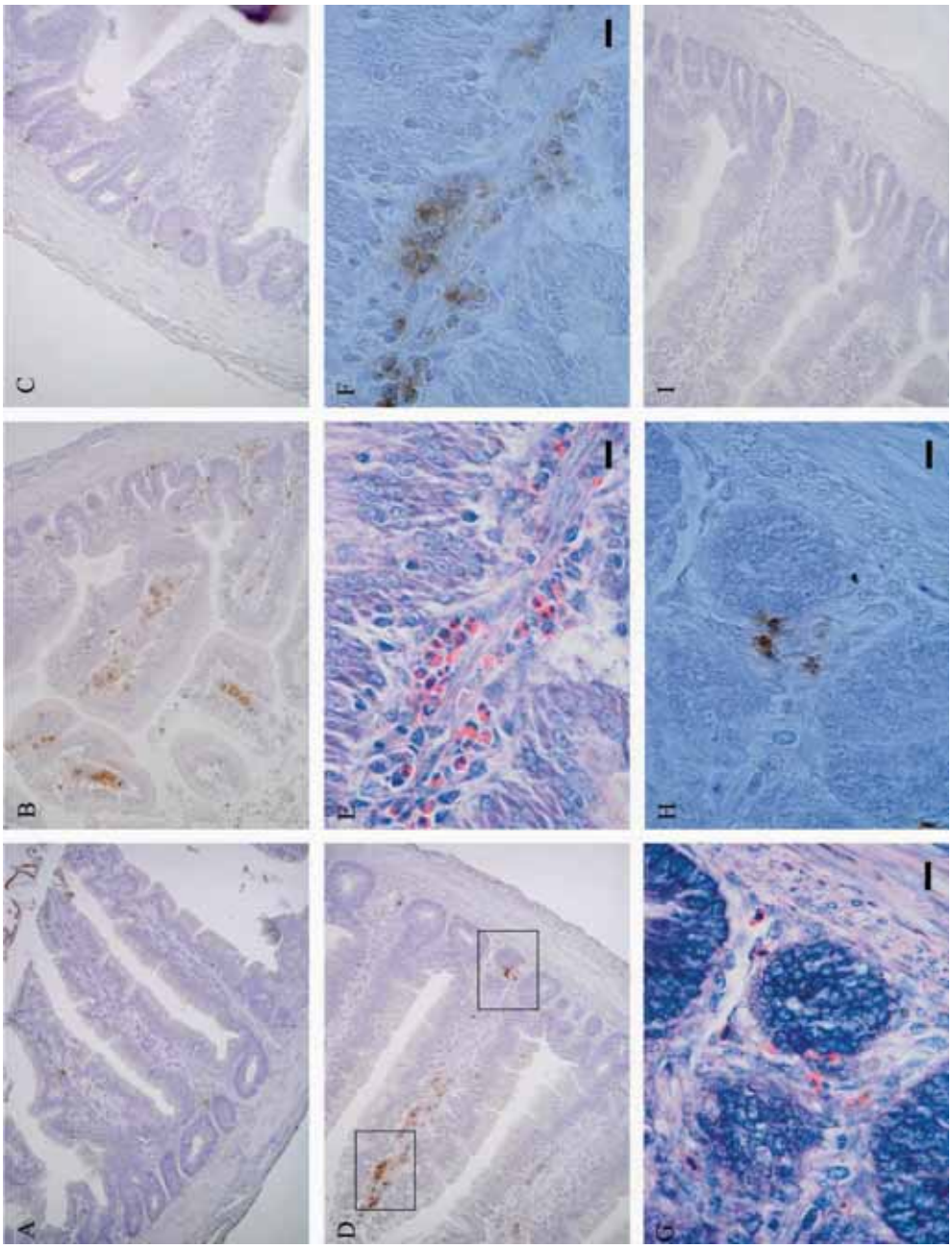
Fig. 1. Immunocytochemical staining of chicken peripheral blood leukocytes. Coated glass slide preparations of heterophil (A, C and E) and mononuclear (B, D and F) cell fractions were first stained with a Giemsa staining technique (C and D) and subsequently immunostained with anti-CMAP27 antibody (A and B). Endogenous peroxidase activity was determined by direct DAB cytochemical staining (E and F). *RBC*, red blood cell; *HG*, heterophilic granulocyte; *Mc*, monocyte; *Lc*, lymphocyte; *Tc*, thrombocyte. Magnification, 1000 ×; Bars indicate 20 µm.

RESULTS

CMAP27 is synthesized by chicken heterophils. Chicken peripheral blood cells were separated into mononuclear cells and granulocyte fractions by density gradient centrifugation which resulted in >95% viability for both fractions as determined by trypan blue exclusion. Microscopic examination of Giemsa and DAB-stained heterophil smears showed peroxidase-negative granulocytes, i.e. heterophils, whereas strong endogenous peroxidase activity was observed for contaminating erythrocytes (Fig. 1E and F). Immunohistochemical staining of granulocytes with anti-CMAP27 antibody showed strong immunoreactivity with peroxidase-negative heterophilic granulocytes (Fig. 1A, C and E). No CMAP27 immunoreactivity was observed for peripheral monocytes, lymphocytes, thrombocytes or erythrocytes obtained from healthy animals (Fig. 1B, D and F).

CMAP27 localization in digestive tract tissues. Immunohistochemical staining with anti-CMAP27 antibody showed that few immunoreactive cells were located in the jejunum lamina propria of 4- and 6-day old non-infected animals (Fig. 2A and C, respectively). In *Salmonella*-infected animals local infiltrations of CMAP27-positive cells were observed to accumulate in the jejunum villus lamina propria at 8 h (Fig. 2B) and 48 h (Fig. 2D, F and H) p.i. Based on their typical morphology observed with Giemsa staining (Fig. 2E, G), i.e. abundant orange-red large rod-shaped granula surrounding a bi-lobed nucleus, and the absence of endogenous peroxidase activity (data not shown), the vast majority of these cells could be identified as heterophilic granulocytes. No detectable CMAP27 expression was observed in intestinal epithelial cells of control animals or in animals after *Salmonella*-challenge. Little difference was observed between liver sections of control (Fig. 3A and C) and *Salmonella*-infected (Fig. 3B and D) animals, since all samples contained low numbers of CMAP27-positive cells. Interestingly, a small group of large lymphoid cells (~20 µm), containing many large granules was observed in the Giemsa-stained liver section of one *Salmonella*-infected animal dissected at 48 h p.i (Fig. 3E).

Fig. 2. Immunohistochemical staining of proCMAP27/CMAP27 in chicken jejunum of 4-day old control and *S. enteriditis*-infected animals, 8 and 48 hours post infection. Anti-CMAP27 immunostained paraffin-embedded jejunum sections of control (A, C) and infected animals (B, D), 8 and 48 hours after inoculation, respectively. Villus lamina propria (E, F) and crypt regions (G, H) of a single chicken jejunum section 48 hours p.i., subsequently stained with Giemsa and anti-CMAP27/hematoxylin, respectively. Immunostained jejunum section in absence of primary antibody (I). Magnification, 200 × (A to D, I) and 1000 × (E to H). Bars indicate 20 µm.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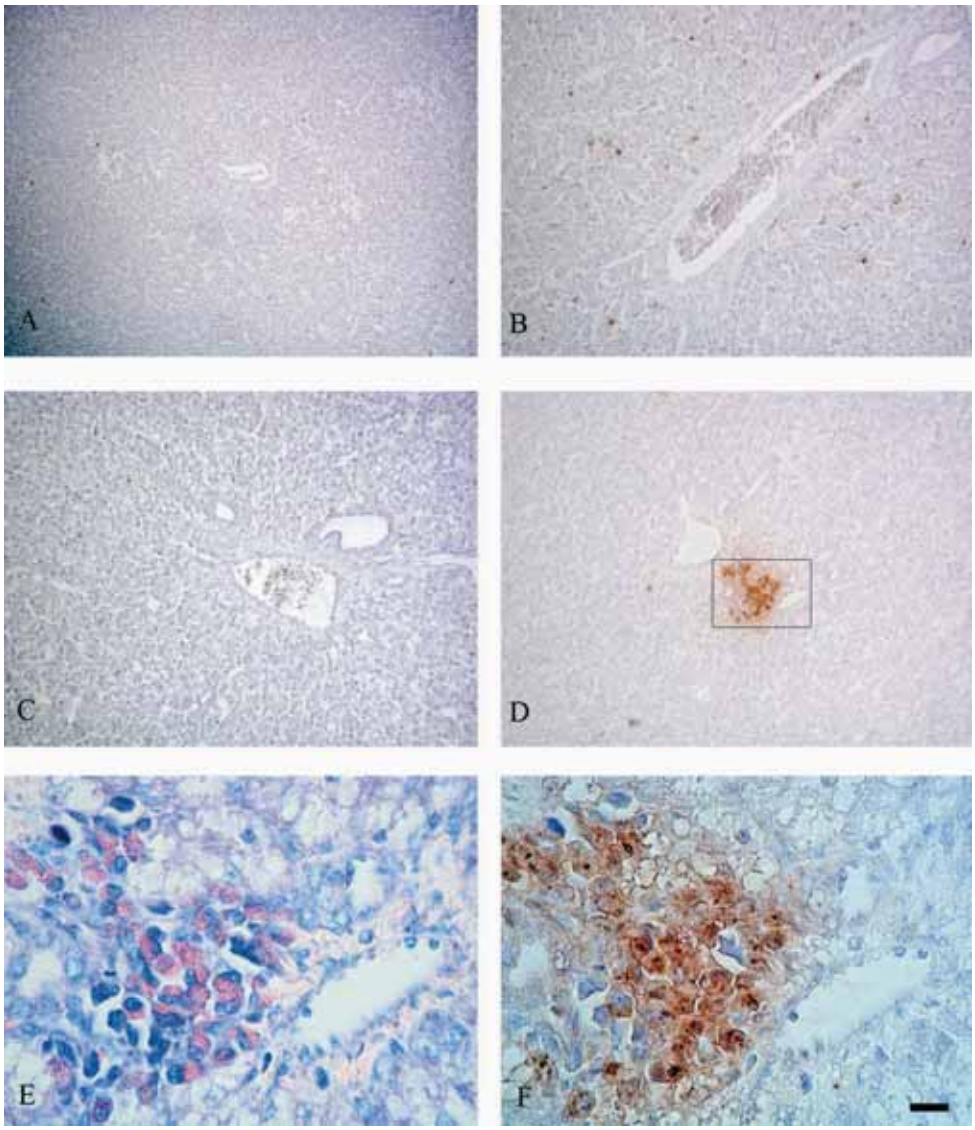


Fig. 3. Immunohistochemical staining of proCMAP27/CMAP27 in chicken liver of 4-day old control and *S. enteriditis*-infected animals, 8 and 48 hours post infection. Anti-CMAP27 immunostained paraffin-embedded liver sections of control (A, C) and infected animals (B, D), 8 and 48 hours after inoculation, respectively. Liver-specific NK (Pit) cells stained with Giemsa (E) and anti-CMAP27/hematoxylin (F), respectively. Magnification, 200 × (A to D) and 1000 × (E to F). Bars indicate 20 µm.

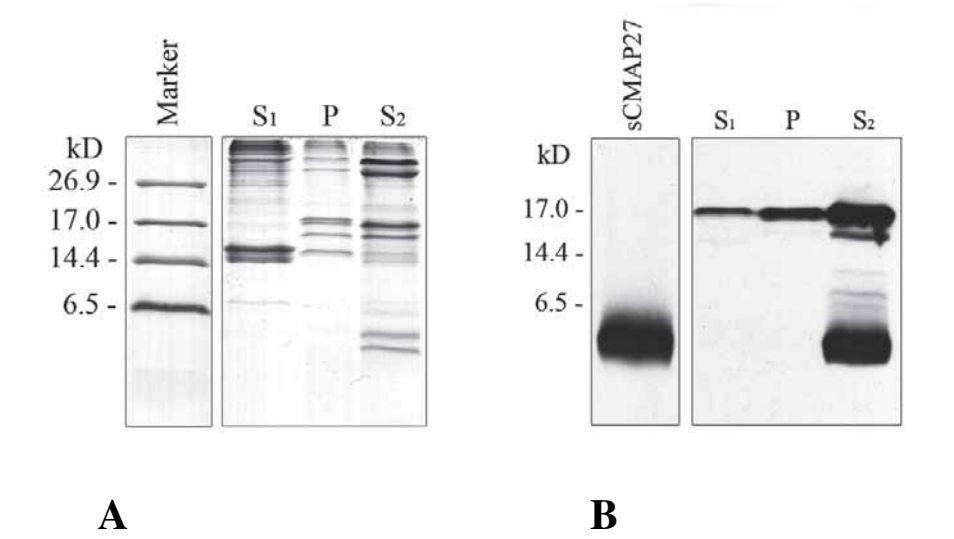


Fig. 4. Western blot analysis of crude leukocyte extracts. Supernatant obtained after leukocyte resuspension in PBSX buffer (S1), supernatant of sonicated leukocyte granule-enriched fraction (S2) and remaining granule pellet fraction (P) were separated by Tris-Tricine SDS-PAGE and subjected to Coomassie staining (**A**) or immunoblotted with anti-CMAP27 antibody (**B**). Synthetic CMAP27 peptide (0.125 µg) was used as positive control.

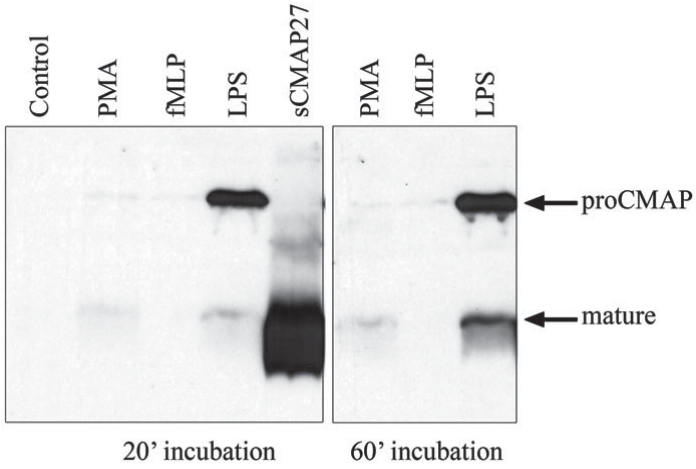
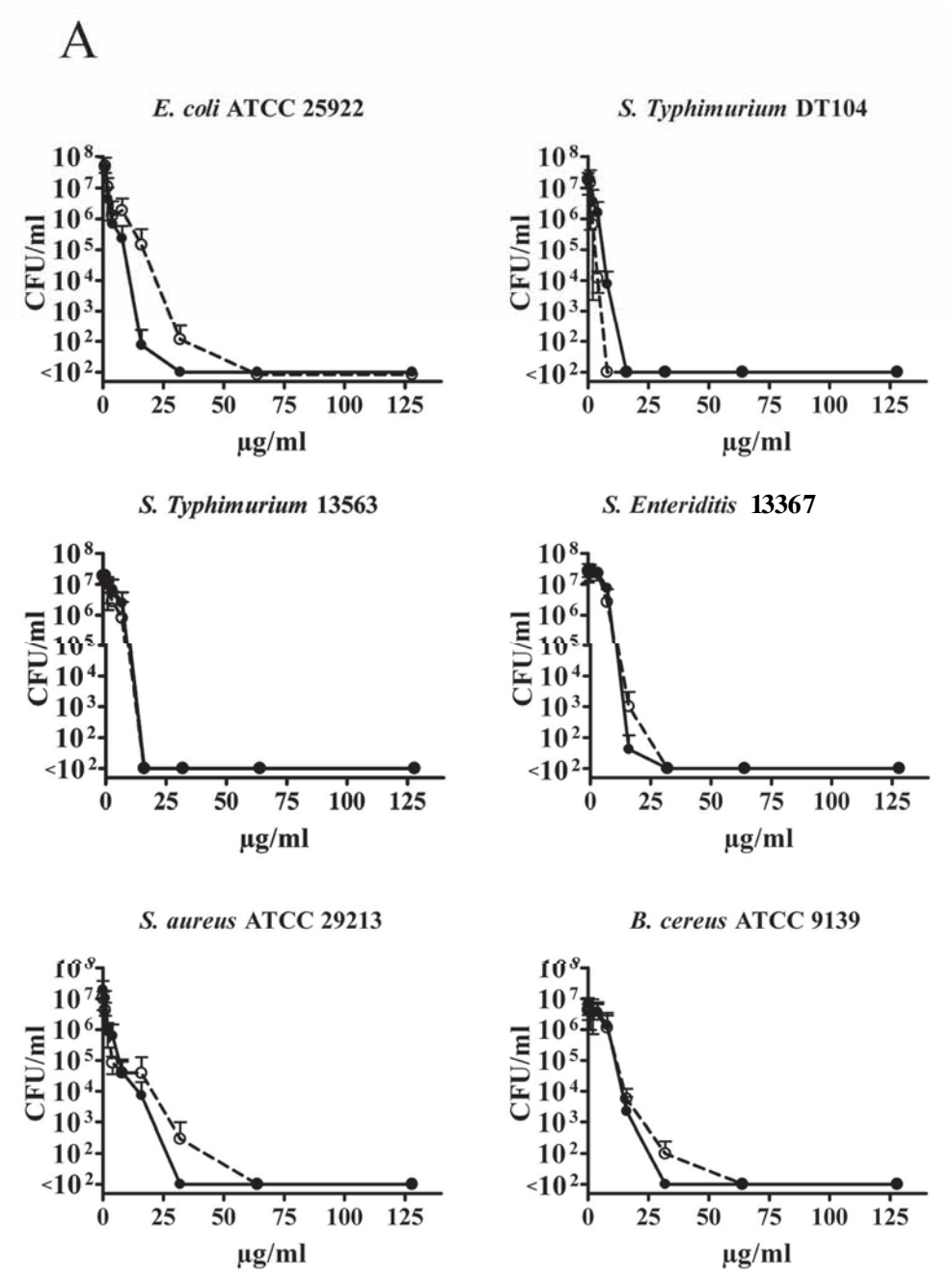


Fig. 5. Exocytosis of CMAP27 from activated chicken heterophils. Heterophils (5×10^6 cells/ml) were stimulated for 20 or 60 min at 37 ºC with 100 µg/ml phorbol myristate acetate (PMA), 100 µg/ml N-formyl-Met-Leu-Phe peptide (fMLP) or 100 µg/ml *S. typhimurium* lipopolysaccharide (LPS). After incubation, cell supernatants (equivalent to 7×10^5 cells) were separated by Tris-Tricine-SDS-PAGE and immunoblotted. Synthetic CMAP27 peptide (0.1 µg) was used as positive control.


A
E. coli ATCC 25922
S. Typhimurium DT104
S. Typhimurium 13563
S. Enteriditis 13367
S. aureus ATCC 29213
B. cereus ATCC 9139
CFU/ml
µg/ml
0
25
50
75
100
125

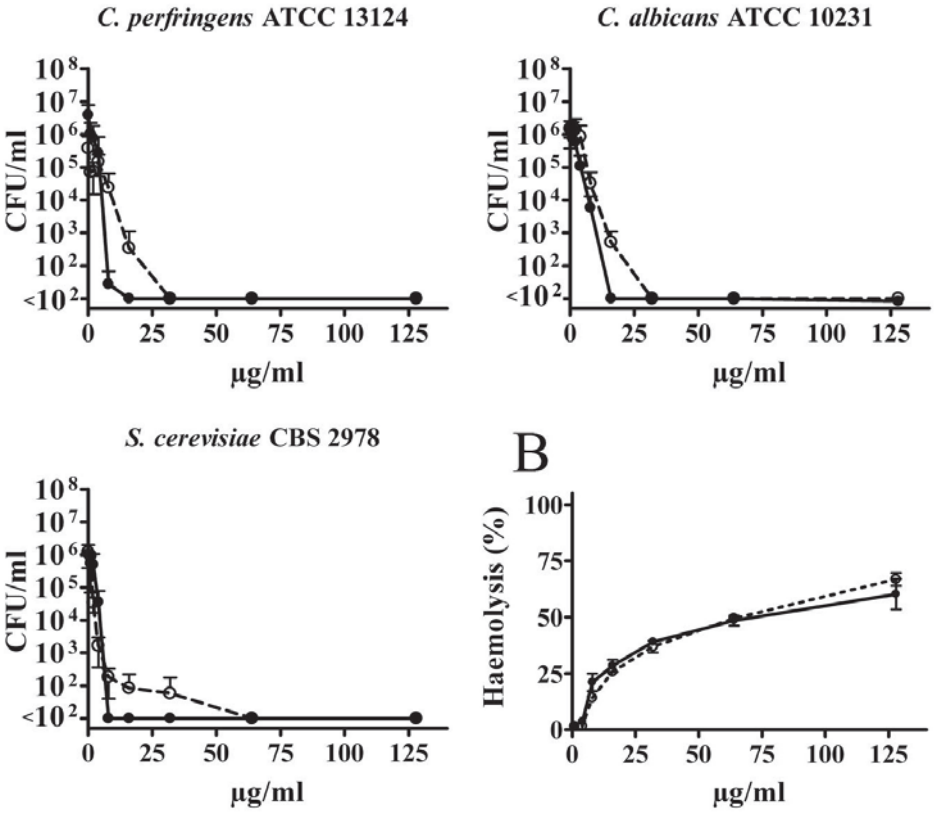


Fig. 6. Concentration-dependent growth of bacteria and yeasts and hemolytic activity against chicken erythrocytes by synthetic chicken myeloid antimicrobial peptide 27 (CMAP27) and synthetic chicken cathelicidin-1 (Cath-1) peptide. (**A**) In colony-counting assays, Gram-negative bacteria, Gram-positive bacteria and yeasts, including chicken isolates *S. typhimurium* 13563 and *S. enteriditis* 13367 were incubated for 3 hours with various concentrations of synthetic CMAP27 (●, continuous line) or Cathelicidin-1 (○, broken line) peptide. *S. cerevisiae* was counted after 48 hrs incubation on agar media at 25 ºC. The other strains were counted after 24 hrs incubation on agar media at 37 ºC. The data are means ± standard deviation, determined in triplicate on two different days (n=6). (**B**) Hemolytic activity of synthetic CMAP27 and Cath-1 peptides against chicken erythrocytes. Chicken erythrocytes (1% suspension) were incubated for 1 hour with various concentrations of synthetic CMAP27 (●) or Cath-1 (○) peptide. Phosphate-buffered Saline served as baseline and a 0.2% (v/v) Triton X-100 solution served as a control for complete lysis. After centrifugation, absorbance of the collected supernatants was measured at 405 nm. Hemolysis (%): (A_{405nm} Sample – A_{405nm} Blank)/(A_{405nm} Triton – A_{405nm} Blank) × 100%.

These cells proved to be strongly immunoreactive with the anti-CMAP27 antibody (Fig. 3D and F) and based on size and morphology identified as Pit cells, liver-specific natural killer cells. CMAP27-positive cells were scarcely detected in crop tissue of non-infected animals and *Salmonella* challenge only marginally increased infiltration of CMAP27-positive cells at 8 and 48 h p.i. (data not shown).

CMAP27 is stored as a precursor and processed upon release. Electrophoresis of chicken peripheral white blood cell extracts and subsequent immunoblotting with anti-CMAP27 antibody showed the presence of an approx. 17 kDa immunoreactive band in both leukocyte supernatants (S1 and S2) and pellet (P) (Fig. 4), while a ~4 kDa immunoreactive band was detected in the granule extract (S2) only.

Chicken heterophils release mature CMAP27 peptide by exocytosis. To determine whether CMAP27 was released extracellularly, heterophils were stimulated with PMA, fMLP or *Salmonella* LPS. Figure 5 shows that within a 20 min incubation period only LPS-stimulation of freshly isolated heterophils resulted in a significant release of CMAP27 precursor and its subsequent processing into a mature peptide. Furthermore, increased mature peptide formation occurred at prolonged incubation with LPS. PMA induced only a minor release and processing of CMAP27 precursor within a 60 min incubation period. Stimulation of heterophils with fMLP resulted in a trace of CMAP27 precursor after 60 min incubation, but mature peptide was not detected. Non-stimulated cell supernatant completely lacked CMAP27 immunoreactivity.

Synthetic CMAP27 displays potent microbicidal activity. The molecular mass of 3,205 and 3,140 ± 4 kDa [M+] determined by MALDI-TOF analysis for synthetic CMAP27 and cathelicidin-1, respectively, were in accordance their calculated molecular mass (CMAP27: 3206 Da; cathelicidin-1: 3141 Da). In colony-counting assays, synthetic CMAP27 peptide inhibited growth of *S. typhimurium* strains 13563 and DT104, *C. perfringens* and both yeast strains to below the detection limit of 100 cells/ml at peptide concentrations lower or equal to 16 µg/ml (Fig. 6). At 32 µg/ml, CMAP27 peptide similarly inhibited the growth of *S. enteriditis*, *E. coli*, *S. aureus* and *B. cereus*. Compared to synthetic cathelicidin-1 peptide, synthetic CMAP27 peptide achieved complete growth inhibition at 2-fold lower peptide concentrations for most strains and at a 8-fold lower peptide concentration against *S. cerevisiae*. Dose-dependent killing of *S. typhimurium* 13563 and *S. enteriditis* was equal for both peptides (16 µg/ml), whereas cathelicidin-1 peptide, as compared to CMAP27 peptide, totally inhibited the growth of *S. typhimurium* DT104 cells at a 2-fold lower (8 µg/ml) peptide concentration. Both peptides exhibited moderate cytotoxicity against chicken erythrocytes with cathelicidin-1 being slightly more cytotoxic at the highest peptide concentrations measured in this study (128 µg/ml). At 16 µg/ml, at which most tested

bacterial strains and both yeast strains were fully inhibited, 26-28% hemolysis of chicken red blood cells occurred.

DISCUSSION

The abundant CMAP27 expression found in chicken heterophils is in line with the prominent localization and function of cathelicidins in their mammalian counterpart, the neutrophil. No detectable CMAP27 expression was observed in other granulocytes or in mononuclear cells. The bovine cathelicidin bactenecin 5 displays a similar type of expression, abundantly expressed in bovine neutrophil large granules, but totally absent in eosinophils, monocytes and lymphocytes (58). In contrast, species expressing a single cathelicidin gene appear to have a much more differentiated expression profile. For instance, human CAP18/LL-37 is expressed by specific monocyte and lymphocyte populations, including monocytes/macrophages, NK cells, γδ T cells and B cells (1), and is also expressed by mucosal epithelia of the respiratory, urogenital and intestinal tracts (4, 20, 23, 34). Similarly, rhesus monkey rhCAP18/rhLL-37 expression was found in granulocytes, lung epithelial cells and alveolar macrophages (3) and in mice, CRAMP is present in neutrophils, lingual epithelium, salivary gland acinar cells and duct epithelial cells (21, 36). The presence of at least one cathelicidin and multiple β-defensins (16, 22), in chicken heterophils indicates that reactive oxygen species (ROS)-independent mechanisms are likely involved in the heterophil-mediated killing of pathogens. Heterophilic granulocytes are considered vital for chicken innate immune defense against bacterial infections (8, 24, 31, 51) and have demonstrated the capacity to kill *Salmonella* by phagocytosis *in vitro* (44). Moreover, the observed efficient killing of phagocytized *S. enteriditis* cells by heterophils was suggested to involve a ROS-independent mechanism (44).

Neonatal chicks are highly susceptible to *Salmonella* infections during the first 4 days post-hatch, but are able to control these infections thereafter (60). This can be explained by a period of immunoincompetence after birth during the maturation of the adaptive immune system. Lymphocyte development in primary immune organs continues for several weeks after hatching (10) and during the first two weeks post-hatch, chickens alter their primarily lipid-based metabolism to promote carbohydrate utilization of their grain-rich diet which directly affects their proliferation and effector functions (41). To investigate the relationship between CMAP27 expression and *Salmonella* infection, we examined by immunohistochemistry the localization of CMAP27 expression in the crop, jejunum and liver tissues of non-infected and *S. enteriditis*-challenged 4-day old chicks. Whereas in several mammalian species, infectious and inflammatory stimuli induced or upregulated tissue-specific cathelicidin

expression (13, 19), no induction of CMAP27 expression was detected in intestinal epithelial cells. At 8 h and 48 h p.i., *Salmonella*-challenged animals showed increased infiltration of CMAP27-positive heterophils in the villi lamina propria of the jejunum and to a lesser extent in crypt regions, whereas in non-infected animals only a marginal population of CMAP27-positive heterophils was observed. Compared to freshly isolated heterophils (Fig.1A), heterophilic granulocytes localized in the villus lamina propria at 48 h p.i. showed diffuse staining with anti-CMAP27 antibody around cells, indicating locally released peptide. After intestinal colonization and translocation through the intestinal epithelium, *S. enteriditis* is capable of colonizing liver within 24 h p.i. (50). However, in our study the *Salmonella*-challenge did not visibly affect the number of CMAP27-positive cells in liver tissue. Interestingly, CMAP27-positive Pit cells were identified in *Salmonella*-infected liver sections. Pit cells are liver-specific natural killer cells, suggested to be a more mature form of circulating NK cells (37). Beside their strong cytotoxic activity towards tumor cells and virus-infected cells (32), NK cells participate in the resistance against bacterial pathogens, which has been shown to involve the secretion of soluble factors, such as perforin and granulysin (40). In addition, activated bovine NK cells display cytotoxic activity against *Mycobacterium*-infected macrophages (15). This implies that chicken liver-specific NK cells may aid in clearance of *Salmonella*-infected macrophages in the liver and that CMAP27 may be part of their antimicrobial arsenal.

On SDS-PAGE, mature peptide co-migrated with synthetic CMAP27 peptide, which has a molecular weight of 3,205 Da, indicating that native mature peptide has a mass similar to that of sCMAP27. The deviation between the calculated molecular weights (3205 Da and 15230 Da) and estimated molecular weights based on migration on SDS-PAGE gels (4 and 17 kDa) for mature peptide and precursor, respectively, reflects the inaccuracy of SDS-PAGE in estimating molecular weights.

Cathelicidins are stored as inactive propeptides in the specific granules of mammalian neutrophils and are intra- or extracellularly released by exocytosis. Therefore, the ~17 kDa immunoreactive bands in leukocyte and heterophil extracts most likely correspond to the CMAP27 precursor without signal peptide (with a calculated mass of 15.2 kDa). Although posttranslational signal peptide processing has been observed for some yeast proteins, higher eukaryotes process signal peptides co-translationally, i.e. immediately or shortly after their translocation into the ER lumen (38). This is corroborated by the findings of Zanetti *et al.* (58) who were able to produce preprobactenecin 5 in artificial cell-free systems, but in bovine neutrophil granules, could only detected probactenecin 5. In our study, mature peptide formation occurred in the supernatant containing granule contents and not in the pellet fraction, which indicates that proCMAP27 is not membrane bound.

Depending on the type of granules they are stored in, agonists of granule exocytosis will differentially affect the release and processing of cathelicidins into the environment. In mammals, cathelicidin propeptides, stored in specific or large granules, are released together with the serine protease-rich contents of azurophilic granules and rapidly cleaved into mature peptides (58). Therefore, the release and processing of CMAP27 precursor by heterophils was investigated using different agonists of exocytosis. LPS-stimulation strongly induced heterophil granule exocytosis and its subsequent processing into mature peptide, whereas even at excessive amounts PMA-stimulation resulted in only a minor release and processing of proCMAP27 and fMLP-stimulation resulted in some release of proCMAP27 after prolonged incubation, but not of mature peptide. Although little is known about avian heterophil exocytosis, this process has been well studied in humans and to some extent in other mammals (17, 18, 35, 43, 55, 58). Phorbol esters are potent stimulators of human neutrophil specific and gelatinase granule exocytosis, and to a lesser extent of azurophilic granules (18, 26). fMLP, a powerful neutrophil chemotactic factor, stimulates the release of gelatinase granules, but mobilizes hardly any specific or azurophilic granules (18, 26, 27). These observations correspond well to our findings for fMLP and PMA-stimulated heterophils, as no mature peptide could be detected after fMLP-stimulations, whereas PMA-stimulation resulted in a time-dependent release of CMAP27 precursor and mature peptide. Chicken heterophils stimulated with *Salmonella*-derived LPS have shown to release a significant proportion of the azurophilic granule marker β-glucuronidase (29), whereas in human neutrophils LPS was seen to induce a significant release of lactoferrin (14), which co-localizes with hCAP18 in specific granules (43). Bovine neutrophils have demonstrated to release bactenecin-5 from their large granules upon stimulation with *E. coli*-derived LPS (46). Interestingly, bovine neutrophils stimulated with *E. coli* or LPS were reported to selectively induce *de novo* synthesis and secretion of large granule-associated cathelicidin bactenecin-5 peptide, but not of BMAP27, BMAP28, BMAP34, indolicidin, cyclic dodecapeptide or Bac7 peptides (46). This suggest that, like mammalian cathelicidins, CMAP27 is stored as a proform in heterophil specific granules, physically separated from its activating proteases, and is activated only when simultaneously released into the environment.

To investigate the antimicrobial and cytotoxic activity of mature CMAP27 peptide, synthetic CMAP27 and cathelicidin-1 peptides were incubated with Gram-negative, and Gram-positive bacteria, yeasts and chicken erythrocytes. In colony-counting assays, synthetic CMAP27 peptide displayed potent bactericidal activity against all tested strains, including a multidrug-resistant *S. typhimurium* DT104 strain. *Salmonella* strains isolated from chicken feces (*S. typhimurium* 13563 and *S. enteriditis* 13367) did not show enhanced resistance towards cathelicidin-mediated killing. Towards most strains, synthetic CMAP27 peptide displayed slightly more potent

bactericidal and fungicidal activity as compared to synthetic cathelicidin-1 peptide. Below 64 μg/ml, synthetic CMAP27 and cathelicidin-1 peptides exhibited similar cytotoxic activity towards chicken erythrocytes, whereas at the highest peptide concentration investigated, sCMAP27 was relatively less cytotoxic. In an earlier study, synthetic fowlicidin-2, a putative alternatively processed CMAP27 peptide containing 5 additional N-terminal aa residues, exhibited less microbicidal activity combined a higher cytotoxic activity against chicken red blood cells when compared to cathelicidin-1 (52). Because structural analysis has revealed comparable hinged helical 3D conformations for all three deduced mature chicken cathelicidin peptides (7, 25, 53), strong functional domain homology among these peptides can be expected. Structure-activity analysis of chicken cathelicidin-1-derived peptides revealed that its flexible N-terminal region contributes to cytotoxic and LPS-binding activity, but not to antimicrobial activity (53). Thus, the absence of these 5 N-terminal aa residues will render the mature peptide less cytotoxic, which is in accordance with our findings.

In conclusion, the potent microbicidal activity of mature CMAP27 peptide against *Salmonella* isolates and its abundant expression in chicken heterophilic granulocytes at the site of infection in *Salmonella*-challenged animals, indicate that cathelicidins play an important role in paratyphoid and other bacterial infections.

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Chapter 6

CMAP27 in chicken uropygial gland secretion and skin injury – Putative role in growth inhibition of feather-degrading microorganisms and wound-repair

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ABSTRACT

Most birds possess a single large sebaceous gland located at the base of the tail, the uropygial gland or preen gland; its secretion is distributed onto plumage during preening activity. We recently showed that chicken preen gland tissue can express moderate to high mRNA levels of antimicrobial peptides (56, 57), suggesting a potential role of the gland in avian skin-related innate immune defense. In this study we investigated the localization of chicken cathelicidin myeloid antimicrobial peptide 27 (CMAP27) in uropygial gland tissue and skin appendages by immunohistochemistry and its antimicrobial activity against skin and plumage associated microorganisms. Immunohistochemistry showed CMAP27 to be restricted to the secretory cells of the glandular epithelium lining the primary and secondary cavities of the uropygial gland, whereas no CMAP27 was detected in healthy skin, comb or wattles. Skin injury was observed to result in infiltration of CMAP27 positive heterophils and local induction of CMAP27 production in keratinocytes around wound edges. Dot blot and Western blot analysis detected variable amounts of CMAP27 precursor protein, but not mature peptide, in sebum from different animals, estimated at >0.25 nmol/g sebum. In colony-counting assay, synthetic CMAP27 peptide exhibited potent microbicidal activity against keratinolytic bacteria *B. licheniformis*, *Kocuria rhizophilae*, *Streptomyces pactum, Streptomyces fradiae* and the keratinolytic fungus *Chrysosporium keratinophilum*, resulting in complete growth inhibition at 4-16 µg/ml. Analysis of the CMAP27 promoter region revealed putative transcription factor binding sites known to be involved in regulation of mammalian cathelicidin expression in skin keratinocytes and myeloid cells. In addition, several binding sites were identified for transcription factors involved in the regulation of lipogenesis. These findings demonstrate that cathelicidins originating from uropygial gland secretions and locally induced cathelicidin expression by epidermal keratinocytes may protect skin and plumage against opportunistic pathogens.

INTRODUCTION

In birds, plumage maintenance is of vital importance as feather condition will greatly affect body thermo-regulation, ability to fly and reproductive success (8, 32, 52). Avian skin is relatively thin and permeable to facilitate thermo-regulation, yet skin infections occur rarely. Birds spend a significant proportion of their time with preening behavior in order to remove dirt, lice and maintain feather quality (38). In addition, birds molt once or twice a year to renew their plumage. Feathers do not accumulate in the environment because of the presence of natural decomposers of feather keratin in soil (31). Feathers are a poorly digestible substrate for most microorganisms as they consist almost entirely of supercoiled β-structures of keratin polypeptide chains cross-linked by disulfide bridges that are highly resistant to degradation by proteolytic enzymes (39). However, common soil bacteria and fungi have been demonstrated to substantially degrade feathers *in vitro* (7, 15, 28) and are commonly found in the plumage of wild and domesticated birds (8, 25, 32, 43), although they do not appear to severely affect plumage condition in healthy animals.

In mammals, skin-related innate immune defense has shown to involve multiple antimicrobial peptides and proteins, including members of the cathelicidin family (11, 16, 17, 20, 36, 48). Cathelicidins are important components of innate skin defense in mice, as demonstrated by their protecting role against necrotic skin infection caused by Group A Streptococci (36). Cathelicidin expression is practically absent in normal skin keratinocytes, but can be induced in response to injury (13, 18) and in inflammatory skin disorders (16). In addition, cathelicidin is constitutively expressed in abundance by human skin eccrine sweat glands and secreted via sweat onto the skin surface (33).

It has been demonstrated that some fatty acids and waxes present in uropygial gland secretion inhibit to some extent the growth of dermatophytes and keratinophilic as well as non-keratinophilic fungi (6, 43, 44), suggesting a role of uropygial gland secretion in the protection of skin and plumage. Recently we reported the discovery of a novel chicken cathelicidin, chicken myeloid antimicrobial peptide 27 (CMAP27), which was found to be highly expressed in uropygial gland tissue, whereas CMAP27 mRNA expression in skin tissue was low (57). Considering the relative importance of antimicrobial peptides in mammalian skin defense, it was hypothesized that antimicrobial peptides play a similar role in the protection of avian skin.

In this investigation we studied the expression of chicken cathelicidin CMAP27 in skin and uropygial gland tissue and uropygial gland secretion, and evaluated its growth inhibiting effects on keratinolytic bacteria and fungi.

MATERIALS AND METHODS

Animals. Healthy, non-infected adult ISA brown layer hens (Gezondheidheidsdienst voor Dieren, Deventer, The Netherlands), 19 weeks of age, were euthanized and dissected. Tissue samples were taken from skin, comb, wattles and uropygial gland, fixed in 10% neutral buffered formalin solution for 24 hours and processed for paraffin-embedding by routine laboratory procedures for light microscopy (3). Down feathers and uropygial gland secretions were collected from adult layer hens of different breeds. Uropygial gland secretions were obtained by manual gland stimulation and stored at -80 °C. Down feathers were stored at -80 °C until needed.

Peptide synthesis. CMAP27 and CysCMAP27 peptides were commercially synthesized by solid phase Fmoc chemistry at JPT Peptide Technologies (Berlin, Germany). Synthetic CMAP27 and CysCMAP27 were produced based on the presence of the putative elastase cleavage site (57), (LVQRG↓RF) at position 127 (Gly_{127} – Arg_{128}) of the CMAP27 prepropeptide. Because of the high probability of posttranslational carboxyamidation of the C-terminal glycine residue (57), CysCMAP27 and CMAP27 peptides were both synthesized with the C-terminal Gly residue substituted by an amide group. Peptide amino acid sequences were RFGRFLRKIRRFRPKVTITIQGSARF-$\mathbf{NH_2}$ (CMAP27), CRFGRFLRKIRRFRPKVTITIQGSARF-$\mathbf{NH_2}$ (CysCMAP27). Synthetic peptides were purified by reversed phase HPLC and characterized by mass spectrometry, electrophoresis on Tris-tricine PAGE gels and dissolved in distilled water.

Immunohistochemistry. Paraffin sections (2-5 µm) of each sample were cut (American Optical 820 microtome, American Optical corporation, Buffalo, NY), mounted on 3-aminoalkyltrietoxysilane (AAS) coated glass slides and dried overnight at 37 °C. Sections were de-waxed in xylene (2 rinses, 5 min each), rinsed in a 100% alcohol bath and were further rehydrated in alcohol 100%, 96%, 70%, and distilled water. Subsequently, slides were Giemsa stained, photographed and kept 2 times 10 min in xylene to removed cover slides. Sections were further washed in alcohol 100% (2 times, 2 min), 96% (2 min), 70% (2 min) and acidified alcohol (1% v/v HCl in 70% v/v ethanol, 5 min). The slides were thoroughly rinsed in running tap, followed by a 2 min rinse in distilled water. Slides were dehydrated in alcohol 70%, 96% and 100%, whereafter endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol. The sections were further rehydrated in alcohol 100%, 96%, 70% and distilled water. Next, sections were thoroughly rinsed (3 rinses, 5 min each) in phosphate buffered saline (PBS) containing 0.05% Tween-20. To reduce non-specific background, slides were blocked with 10% normal goat serum (Jackson Immunoresearch, West Grove, PA) for 15 min at room temperature. Blocking serum was removed by tapping the slides, which were then incubated with the primary

antibody anti-CMAP (1:100) for 60 min at room temperature. After another three washes (5 min each) with PBS/Tween, the sections were incubated for 30 min at room temperature with a biotinylated goat anti-rabbit antibody (Vector Laboratories Inc., Burlingame, CA). After three further 5 min washes in PBS/Tween, sections were flooded for 30 min with ABC reagent (Vector Laboratories Inc.) and subsequently washed with PBS. Finally, sections were incubated with 0.05% DAB in 0.05 M Tris buffer (pH 7.6) containing 0.03% hydrogen peroxide for 10 min. For negative controls primary antibody was omitted and replaced by PBS. Sections were counterstained with Mayer's hematoxylin for 15-30 s, dehydrated and mounted in Pertex (Klinipath, Duiven, The Netherlands). For negative controls primary antibody was omitted and replaced by PBS. For determination of endogenous peroxidase activity in cells and tissue sections, after dewaxing, the sections were rinsed in PBS (3 rinses, 5 min each). Subsequently, slides were placed in 0.05% DAB in 0.05 M Tris buffer (pH 7.6) containing 0.03% hydrogen peroxide solution for 10 min and counterstained with Mayer's hematoxylin for 15-30 s.

Extraction of uropygial gland secretion. Collected uropygial gland secretions from three animals were used for the extraction of proteinaceous material as follows: three ml of chloroform was added to approximately 120 mg of secreted wax per animal, vortexed and kept for 30 min at 4 °C. An equal volume of a 60% (v/v) acetonitrile, 1% (v/v) trifluoroacetic acid mixture in water was added, the suspensions were vortexed and kept for 30 min on a rollerbench (4 °C). After centrifugation (10 min, 4000 × g, 4 °C), the upper layer (ACN/TFA fraction 1) of each suspension was collected and stored on ice. Three ml of ACN/TFA solution was added to the remaining chloroform (bottom) layer, vortexed and centrifuged (10 min, 4000 × g, 4 °C). Both upper (ACN/TFA fraction 2) and bottom layers (chloroform fraction 1) were collected and stored on ice. The remaining pellet was resuspended in ACN/TFA solution by vortexing, centrifuged, after which the upper layer was collected (ACN/TFA fraction 3) and stored on ice. Per animal, lyophilized ACN/TFA fractions were resuspended in 150 µl distilled water. Chloroform fractions were applied on chloroform-activated 3 ml Silica cartridges (Waters, Milford, MA) and rinsed with chloroform to remove unbound material. Bound material was eluted from the cartridges using basic methanol (99.75 % MetOH, 0.25% NH_4OH), dried with N_2 gas and resuspended in 150 µl distilled water.

Dot blot and Western blot analysis. For dot blot analysis, 5 µl aliquots were spotted onto nitrocellulose membrane and air-dried. For western blotting, 8 µl aliquots were dissolved in SDS sample buffer, heated for 5 min at 95 °C. Synthetic CMAP27 peptide was used as a positive control. The samples were separated on a 18% Tris-tricine-SDS gel (45) and electroblotted on Protran® nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Immunostaining was performed using

affinity purified rabbit polyclonal anti-CMAP27 antibody. Nitrocellulose membranes were blocked in 3% milk powder containing TTBS buffer (20 mM Tris, 0.5 M NaCl, 0.05 % Tween-20, pH 7.5) overnight at 4 ºC and subsequently incubated at room temperature for 1 hour with polyclonal rabbit anti-CMAP27 antibody (1:1000) diluted in blocking solution. Thereafter, membranes were washed three times for 10 min with TTBS buffer and incubated at room temperature for 1 hour with horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody (1:5000; Nordic, Tilburg, The Netherlands) diluted in blocking solution. Finally, excess secondary antibody was removed by washing 2 × 10 min in TTBS buffer, followed by a single 10 min wash step in TBS buffer (20 mM Tris, 0.5 M NaCl, pH 7.5). Immunoreactive protein bands were detected by enhanced chemiluminescence (ECL) using an Amersham ECL Western Blotting Detection Reagents kit (GE Healthcare Bio-Sciences, Uppsala, Sweden). Membranes were incubated at room temperature with ECL reagent for 1 min and exposed to CL-XPosure film (Pierce, Rockford, IL).

Bacterial and fungal cultures. Reference strains *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853 were obtained from the American Tissue Culture Collection. *Bacillus licheniformis* ATCC 21424, *Kocuria rhizophilae* ATCC 9341, *Streptomyces pactum* ATCC 27456, *Streptomyces fradiae* ATCC 14544, *Chrysosporium keratinophilum* ATCC 14862 and *Scopulariopsis brevicaulis* NRRL1103 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellculturen Gmbh (Braunschweig, Germany). *E. coli*, *S. aureus* and *P. aeruginosa* were grown at 37 °C overnight to stationary phase in Trypticase Soy Broth (TSB; Oxoid Limited). *B. licheniformis* and *K. rhizophilae* were cultured for two days at 30 °C in nutrient broth (NB; Oxoid) and *Streptomyces sp.* were cultered for several days at 30 °C in Glucose Yeast Maltose broth (GYM; 4% glucose, 4% yeast extract, 10% malt extract, 2% $CaCO_3$, 12% agar, pH 7.2). The keratinolytic fungi were grown on Sabouraud Dextrose agar plates (SDA; 10% peptone, 40% dextrose, 20% agar) at 26 °C.

Feather-degradation. Fungal conidia were harvested from SDA plates as follows: 5 ml of a 1% Tween-20 solution was applied on the plate surface and conidia and hyphae were scraped of the surface with a cotton swab. The supension was then transferred into a sterile tube, vortexed at high speed and filtered through sterilized glass wool to separate the conidia from hyphae. The isolated conidia were then washed and diluted in 1000-fold diluted Sabouraud Dextrose broth (0.01% peptone, 0.04% dextrose) and counted in a Fuchs-Rosenthal hemocyte meter. Bacterial suspensions were washed in 1000-fold diluted TSB or NB medium. To investigate if the selected bacterial and fungal strains possessed true keratinolytic activity, feathers were sterilized without degradation-facilitating treatments, such as UV-radiation or heating, by a procedure that simultaneously removed old gland wax. Chicken down feathers

were de-fatted and sterilized by 3 times washing in chloroform/methanol solution (1:1), rinsed 3 times in distilled water and air dried. Single feathers were inserted in cell culture flasks, containing 25 ml of 10^6 CFU/ml bacterial cells or fungal conidia in medium A (0.7 g/L KH_2PO_4, 1.4 g/L K_2HPO_4, 0.5 g/L NaCl, 0.06 g/L $CaCl_2$, 0.1 g $MgCl_2 \cdot H_2O$, pH 7) (27) or medium B (1.5 g/L K_2HPO_4, 0.015 g/L $FeSO_4 \cdot 7H_2O$, 0.005 g/L $ZnSO_4 \cdot 7H_2O$, 0.025 g/L $MgSO_4 \cdot 7H_2O$, 0.025 g/L $CaCl_2$, pH 8) (15, 25) and incubated during 4 weeks at 30 °C while gently shaking.

Antimicrobial assays. The antimicrobial activity of synthetic CMAP27 (sCMAP27) peptide towards bacterial and fungal strains was investigated using colony-counting assays as described previously (56). Bacterial concentrations were determined by measuring the optical density and were grown to an OD_{620nm} of 0.4 – 0.7 and diluted to ~2.5×10^6 CFU/ml in 1000-fold diluted TSB or NB medium. Conidia suspensions obtained from filamentous fungi were diluted in 1000-fold diluted SD medium (0.01% peptone, 0.04% dextrose) to 5×10^4 conidia/ml. Microorganisms were incubated in triplicate at 30 ºC for 3 h with 0-256 µg/ml sCMAP27 peptide in 50 µl of minimal (i.e. 1000-fold diluted) medium. After incubation, the well content was diluted 10-, 100- and 1000-fold in minimal medium and 100 µl of the solutions were spread on agar plates. For each dilution, the number of colony-forming units (CFU) was determined after 24-48 h of incubation at 30 ºC for bacteria and 72-96 h of incubation for filamentous fungi at 30 ºC and expressed as the number of surviving CFU per ml.

Promoter analysis. The published cDNA contig sequence containing the CMAP27 gene was used to investigate the 5' flanking sequence for putative transcription factor binding sites, using the JASPAR (http://jaspar.genereg.net/) and MatInspector software (http://www.genomatix.de/). A putative transcription start site (TSS) was predicted using neural netwerk promoter prediction software (Berkeley Drosophila Genome Project, http://www.fruitfly.org/seq_tools/promoter.html).

RESULTS

Localization of proCMAP/CMAP27 in uropygial gland tissue. The chicken uropygial gland consists of two pear-shaped lobes containing a large primary cavity in which sebum is collected and discharged through separate single ducts onto the gland exterior (Fig. 1A). The lobe walls are formed by radially oriented secretory tubules, where along the tubule length, the layer thickness of cells containing lipoid bodies gradually increases (30). The tubule interior is composed of a thin layer of basal cells (germinative layer), covered by a secretory area that formes and stores lipoid secretion granules (secretory area) and a subsequent degenerative (transitional) layer where cells become hypertrophic. In the latter layer lipoid granules coalesce into secretory spheres

and nuclei become pycnotic. When cells reach the innermost part of the tubule, cell walls rupture and granule contents plus cell debris form a keratinized layer (corneus laminiae) that is sloughed off into the tubule lumen and migrates towards the secondary cavities. Where tubules emerge in the primary cavity, a network of variably sized trabeculae is formed by the tubule walls (Fig. 1B) and produced sebum is emptied in the spaces in between (i.e. secondary cavities) and eventually stored in the primary cavity (30). Immunohistochemical staining of chicken uropygial gland frontal sections with anti-CMAP27 antibody showed ubiquitous expression of (pro)CMAP27 in glandular epithelial cells lining the trabeculae network surrounding the secondary cavities (Fig. 2A, C) and primary cavity wall (Fig. 2E, G). Near secondary cavities and at the bottom of the two primary cavities, anti-CMAP27 staining was localized in a thin layer of secretory cells with distinct spheres of secretion, visible as empty spaces (Fig. 2A-D). In the glandular epithelial layer covering the primary cavity side walls, anti-CMAP27 staining was dispersed throughout the secretory en transitional area (Fig. 2E-H). No immunoreactivity was observed in uropygial gland primary ducts (not shown).

ProCMAP/CMAP27 is not expressed in healthy skin or skin derivatives. In all investigated animals, ventral or dorsal skin epidermis sections, comb and wattle sections did not exhibit immunoreactivity with anti-CMAP27 antibody (Fig. 3).

ProCMAP/CMAP27 is involved in wound healing. The uropygial gland sections of one animal showed a recent injury of the papilla epidermal surface. Immunostaining with anti-CMAP27 antibody and Giemsa staining showed a massive infiltration of CMAP27-positive granulocytes at the site of injury (Fig. 4A-D), identified by the absence of endogenous peroxidase activity as heterophils (Fig. 4E). In addition, (pro)CMAP27 expression was locally induced in neighboring keratinocytes (Fig. 4B, D).

ProCMAP27 protein is secreted via uropygial gland sebum. Immunospecificity of the anti-CMAP27 antibody in dot blot and Western blot analysis was confirmed by the absence of detectable staining for synthetic cathelicidin-1 peptide on dot blots (Fig. 5A), and prestained benchmark plus 2 (Invitrogen) and polypeptide markers (Bio-rad Laboratories) on Western blots (not shown). Dot blot analysis showed weak to moderate anti-CMAP27 immunoreactivity in the ACN/TFA extracted sebum, while immunoreactivity was minimal or absent in chloroform fractions (Fig. 5B). Immunoblotting of fractions separated on a one-dimensional Tris-Tricine-SDS gel showed a single band corresponding to the CMAP precursor in ACN/TFA fractions obtained from animal 2 and 3 only. Mature CMAP27 was not detected (Fig. 5C). Based on band intensity comparison with sCMAP27 peptide (0.8 and 8 pmol), the amount of proCMAP27 present in these lanes was estimated to be >1.6 pmol, which corresponds to >0.25 nmol proCMAP27/g uropygial gland wax.

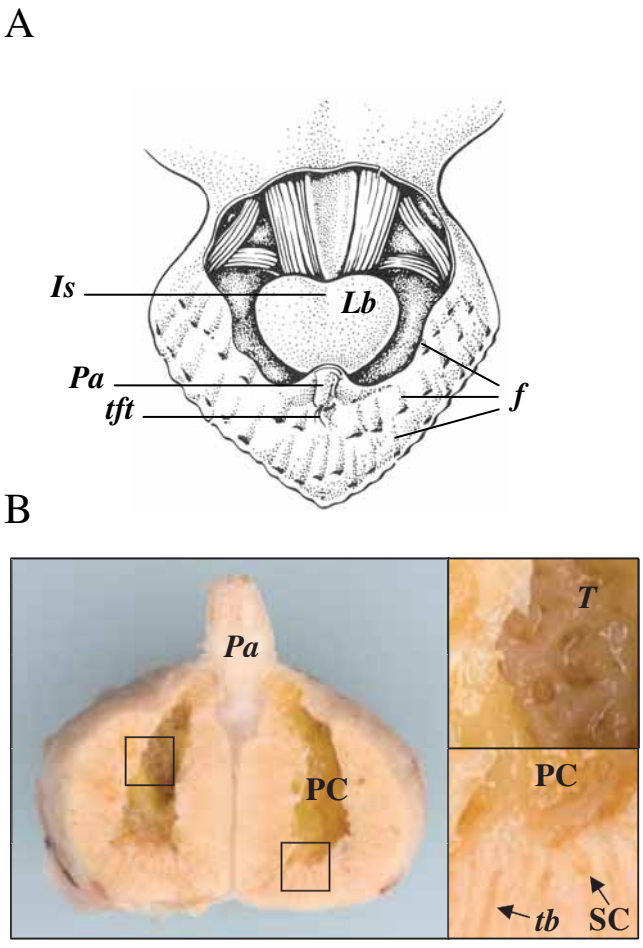

Fig. 1. (A) Dorsal view of a dissected chicken tail with exposed uropygial gland and its environment. (B) Frontal section of the chicken uropygial gland. *Is*, interlobular septum; *f*, feather follicles; *Lb*, lobe; *Pa*, papilla; *PC*, primary cavity; *SC*, secondary cavity; *T*, trabeculae; *tb*, tubule; *tft*, tuft.

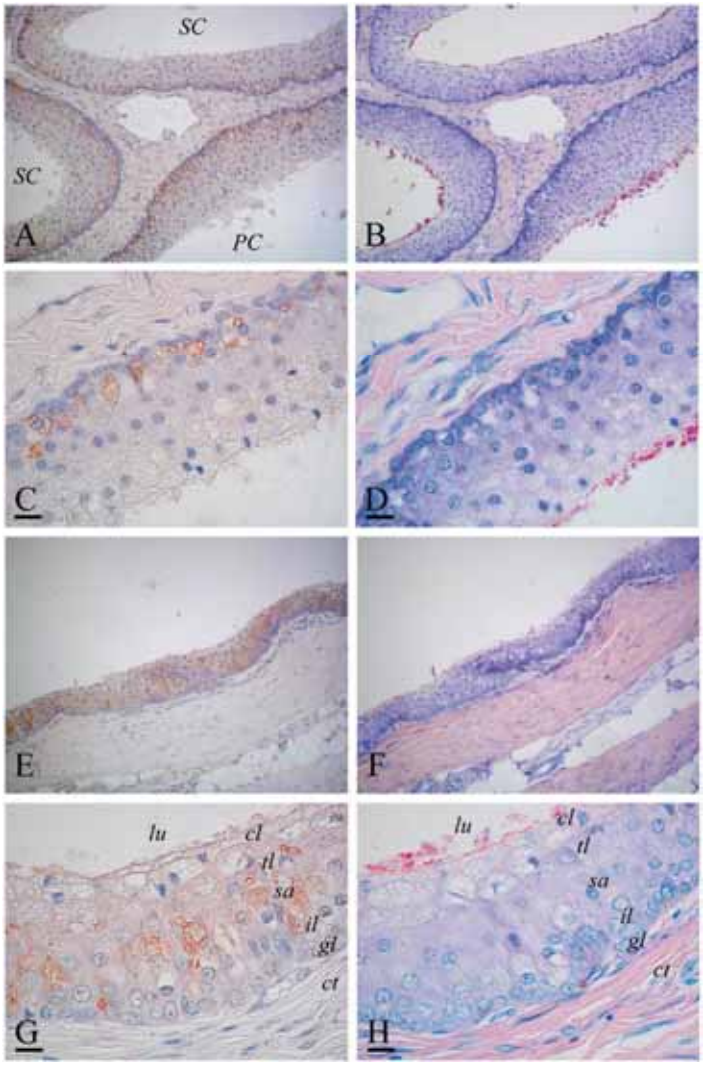

Fig. 2. CMAP27 is expressed by uropygial gland glandular epithelium. Paraffin-embedded sections of the uropygial gland lobe basement (A to D) and wall (E to H), were Giemsa-stained (B, D, F and H) or immunostained with anti-CMAP27 antibody (A, C, E, and G) using hematoxylin as counter stain. proCMAP/CMAP27 expression was detected in the secretory cells lining the network of trabeculae surrounding the secondary cavities. PC, primary cavity; SC, secondary cavity; *ct*, connective tissue; *gl*, germinative layer; *il*, intermediate layer; *sa*, secretory area; *tl*, transitional layer; *cl* corneus lamina; *lu*, lumen. Magnification, 200 × (A, B, E, F) and 1000 × (C, D, G, H). bars indicate 20 µm.

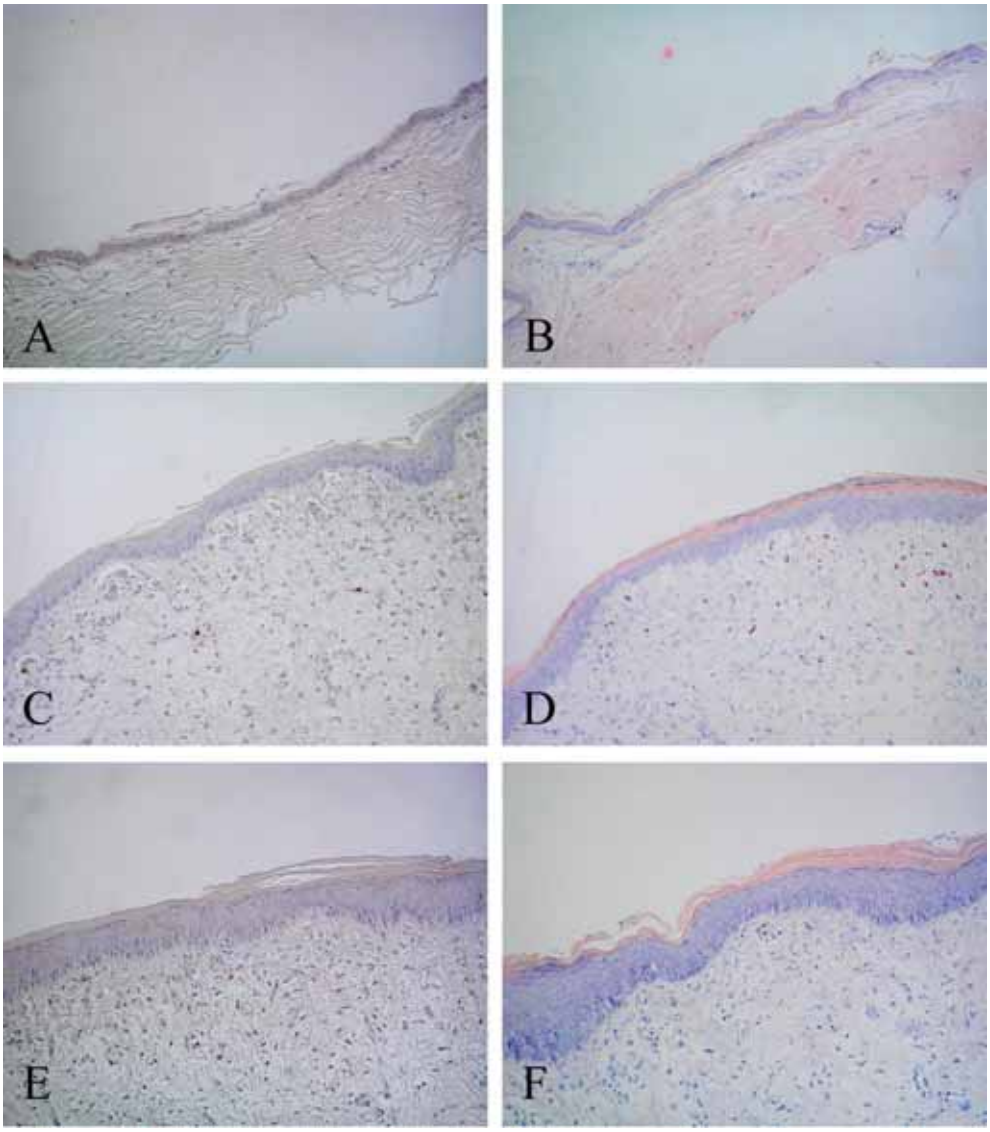

Fig. 3. proCMAP/CMAP27 is not expressed in healthy skin or skin derivatives. Paraffin-embedded integument sections of skin (A, B), comb (C, D) and wattle (E, F) were stained with anti-CMAP27 antibody using hematoxylin as counter stain or Giemsa-stained, respectively. Magnification, 200 ×.

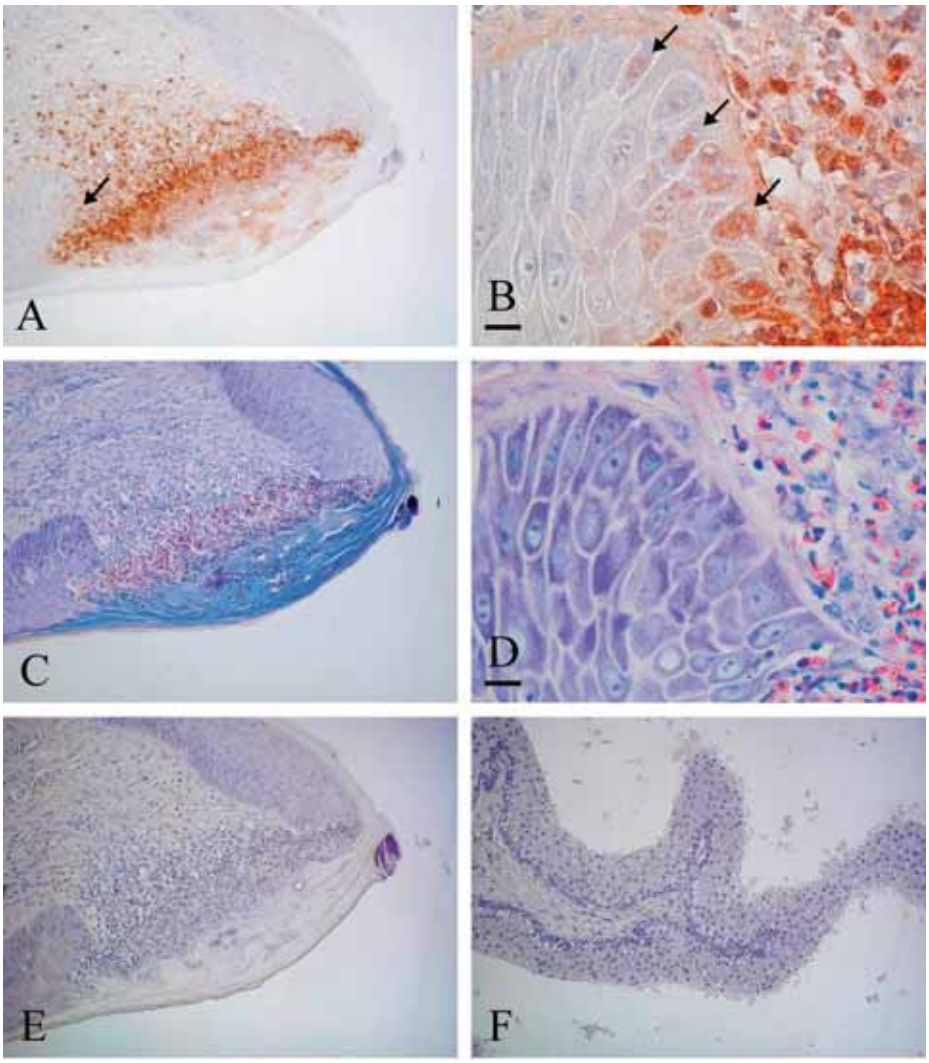

Fig. 4. CMAP27 in granulocytes and keratinocytes during wound repair. Paraffin sections of a damaged uropygial papilla were stained with anti-CMAP27 antibody using hematoxylin as counter stain (A, B), Giemsa solution (C, D) or directly stained with DAB for endogenous peroxidase activity (E, F). ProCMAP/CMAP27 expression is detected in keratinocytes (A and B; arrows) and infiltrating granulocytes at the site of injury (A, C). The infiltrating granulocytes were identified as heterophilic granulocytes based on morphology (D) and absence of endogenous peroxidase activity (E). For negative controls, immunostaining was performed on gland sections in the absence of primary antibody (F). Magnification, 200 × (A, C, E, and F) and 1000 × (B and D). Bars indicate 20 µm.

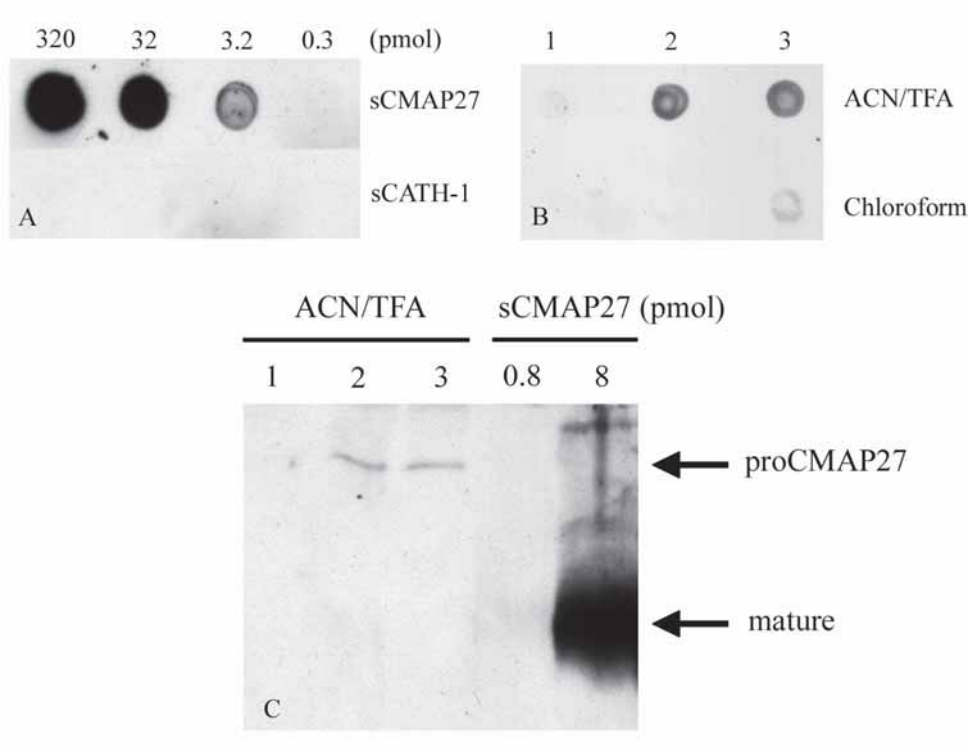

Fig. 5. Chicken uropygial gland wax contains proCMAP27. (A) Anti-CMAP27 antibody specificity was evaluated by immunostaining of dot blots spotted with 1 ng to 1 µg sCMAP27 or sCathelicidin-1. Uropygial gland wax extracts were evaluated by dot blot (B) or western blot (C) followed by immunostaining with anti-CMAP27 antibody. Approximately 120 mg uropygial gland secretion was dissolved in chloroform, extracted with several washes of 60% acetonitrile/1% trifluoroacetic acid (ACN/TFA) and, after lyophilization, resuspended in 150 µl distilled water. Chloroform fractions were passed through solid phase extraction (SPE) cartridges, eluted with basic methanol, dried and resuspended in 150 µl distilled water. (B) Five µl aliquots of each fraction were spotted on nitrocellulose membrane for dot blot analysis. (C) Eight µl aliquots of the ACN/TFA fractions were mixed with 16 µl sample buffer, separated by Tris-tricine-SDS-PAGE and evaluated on Western blot. Acetonitrile/TFA extracts of ~120 mg gland secretion obtained from three animals (lanes 1 to 3) compared to 2.5 ng (lane 4) and 25 ng (lane 5) of synthetic CMAP27 peptide.

Common soil microorganisms degrade feathers. Feather degradation was assessed in both media for all investigated bacterial and fungal strains. In general, fungi showed increased feather-degrading capacity in medium B, whereas the feather-degrading capacity of bacterial strains was medium-independent. Modest feather

degradation was observed for *B. licheniformis* incubated with de-fatted down feathers in medium A at 30 °C during the first two weeks of the 4 week incubation period (Fig. 6A); after 4 weeks feathers were degraded to a large extent. Feathers were not visibly degraded by *K. rhizophilae* if incubated with 10^6 CFU/ml in medium A (Fig. 6B), while feathers in medium A that were directly inoculated with ~10^8-10^9 CFU/ml resuspended in culture medium were completely broken down (data not shown). Although *S. pactum* displayed only moderate keratinolytic activity in medium A, substantial structural damage was observed after 28 days (Fig. 6C). The keratinolytic fungi, *C. keratinophilum* (Fig. 6D) and *S. brevicaulis* (Fig. 6E), degraded feathers within 4 days of incubation in medium B, followed by complete structural degradation within 14 days of incubation.

Synthetic CMAP27 inhibits growth of keratinolytic microorganisms. In colony-counting assays, synthetic CMAP27 peptide strongly inhibited the growth of keratinolytic bacteria, including actinomycetes. At peptide concentrations of 16 µg/ml, the survival of *B. licheniformis*, *K. rhizophilae* (Fig. 7A), *S. pactum* and *S. fradiae* (Fig. 7B) and the keratinolytic fungus *C. keratinophilum* (Fig. 7C), were reduced to below the detection limit of 100 cells/ml. Likewise, sCMAP27 completely inhibited the growth of *E. coli* and *S. aureus* (Fig. 7D) at a peptide concentration of 32 µg/ml, whereas a two-fold higher peptide concentration was needed to fully inhibit the growth of *P. aeruginosa* cells (Fig. 7D). Although a 1 LOG decrease in *S. brevicaulis* survival was observed in the presence of 64 µg/ml sCMAP27, its survival did not further decrease at higher peptide concentration, (Fig. 7C).

CMAP27 promoter analysis. The genomic region upstream of the CMAP27 gene was searched for the presence of putative transcription factor binding sites known to be associated with cathelicidin regulation. A putative transcription start site (TSS) was found at 11 bp upstream of the ATG start codon, preceded by a TATA box at -26 bp (Fig. 8). Transcription factor analysis using MatInspector software indicated putative binding sites for nuclear factor kappa beta (NFκβ; -174 bp, -242 bp), activator protein 1 (AP-1; -53 bp, -158 bp), nuclear factor interleukin-6 (NF-IL6 or c/EBPβ, -45 bp, -137 bp, -756 bp, -908 bp), CCAAT box (-440 bp, -626 bp), and vitamin D receptor/retinoid X receptor heterodimer (VDR/RXR; +64 bp, -51 bp, -236 bp, -465 bp). In addition, multiple binding sites were found for transcription factors involved in the regulation of lipogenesis: androgen response element (ARE, -245 bp, -696 bp), retinoic acid receptor (RAR, -155 bp, -418 bp), sterol response element binding protein (SREBP, -475 bp, -655 bp, -752 bp, -997 bp), and peroxisome proliferator-activated receptor/retinoid X receptor heterodimer (PPAR/RXR, -624 bp, -724 bp).

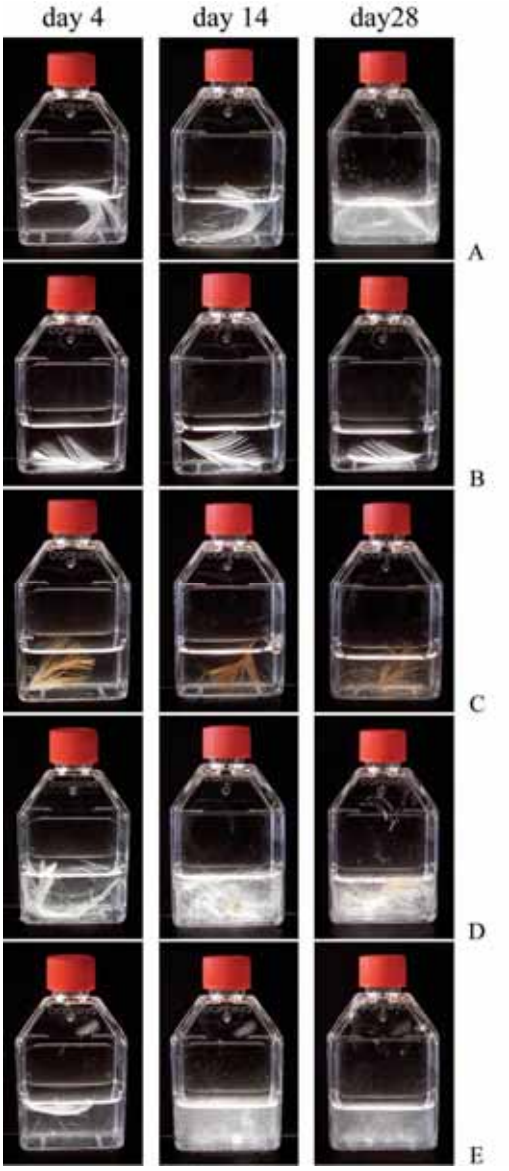

Fig. 6. Chicken feathers are degraded by keratinolytic microorganisms. Defatted chicken feathers were inoculated with 10^6 cfu/ml and incubated for several weeks at 30 °C in buffered nutrient-poor media containing common soil microorganisms. (A) *B. licheniformis* ATCC 21424, (B) *K. rhizophilae* ATCC 9341 , (C) *S. pactum* ATCC 27456, (D) *C. keratinophilum* ATCC 14862 and (E) *S. brevicaulis* NRRL 1103.

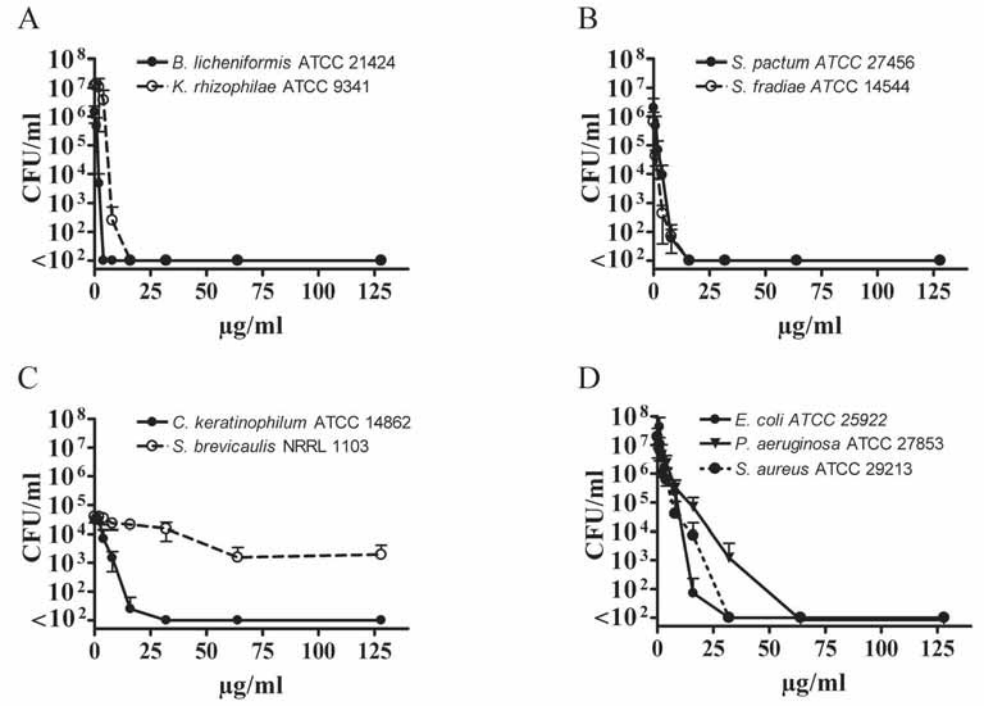

Fig. 7. Growth inhibition of keratinolytic microorganisms by sCMAP27. In colony-counting assays, sCMAP27-mediated growth inhibition was investigated against keratinolytic bacteria (A), keratinolytic actinomycetes (B), keratinolytic fungi (C), and opportunistic bacterial pathogens (D).

Fig. 8. Transcription factor analysis of the CMAP27 promoter region. Nucleotide sequence of the CMAP27 gene promoter deduced from the published cDNA sequence (accession no. DQ092350). Nucleotides are numbered relative to the predicted TSS (arrow). The partial first exon sequence is shown in uppercase, and the putative 5' untranslated region is shown in italics. Putative transcription factor binding factors are in boldface type and underlined.

DISCUSSION

Immunohistochemistry showed that CMAP27 immunoreactivity in the uropygial gland was restricted to the most basal cell layers in the secretory area of the proximal tubular regions, secondary cavities and lower part of the primary cavities, whereas in the thinner upper parts of the primary cavities immunoreactivity was seen throughout the secretory and transitional area. A similar pattern has been reported for the expression of β-defensin-1 and -2 in the human philosebaceous units (10, 40), where marked defensin expression was found in basal cells covering sites containing stem cell populations and suprabasal defensin expression in regions highly exposed to microbial invasion and skin microflora. These observations, together with the absence of anti-CMAP27 immunoreactivity in the distal tubule regions, supports a role of CMAP27 in the protection of the germinative layer in the gland regions most exposed to microorganisms.

In mammals, cathelicidin and other antimicrobial peptides secreted by sweat glands contribute to skin innate immune defense (33, 48). Human β-defensins-1 and -2 transcripts and proteins have been detected in sweat glands and sebaceous glands (1, 10, 40). LL-37, α-defensins and several other antimicrobial proteins are also present in human vernix caseosa, a lipid-rich substance derived from sebaceous sources and stratum corneum, that covers the skin of the fetus and newborns (63). Because birds lack sweat glands, we speculated that birds uropygial gland secretions might also contribute to skin innate immune defense. Therefore, proteinaceous extracts were prepared from gland wax and analyzed for the presence of anti-CMAP27 immunoreactivity. Dot blot analysis and Western blot analysis showed that low amounts of CMAP precursor were present in uropygial gland wax, conservatively estimated at >0.25 nmol/g secretion. It should be noted that, although white blood cells were scarcely seen in the gland sections used for immunohistochemistry, that some contamination by blood cells can not be completely excluded. In comparison, human cathelicidin hCAP18/LL-37 concentrations in sweat secretion were shown to be considerably variable between individuals (~1 μM on average), of which ~10% is processed into mature peptide, at least 20-fold lower then necessary for antimicrobial activity (33). In human skin, cathelicidin precursor hCAP18 is processed by the serine proteases stratum corneum tryptic enzyme (SCTE, kallikrein 5) and stratum corneum chymotryptic enzyme (SCCE, kallikrein 7) into LL-37 and smaller mature peptides (62). Thus, it is possible that proCMAP27 undergoes similar proteolytic processing when deposited onto skin. In addition, the previously reported expression of moderate β-defensin mRNA levels in uropygial gland tissue extracts (56) suggest a possible synergistic action between cathelicidin and defensin peptides (34) when simultaneously released onto the skin surface.

Morphologically, chicken epidermis and uropygial gland epithelium are similar, both gland and skin cells of the translational layer show hypertrophy and develop cytoplasmic vacuoles containing sebaceous secretions (30). Considering this fact, sections of ventral and dorsal skin were investigated by immunohistochemistry. As observed for murine and human skin (13, 16), CMAP27 expression was not detected in normal chicken epidermis, nor was it detected in skin appendages, such as comb or wattles. However, chicken skin injury resulted in a massive infiltration of CMAP27-positive heterophils at the site of injury and local expression of CMAP27 in keratinocytes near the site of re-epithelialization. Although numerous granulocytes were seen localized along the wound perimeter, infiltration of the keratinocyte layer was limited. Investigation of multiple Giemsa-stained sections of this sample did not show co-localization of granulocytes with CMAP27-positive keratinocytes. Furthermore, epidermal CMAP27 expression was restricted to the wound edge, in line with reported induced LL-37 expression by human skin keratinocytes upon injury (13). Although the detection of CMAP27 in the epidermal layer is likely the result of *de novo* cathelicidin synthesis by keratinocytes, this will have to be confirmed by *in vitro* stimulation of chicken epidermal keratinocytes.

Particularly ground-foraging birds, such as domesticated chickens, are constantly exposed to soil-derived microorganisms. Feather (mainly β-) keratin is a poor substrate for most microorganisms and highly resistant to proteolytic enzymes (28, 51, 59), yet common soil bacteria and fungi have the capacity to degrade feathers *in vitro* (15, 26, 31, 37, 53). Keratinolytic bacteria are wide-spread in the plumage of wild birds and mostly belong to *Bacillus* species, predominantly *B. licheniformis* (8). Keratinolytic fungi have been isolated from bird plumage (22, 23, 41, 42) or old nests (22). In domestic fowl, the breast skin surface was shown to be predominantly colonized by *Staphylococcus epidermidis* and to a lesser extent *Kocuria rhizophilae* and *Streptomyces* sp. (4), whereas multiple keratinolytic fungal species were found, among which *Chrysosporium keratinophilum* and *Scopulariopsis brevicaulis* exhibited the strongest keratinolytic activity (14, 24, 25). Interestingly, it was found that extirpation of the uropygial gland resulted in a significant shift in the skin microflora, i.e. increased colonization of the skin surface by *S. aureus* and a *Bacillus* sp.*, Aspergillus* sp. *and S. brevicaulis* , whereas *S. epidermis*, *Streptomyces* sp. populations were severely depleted (4, 5). Our findings demonstrate that, *in vitro*, native feathers were partially or completely degraded by *B. licheniformis*, *S. pactum*, *C. keratinophilum* and *S. brevicaulis* strains. In this study we also demonstrated that the vast majority of the tested feather-degrading bacterial and fungal strains were inhibited in the presence of CMAP27. CMAP27 displayed potent microbicidal activity against opportunistic skin pathogens, keratinolytic bacteria and the keratinolytic fungus *C. keratinophilum*, while germination and growth of *S. brevicaulis* conidia was not greatly

affected. Interestingly, it is now known that in many filamentous fungi, the spores, fungal walls and the surface of fruiting bodies are covered by considerable amounts of hydrophobins, a multigene family of small (~6 – 10 kDa) amphipathic proteins, and that certain hydrophobins have been implicated in fungal pathogenesis (29, 60). Furthermore, under conditions of nutrient deprivation, such as in our colony-counting assays, hydrophobin expression has been observed to be up-regulated (49). Importantly, within a species, different hydrophobins seem to fulfill specific roles, meaning that only some types of hydrophobins are predominantly found in spore walls as well as secreted at high concentrations into the environment (29). Moreover, the hydrophobin pattern in mycelium as well as spore walls is highly variable at the species and sub-species level (35). Thus, although we investigated only two species, we postulate that the amphipathic properties of cell wall and secreted hydrophobins, could render some fungi less susceptible to AMP-mediated killing and thereby contribute to their pathogenicity.

Because sebum production is continuous and known to be controlled by hormones, retinoids and growth factors (64), we investigated if the CMAP27 promoter region contained putative binding sites associated with transcription regulation of sebum production. Indeed, multiple putative binding sites were found for c/EBPβ (NF-IL6), PPAR/RXR, SREBP, AR and RAR (Fig. 8). Androgen receptors (AR) are localized in the chicken comb and bottom half of the uropygial gland tubules, yet not in the proximal part of tubules, secondary or primary cavities (50). SREBP-1, an important regulator of cholesterol and fatty acid biosynthesis, is highly expressed in the chicken uropygial gland and in human sebocytes (2, 21). Likewise, high PPARα expression was also found in the chicken uropygial gland (12). These are important observations since CCAAT enhancer binding proteins (c/EBPs) and peroxisome proliferator-activated receptors (PPARs) are crucial for terminal differentiation of adipocytes (21) and are known to be highly expressed in human sebocytes (9). These findings implicate that under non-pathogenic conditions, a tissue-specific coordination of CMAP27 biosynthesis and sebum production must exist which is most likely governed by key regulators of lipogenesis.

The observed CMAP27 induction in keratinocytes and variablility of CMAP27 expression observed in immunohistochemical, dot blot and Western blot analyses of uropygial gland tissue, or secretion, implicates a possible inducibility of CMAP27 in this gland. Investigation of the upstream region of the CMAP27 gene contained several putative transcription factor binding sites involved in mammalian cathelicidin regulation: NFκβ, AP-1, NF-IL6 (c/EBPβ), RAR and VDR/RXR binding sites. Potential binding sites for NFκβ, AP-1, NF-IL-6 have also been found in the porcine cathelicidin PR-39 and bovine bactenecin-5 promoter regions, cathelicidins that, like CMAP27, are highly expressed and stored in neutrophils (54, 61). Cathelicidin

expression has been shown to be induced in mouse and human keratinocytes upon skin injury (13, 47). In humans, vitamin D responsive elements (VDREs) have been shown to play a pivotal role in this local induction of cathelicidin expression (58). Despite the significant similarity between the human and primate CAMP and mouse CRAMP promoter regions, the conserved region of the human and chimpanzee promoter in which a vitamin D response element (VDRE) is located, is lacking in the mouse CRAMP promoter region (19), explaining why CRAMP induction in keratinocytes is not regulated by $1,25(OH)_2VitD_3$ (46). It is evident that in animals that have their skin covered with a dense complexion of hairs or feathers, conversion of vitamin precursor by UV-B light is more complicated, which perhaps explains why VDREs are absent in the murine, rat and dog CAMP promoter regions (19). Interestingly, in fowl, the uropygial gland is the main site of synthesis and storage of the vitamin D_3 precursor 7-dehydrocholesterol (55). Both vitamin D3 and 7-dehydrocholesterol are present in the uropygial gland and to a lesser extent in the unfeathered skin of the legs and feet, while only low amounts are found in the back epidermis (55). The presence of multiple VDREs in the chicken CMAP27 promoter regions, makes it temping to speculate about a possible VDR-dependent induction of CMAP27 in keratinocytes upon skin injury.

In summary, this study gives new insights in the immune-related functions of the avian uropygial gland in relation to the protection of skin and plumage. To our knowledge, this is the first report of antimicrobial peptide production by the avian uropygial gland. Further studies will focus on the relative contribution of antimicrobial peptides such as cathelicidins and defensins to integument homeostasis and wound repair in birds.

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Chapter 7

General Discussion

INTRODUCTION

Since the mid 1940's, animal feed has been supplemented with subtherapeutical doses of antibiotics, so-called growth-promoting antibiotics (GPAs), mainly to improve growth performance (11). However, nowadays due to modernization of the animal production process, GPAs only marginally affect growth performance (19). Furthermore, the concomitant increase of antibiotic-resistant bacteria in production animals (70) and in nosocomial infections (18) became a major public health concern. Finally, the risks for development of antibiotic resistance in human pathogens and the transmission thereof led to a total European ban on the use of antibiotics in animal feeds to promote growth (40). Hence, alternative strategies were needed to improve food safety and promote growth in production animals.

Modulation of natural defense mechanisms, in particular those affecting the gastrointestinal (GI) tract, has now become the focus of attention. Several strategies have aimed at shifting the delicate balance between residential "non-pathogenic" and potential pathogenic microorganisms with the use of prebiotics, probiotics, organic acids, enzymes or herbs (65), but fundamental knowledge on their working mechanisms, and more important, their effects on host defense mechanisms, are lacking.

In mammals, antimicrobial peptides (AMPs) have demonstrated to play an important role in the host defense of skin and mucosal surfaces (36, 47, 69). Experimental evidence suggests that altered mammalian AMP expression levels in specific tissues may result in augmented disease resistance (42).

In stark contrast to mammals, little is known about the repertoire and biological functions of avian antimicrobial peptides. However, avian tissue-specific AMP expression and its regulation may largely reflect those described for mammalian species. Hence, modulation of antimicrobial peptide expression, for instance by dietary stimulation, could be a promising approach to improve animal health and welfare, as well as to promote animal growth.

In this thesis, novel members of known antimicrobial peptide families were sought using *in silico* analysis. After sequencing, tissue-specific expression of selected antimicrobial peptides was analyzed. Finally, properties of these peptides were determined with the use of synthetic or recombinant peptides. The results described in Chapters 3 to 6 are summarized below per chapter, followed by a general discussion on biological functions of avian defensins and some of their possible applications.

SUMMARY OF RESULTS

In **Chapter 3**, the localization of chicken AvBD9 (Gallinacin-6) and its antimicrobial properties were investigated. Using *in silico* analysis we discovered in total 7 novel chicken β-defensins, including AvBD9. Investigation of mRNA expression profiles showed breed-independent high expression levels of AvBD9 in crop and esophagus tissue. The high variability of AvBD9 mRNA levels in crop tissue of 13 day-old broiler chickens and the presence of putative binding sites for transcription factors known to be involved in mammalian β-defensin regulation, suggested an inducible nature of the AvBD9 gene. Fast killing kinetics were observed, and even at low synthetic AvBD9 concentrations, incubation with bacterial cells resulted in distinct morphological ultrastructural changes. The relatively salt-insensitive broad bactericidal and fungicidal activity of AvBD9 in colony-counting assays indicated an important role of AvBD9 in chicken innate immunity.

In **Chapter 4**, the discovery of CMAP27, a chicken cathelicidin, is described. The CMAP27 gene was found by *in silico* analysis and cloned from chicken bone marrow cells. Based on a putative elastase cleavage site between the cathelin-like domain and C-terminal domain, a 27 amino acid residue long, highly cationic (+10) α-helical mature antimicrobial peptide was predicted with a surprising similarity to mammalian myeloid α-helical cathelicidin peptides. RT-PCR studies confirmed that, like mammalian peptides, CMAP27 mRNA was highly and predominantly expressed in myeloid and lymphoid tissues. These findings indicated the evolutionary importance of the α-helical cathelicidin peptide subclass and support the theory of an ancestral cathelicidin gene.

In **Chapter 5**, the biosynthesis and release of CMAP27 in chicken granulocytes and presence in tissues of the digestive tract were examined in relation to a *Salmonella* challenge. Using immunohistochemistry, abundant CMAP27 expression was detected in heterophilic granulocytes, but not in other peripheral blood cells. Western blots of total leukocyte fractions localized proCMAP27 in granule contents. Stimulation of heterophils with different agonists of exocytosis indicated that CMAP27 is stored as an inactive proform in specific granules, separated from its activating proteases, and activated after extracellular release via exocytosis. Considerable infiltration of CMAP27 containing heterophils was observed in the jejunum of *Salmonella*-challenged 4 day-old chicken broilers at 8 and 48 hours post infection as compared to non-infected animals; however, CMAP27 expression was not induced in intestinal epithelial cells. In addition, abundant CMAP27 expression was also detected in liver-specific natural killer (Pit) cells present in the liver of a *Salmonella*-infected animal. Synthetic CMAP27 and cathelicidin-1 peptides displayed broad spectrum bactericidal and fungicidal activity against the tested strains, including chicken-specific

Salmonella isolates. The abundant expression of CMAP27 in heterophils and its potent antimicrobial properties indicate a pivotal role for cathelicidins in heterophil-mediated killing.

The putative protective function of CMAP27 from the chicken uropygial gland and its role in skin wound repair are described in **Chapter 6**. Immunohistochemistry showed CMAP27 expression to be restricted to the secretory cells of the uropygial gland epithelium in the primary cavity, secondary cavities and proximal tubule regions, suggesting a role in the local protection of glandular epithelial stem cells. ProCMAP27, but not mature peptide, was also detected in gland secretion, conservatively estimated at >1 µg/g sebum. CMAP27 expression was absent in normal skin and skin appendages, but found to be induced in keratinocytes upon skin injury with accompanying massive infiltration of CMAP27 containing heterophils. Synthetic CMAP27 peptide displayed potent bactericidal activity against feather-degrading bacteria and the feather-degrading filamentous fungus *C. keratinophilum,* all known to be present in the plumage of wild and domesticated birds. Analysis of the CMAP27 gene promoter region revealed multiple putative transcription factor binding sites for key regulators of lipogenesis, indicating that constitutive uropygial gland CMAP27 expression is most likely linked to sebum (wax) production. In addition, putative binding sites were found that are known to be involved in regulation of cathelicidin expression in skin keratinocytes and myeloid cells. These findings indicate that cathelicidins, and perhaps other antimicrobial peptides, may contribute to the protection of skin and plumage in birds.

DISCUSSION

A role for the avian uropygial gland in the protection of skin and plumage?

The discovery of at least one cathelicidin peptide, and possibly also defensin peptides, in chicken uropygial gland secretions, is intriguing. The biological functions of this gland are still largely the subject of debate and appear to vary considerably between avian species. For instance, the uropygial gland of the Eurasian Hoopoe (*Upupa epops*) and South African Woodhoopoe (*Phoeniculus purpureus*), is able to produce a repellent black malodorous secretion that is sprayed onto predators as a means of defense (68), whereas in Greater hornbills (*Buceros bucornis*) the uropygial gland secretion is used to stain parts of its body yellow for signalling during threat displays (13). Due to the occurrence of relatively large uropygial glands in waterbirds and the water-repellent properties of secreted gland waxes, the uropygial gland has long been implicated in the water proofing of plumage (13). However, similar gland sizes have been observed in terrestrial birds of which Galliformes possess relatively large

uropygial glands (26). Thus, it appears that during avian evolution, the functions of this gland have adapted to the habitat and needs of individual avian species. In the case of the ancestor of the domesticated chicken, the red jungle fowl, a ground-foraging bird that lives in the tropical rain forests of Malaysia and India (15), the ubiquitous presence of soil keratinolytic fungi and bacteria in combination with a warm and humid climate may have steered adaptation of this gland towards a skin-related immune function.

The question remains if and how birds use this potential defense mechanism to protect their skin and plumage. Based on our findings, its primary function may be the protection of glandular epithelial stem cells when constitutively expressed. In Chapter 6 it is shown that, under "normal" circumstances, transcriptional regulation of CMAP27 expression is most likely linked to sebum production via transcription factors that regulate lipogenesis. The occurrence of hormone receptors, local hormone production in this gland and presence of steroid hormone binding sites in the CMAP27 promoter indicate the possibility of hormone-mediated CMAP27 upregulation. This is supported by the finding that mallard ducks (*Anas platyrhynchos*), red knots (*Calidris canutus*) and many species of sandpipers (*Scolopacidae*) have demonstrated to be subject to seasonal hormone-induced variations in uropygial gland wax composition (29, 43-45, 57). Outside the breeding season their secretion is predominantly composed of mono-ester waxes, but is changed to a less volatile di-ester wax composition (thought to be less noticeable to the olfactory organs of predators) at the onset of courting and remains so during the breeding season.

Another example of breeding season-depending regulation of antimicrobial expression has been observed in male king penguins. During the breeding season of king penguins only fasting males that await the return of their partner protect their stomach contents, in part, with upregulated AMP expression and secretion into the stomach contents, enabling them to feed theirs chicks after hatching (60). Thus, the observed seasonal dependence of uropygial gland wax composition and tissue-specific AMP upregulation in breeding king penguins indicate the possibility of enhanced AMP expression and secretion of selected tissues during the breeding season. It would therefore be interesting to investigate the role of steroid hormones in upregulation of uropygial gland AMP expression during the breeding season of birds and its impact on microbial populations on avian skin and in plumage.

Biological functions of avian AMPs in wound-repair

Upon epithelial injury, repair mechanisms are initiated that involve migration, proliferation and, finally, differentiation of keratinocytes. In mammals, cathelicidin was shown to be involved in re-epithelialization of wounds in skin and airways (23, 55). Similarly, human neutrophil defensins have been observed to stimulate wound

closure through induction of fibroblast and epithelial migration and proliferation (1, 33). The findings in this thesis show that at least one cathelicidin, CMAP27, is involved in wound-repair in birds (Chapter 6). In addition, recruitment of inflammatory cells resulted in a massive infiltration of CMAP27-containing heterophils at the site of injury. This may serve a dual purpose. Firstly, to detect and rapidly clear microorganisms from the wound by phagocytosis and extracellular release of bactericidal granule contents. Secondly, released cathelicidin(s) and defensins may stimulate epithelial cell migration and proliferation to augment wound closure.

In skin, epidermal $1,25(OH)_2D_3$ together with calcium regulates differentiation by sequentially switching on and off genes encoding elements as well as of enzymes required for differentiation (4). The discovery of putative vitamin D response elements in the CMAP27 promoter region suggests that the regulatory mechanisms controlling CMAP27 induction in keratinocytes may reflect those described for human skin, i.e. $1,25(OH)_2D_3$-mediated. Despite being almost completely covered with feathers, birds are able to generate sufficient vitamin D_3 (61, 62), which is taken up via the skin into the circulation and further processed to $25(OH)D_3$ in the liver (37). Keratinocytes have the full machinery to convert $25(OH)D_3$ into the active metabolite $1,25(OH)_2D_3$. The role of the uropygial gland in this appears to be species-specific and although the uropygial gland is the main site of vitamin D_3 precursor synthesis in birds, the observed vitamin D_3 production in unfeathered chicken skin does indicate a certain extent of redundancy (61). In conclusion, it is anticipated that avian cathelicidins as wells as defensins may be involved in wound-repair by stimulation of epithelial cell migration and proliferation and that local cathelicidin induction in keratinocytes during this process is possibly Vitamin D_3-mediated.

Biological functions of AMPs in avian mucosal innate immune defense

Gene expression profiles show abundant expression of avian cathelicidins and defensins in myeloid tissues and/or cells (Chapters 2, 3 and 4) and highly variable tissue-specific expression of β-defensins was observed at the mucosal surfaces of the respiratory, intestinal and urogenital tracts. In addition, several β-defensins, such as king penguin AvBD103b, chicken AvBD3, and possibly chicken AvBD9, showed inducible expression in response to bacterial challenge. Synthetic peptides based on avian cathelicidins and defensins, with the exception of chicken AvBD13, demonstrated potent microbicidal activity against a variety of microorganisms (Chapters 3, 5 and 6). However, real expression levels on a protein basis of these peptides and their cell- and tissue-specific localization at mucosal surfaces are unknown.

It is anticipated, and partially demonstrated (LPS binding activity of chicken cathelicidins (53)), that avian AMPs are multifunctional molecules and, in addition to their direct antimicrobial properties, form a link between innate and adaptive immunity. Furthermore, numerous avian "homologues" have been described of receptors and adaptor proteins in signalling pathways, but most of these possess only little sequence identity to their mammalian counterparts, suggesting alternative signalling pathways. Thus, regulation of AMP expression in avian immune cells and mucosal tissues and their additional biological functions may to some extent differ from those described for mammals.

Therapeutical applications

The development and persistence of microbial resistance against classical antibiotics remains problematic. The arsenal of effective antibiotics against nosocomial pathogens is limited and the rate of microbial adaptation to treatments exceeds the developmental rate of new classes of antibiotics. Therefore, antimicrobial peptides may form a promising 'new' class of broad-spectrum antibiotics. The broad-spectrum antimicrobial activities of CMAP27, AvBD9 and other avian cathelicidins and defensins described in this thesis and reported by others (Chapter 2, Table 3), renders these peptides useful for practical applications. However, there are several pitfalls to be anticipated if AMPs are to be used as therapeutical tools.

Firstly, AMPs are widely spread in plants and animals (74), but microbial resistance against AMPs is limited. In time bacteria and fungi have adapted their cell membrane in various ways to counteract AMP-mediated growth inhibition, yet most microorganisms remain susceptible to their actions (39). Some bacterial species, including *Pseudomonas aeruginosa* and *Burkholderia cepacia,* are highly resistant to single AMP-mediated killing (32, 46), while other species may acquire partial AMP-resistance after as many as several hundreds of generations of exposure to sub-lethal AMP concentrations (8). *In vivo*, microbes are predominantly exposed to AMPs if they pose as a threat i.e. aim to adhere and invade. Only then, locally or systemically, stored AMPs are mobilized and released upon arrival or are induced locally. Thus, in general, the exposure of microbes to AMPs *in vivo* is kept at minimal levels at most body interfaces. Bacteria have cohabitated with *Streptomyces sp.* and *Penicillium* moulds, producers of the natural antibiotics Streptomycin and penicillin for millions of years, and resistance against these antibiotics was low prior to their widespread clinical use, but has skyrocketed since (8). Thus, development of AMP resistance may be inevitable if similar strategies are used for the therapeutical use of AMPs.

Secondly, it is not known if continuously enhanced AMP expression may result in a local or systemic chronic inflammatory condition, which may result in loss

of tissue or organ function. Inflammation is a complex process in which a diversity of pro-inflammatory cytokines, chemokines and anti-inflammatory cytokines are produced and both activating and inhibitory receptors are expressed on inflammatory cells (30). The exact role of cathelicidins and defensins in delayed resolution of inflammation is not known. Mammalian cathelicidins and defensins have shown to be attractants of inflammatory cells, including neutrophils, immature dendritic cells, T cells and mast cells (24, 34, 72, 73) and to promote a Th1-directed adaptive immune response. Furthermore, AMPs may also indirectly facilitate the recruitment of phagocytes, immature dendritic cells and T cells through induction of chemokines and corresponding receptors (71). Human LL-37 matured dendritic cells show enhanced secretion of Th1-inducing cytokines IL-6 and IL-12 with a concomitant decreased IL-4 expression (10) and Th1 responses are stimulated by LL-37 *in vitro* (10). Similarly, a Th1-polarizing cytokine profile of IL-1α, IL-1β, IL-6 and IL-12, has been observed for mouse BD-2 matured dendritic cells (5). Because activated mature dendritic cells can determine and maintain Th1/Th2 polarization of T and B cells (2, 38), both cathelicidins and defensins may be able to regulate the type of immune responses (71). Human BD-2 has been shown to induce the release of the proinflammatory mediators histamine and prostaglandin from rat mast cells (35). In contrast to the events and mediators involved at the onset of inflammation, the mediators and mechanisms responsible for the resolution of inflammation have largely been ignored until recently (31). Thus, experimental *in vivo* trials with prolonged induced AMP expression will have to be carried out to investigate the consequences of 'permanently' elevated AMP levels.

Modulation of endogenous antimicrobial peptide expression

Unfortunately, induction of AMP expression has been mainly demonstrated using immortalized cell-lines and isolated cells, whereas little data has been obtained from experimental infection models. Modulation of endogenous AMP expression levels has been shown for short-chain fatty acids (22, 51), amino acids (14, 56), flavonoids (51), vitamin D_3 analogs (16, 66), probiotics (67) and 'probiotic' cell wall components (7):

Short-chain fatty acids. Short-chain fatty acids (SCFAs) are produced by bacterial fermentation of dietary fibers in the human colon (9). Administration of 2 mM has been shown to induce β-defensin expression of (hBD1, 5-fold; hBD2, 15-20-fold) in colonic epithelial cell and monocyte lines, but not keratinocytes, within 24 hours (49). Similarly, 2-5 mM of propionate, butyrate or isobutyrate, but not acetate or lactate, was shown to induce cathelicidin expression (LL-37, 70-fold) in colonic epithelial cell lines within 24-48 hours (22, 49, 51). Furthermore, treatment of *Shigella*-infected rabbits with 0.14 mmol butyrate/kg body weight twice daily for 3 days has

demonstrated to improve the outcome in *Shigellosis* (41, 42). Butyrate-induced AMP is independent of SCFA-mediated effects on cell differentiation (51), and is thought to augment transcription of specific genes through inhibition of histone deacetylases (HDACs) (20, 27, 50, 51, 54), although its observed MAP kinase pathway dependency indicates a more complicated working mechanism (27, 75).

Amino acids and proteins. The branched essential amino acid L-isoleucine and analogs thereof, are able to induce β-defensin expression in enteric β-defensin cotransfected Madin-Darby Bovine Kidney cells (MDBK) (10 to 12-fold at 3-12.5 µg/ml within 24 hours) and human colonic epithelial cells (hBD1; 2-fold at 200 µg/ml within 6 hours) (14, 56). Likewise, 250 µg/ml arginine or 1% bovine serum albumin was shown to specifically induce β-defensin expression 2 to 3-fold in human colonic cell lines (56).

Flavonoids. Flavone (150 µM) significantly induced LL-37 expression in colonic epithelial cells after 24 hours, without affecting cell differentiation (49). The mechanisms underlying flavone-induced cathelicidin expression are not known.

Vitamin D_3. The vitamin D_3 metabolites $1,25(OH)_2D_3$ and $25(OH)D_3$ were shown to significantly induce LL-37 expression within 24 hours in primary keratinocytes, epithelial cell lines and myeloid cells (66). A 140-fold upregulation was observed in keratinocytes (49), whereas cathelicidin expression in colonocytes was not affected. Interestingly, $1,25(OH)_2D_3$-mediated cathelicidin induction was even more profound if cells were simultaneously exposed to LPS (66) or sodium butyrate (17). $1,25(OH)_2D_3$ significantly enhanced β-defensin expression in epithelial cell lines, but not in neutrophils or monocytes (66). Vitamin D_3 metabolites act directly on the promoter regions of specific genes through binding to soluble vitamin D receptors that subsequently translocate to the nucleus and bind to vitamin D responsive elements in promoter regions of specific genes, including cathelicidins and defensins (6, 48).

Probiotics. A variety of probiotic bacteria, including *E. coli* Nissle 1917 and *Lactobacillus* spp. have demonstrated to dose-dependently induce β-defensin expression in Caco-2 intestinal epithelial cells (67). Exposure to living *E. coli* Nissle 1917 cells (10^8 CFU/ml) was shown to upregulate hBD2 expression 68-fold in Caco-2 cells. The highest bacterial concentration tested (heat-killed, 5×10^9 CFU/ml) induced hBD2 expression 362-fold. However, induced hBD2 levels peaked after 3 to 6 hours of exposure, but returned to basal levels after 12 hours. In contrast, uropathogenic *E. coli* (UPEC) induced a 6-fold increase in hBD2 mRNA, whereas several other *E. coli* strains failed to significantly induce hBD2 expression. Beta-defensin induction by *E. coli* Nissle 1917 was mediated through flagellin (52), not by LPS (67) and was shown to require NFκβ and AP-1 binding sites for maximum promoter activity in Caco-2 cells. In contrast, administration of 10^9 or 10^{11} CFU/ml *E. coli* Nissle 1917 to young

pigs during a 21 day period did not affect the expression levels of PR39, protegrins or pBD1 (12).

"***Probiotic" cell wall components.*** Oral administration of lysozyme-modified probiotic components prepared from *Lactobacillus acidophilus* ATCC 53103 (LzMPC; 5 days gavage prior and 3 days post CLP with10 ml/kg, one ml derived from 10^9 cells) was shown to protect rats against lethality from microbial sepsis induced by cecal ligation and puncture (CLP) (7), a model that mimics the clinical course of sepsis in humans. *In vitro*, LzMPC upregulated rat cathelicidin-related antimicrobial peptide (CRAMP) expression in macrophages and enhanced their bactericidal activity. *In vivo*, oral administration of LzMCP restored decreased liver CRAMP expression levels due to CLP or surgical stress, upregulated CRAMP expression in macrophages (>14-fold), but did not affect macrophage TNFα secretion or cecal bacterial counts (7).

In conclusion, some SCFAs, amino acids, proteins, vitamin metabolites, prebiotics and probiotics are able to stimulate AMP expression to various degrees *in vitro* and *in vivo*. Clinical trials have demonstrated that induced AMP expression may coincide with improved clinical outcome. Particularly, infections in which pathogens, such as *Shigella* spp., manipulate the innate immune barrier by downregulation of specific AMP expression to facilitate invasion, are interesting targets for this approach. Whereas some substances, including SCFAs, appear to influence basic, evolutionary well-conserved mechanisms to enhance expression and are therefore likely to affect multiple species, others (vitamin D_3 metabolites), clearly use species- and tissue-specific pathways to enhance AMP expression. However, no or only limited data, is available on prolonged exposure to the above mentioned substances. Vitamin D_3 in skin keratinocytes is strictly regulated under 'normal' circumstances. The observed temporal limitations for *E. coli* Nissle-mediated AMP induction suggest that initial powerful AMP induction may be short-lived due to feedback regulation mechanisms. Thus, more research is necessary to identify and test novel substances regarding their effects on maintenance of innate immune homeostasis and their relation to endogenous antimicrobial peptide expression.

Applications in poultry

The use of probiotics seems promising for both animal health and food safety, in particular when administered to newly hatched chicks. The digestive tract of these chickens is rapidly colonized by a variety of microorganisms, but the number of *Lactobacilli* in crop and ceca is highly variable. These bacteria are generally only found in high numbers throughout the digestive tract after 3 days of feeding (3), whereas in adult chickens, *Lactobacillus* spp. are predominant at the crop surface (21). It has been demonstrated that mixtures of microorganisms obtained from healthy adult

animals when given to newly hatched chicks could prevent cecal colonization of *Salmonella* (3). In addition to competition for binding places, 'healthy' digestive tract microbiota may suppress other microorganisms by production of bacteriocins (58). The observed AMP upregulation by living *Lactobacilli* in cell lines and in other species indicates that local interactions between residential digestive tract microbiota and crop mucosa may also trigger and maintain local AMP expression in the gut. The use of SCFAs as feed additive in poultry has long been based on their antibacterial activity outside the digestive tract (64). Intestinal butyrate concentration were also shown to positively correlate with lactic acid bacterial (lactobacilli and bifidobacteria) counts (28) and to negatively correlate with *Salmonella* invasion (63). *In vivo* experiments have shown that SCFA addition to poultry feed results in increased SCFA concentrations in crop and gizzard, but not in the intestine (25, 59). Moreover, the concomitant decline of lactic acid production in the crop suggests that accumulation of SCFAs in the crop negatively affects the residential lactobacillus population (59). Thus, SCFAs should be administered in coated form or as digestible fibers that release butyrate or other SCFAs upon fermentation in the ceca. The presence of vitamin D responsive elements in the CMAP27 promoter region warrants further investigation on their functionality. Vitamin D metabolites, SCFAs, probiotics and other feed additives will have to be tested to determine if they can be applied to increase intestinal health by upregulation of the antimicrobial peptides described in this thesis.

Concluding remarks

The work in this thesis has revealed the existence of several, to date unknown, biological mechanisms that are involved in avian innate immunity. Evidence has been presented of abundant antimicrobial peptide expression in innate effector cells and tissues of the digestive tract, their release upon infection as well as their potent antimicrobial properties. Furthermore, putative roles of antimicrobial peptides and underlying mechanisms in wound-repair and the protection of skin and plumage have been uncovered. Although we have only started to unveil the biological mechanisms involved in avian AMP regulation, their apparent evolutionary conservation in vertebrates and the promising results with mammalian AMP modulation suggest that dietary modulation of endogenous antimicrobial peptides could be a feasible strategy to improve animal health and growth in poultry.

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Samenvatting in het Nederlands

Sinds de jaren ‘40 worden antibiotica in sub-therapeutische hoeveelheden aan diervoeders voor varkens en pluimvee toegevoegd, voornamelijk om de groei te bevorderen. Tegenwoordig is de invloed van deze zogenaamde groeibevorderaars op de groeiprestatie marginaal, dankzij de modernisatie van dierproductieprocessen. De risico’s van ontwikkeling van antibiotica-resistentie in humane pathogenen en de mogelijke transmissie daarvan hebben uiteindelijk geleid tot een Europees verbod (in 2006) op het gebruik van antibiotica in diervoeders. Er is nu behoefte aan mogelijkheden om de gezondheid, en met name darmgezondheid, van landbouwhuisdieren en daarmee de voedselveiligheid op een natuurlijke wijze te verbeteren. Modulatie van natuurlijke afweermechanismen door voederadditieven kan één van die mogelijkheden zijn. Selectie van dieren die beter toegerust zijn wat betreft hun natuurlijk afweersysteem kan een andere mogelijkheid zijn. Diverse strategieën zijn gericht op het verschuiven van de delicate balans in de darm tussen residente “niet-ziekteverwekkende” en potentiële ziekteverwekkende micro-organismen m.b.v. prebiotica, probiotica, organische zuren, enzymen en kruiden. Echter, fundamentele kennis over hun werkingsmechanismen en, nog belangrijker, hun effect op het immuunsysteem van de gastheer, ontbreekt.

In zoogdieren is aangetoond dat antimicrobiële peptiden (AMP’s) een belangrijke rol spelen in de verdediging van huid en slijmvliezen. Op basis van experimenteel bewijs in zoogdieren kan worden aangenomen dat verhoogde AMP expressieniveaus mogelijk kunnen leiden tot een betere weerstand tegen ziekte.

In tegenstelling tot zoogdieren is weinig bekend over het repertoire aan AMP’s en de biologische functies daarvan in vogels. Voor een deel zal de weefselspecifieke AMP expressie en haar regulatie in vogels overeenkomen met die van zoogdieren. Daarom kan modulatie van endogene antimicrobiële peptide expressie, bijvoorbeeld m.b.v. voederadditieven, een veelbelovende aanpak zijn om diergezondheid en -welzijn als ook dierlijke groei te bevorderen.

In dit proefschrift werd in eerste instantie m.b.v. *in silico* analyse gezocht naar tot dan toe onbekende vertegenwoordigers van bekende antimicrobiële peptide families. Na het bepalen van nucleotidesequenties van nieuwe genen werd de weefselspecifieke distributie van een geselecteerd aantal antimicrobiële peptiden verder onderzocht. Bovendien werden de eigenschappen van deze peptiden onderzocht door gebruik te maken van synthetische of recombinante peptiden.

Hoofdstuk 1 beschrijft de achtergronden en probleemstelling die aanleiding waren voor het onderzoek wat in dit proefschrift is beschreven.

Hoofdstuk 2 geeft een overzicht van de huidige kennis van vogels m.b.t. defensines en cathelicidines, twee belangrijke families van antimicrobiële peptiden. Naast een beschrijving van gen- en peptidestruktuur, biosynthese, weefsel-specifieke genexpressie en antimicrobiële aktiviteit worden ook regulatie van genexpressie en mogelijke werkingsmechanismen beschreven.

In **hoofdstuk 3** worden lokalisatie en antimicrobiële eigenschappen van kippen β-defensine AvBD9 (Gallinacin-6) beschreven. Door middel van *in silico* analyse ontdekten we 7 tot dan toe onbekende kippen β-defensines, inclusief AvBD9. Ras-onafhankelijke hoge AvBD9 mRNA expressie werd gevonden in krop en slokdarm weefsel. De variabele AvBD9 mRNA expressie in krop weefsel van 13 dagen oude kuikens en de aanwezigheid van bindingsplaatsen voor transcriptiefactoren die betrokken zijn bij de regulatie van zoogdier β-defensines duiden op een mogelijke opregulatie van het AvBD9 gen. Tijdens incubatie van bacteriën met synthetisch AvBD9 peptide werden zelfs bij lage concentraties peptide duidelijke veranderingen in de bacteriële morfologie waargenomen. De relatieve ongevoeligheid voor zout en het brede spectrum aan bacteriedodende en schimmeldodende activiteit van AvBD9 in "colony-counting assays" duiden op een belangrijke rol voor AvBD9 in de aangeboren immuniteit van kippen.

In **hoofdstuk 4** wordt de ontdekking van kippen cathelicidine CMAP27 beschreven. Het CMAP27 gen werd ontdekt m.b.v. *in silico* analyse en gekloneerd uit kippenbeenmerg cellen. Gebaseerd op de aanwezigheid van een elastase knipplaats tussen het catheline-domein en C-terminale antimicrobiële domein, werd een sterk cationisch (+10), antimicrobieel peptide voorspeld met een α-helix struktuur en een sterke gelijkenis met de myeloïde α-helix peptiden beschreven voor zoogdieren. CMAP27 mRNA bleek, net als in zoogdieren, voornamelijk en in hoge mate tot expressie te komen in myeloïde and lymfoïde weefsels. Deze bevindingen geven het evolutionaire belang van de α-helix subklasse van cathelicidine peptiden aan en ondersteunen de hypothese van een voorouderlijk cathelicidine gen waaruit andere cathelicidine genen zijn onstaan.

In **hoofdstuk 5** werden de biosynthese en verspreiding van CMAP27 in kippen granulocyten en de aanwezigheid van CMAP27 in het spijsverteringskanaal van de kip in relatie tot *Salmonella* besmetting onderzocht. Met behulp van immunohistochemie werden hoge CMAP27 concentraties ontdekt in heterofiele granulocyten, maar niet in andere perifere bloedcellen. Western blot analyse van het totale aantal leukocyten lokaliseerde de aanwezigheid van proCMAP27 in de inhoud van leukocyt granules. Stimulatie van heterofiele granulocyten met verschillende exocytose agonisten toonde aan dat CMAP27 was opgeslagen als inactief propeptide in

specifieke granules, gescheiden van zijn activerende proteases. Na extracellulaire uitscheiding via exocytose van zowel propeptide als activerende proteases wordt de CMAP27 precursor geaktiveerd door het afsplitsen van het C-terminale antimicrobiële domein. In vergelijking met niet-besmette dieren, werd een aanzienlijke infiltratie van CMAP27 bevattende heterofiele granulocyten waargenomen in het jejunum van *Salmonella*-besmette 4 dagen oude vleeskuikens, 8 en 48 uur na inoculatie. Er werd echter geen inductie van CMAP27 expressie waargenomen in darmepitheelcellen. Wel werd er hoge CMAP27 concentraties ontdekt in lever-specifieke natural killer (Pitt) cellen in de lever van een met *Salmonella* besmet dier. Zowel het synthetische CMAP27 peptide als het synthetische cathelicidin-1 peptide vertoonden een breed spectrum aan bacteriedodende en schimmeldodende aktiviteit tegen de geteste stammen, inclusief kip-specifieke *Salmonella* stammen. De overvloedige aanwezigheid van CMAP27 peptide in heterofiele granulocyten en krachtige antimicrobiële eigenschappen van CMAP27 duiden op een belangrijke rol voor cathelicidine peptiden in de microbicidale activiteit van heterofiele granulocyten.

De mogelijke beschermende functie van CMAP27 afkomstig uit de stuitklier en de rol van CMAP27 in het herstel van huidweefsel zijn beschreven in **hoofdstuk 6**. Met behulp van immunohistochemie werd aangetoond dat de CMAP27 expressie in de stuitklier zelf beperkt was tot de secretoire cellen in het klierepitheel van de primaire en secundaire holtes en de proximale einden van de hierin uitmondende klierkanaaltjes hetgeen in overeenstemming is met een rol voor CMAP27 in de bescherming van epitheliale klierstamcellen. ProCMAP27, maar niet het mature CMAP27 peptide, werd ook gevonden in het door deze klier uitgescheiden sebum (was) en werd behoudend geschat op >1 µg/g sebum. CMAP27 expressie bleek afwezig te zijn in normale huid en huidaanhangsels maar bij huidbeschadiging te worden geïnduceerd in keratinocyten met bijkomende infiltratie van CMAP27-bevattende heterofiele granulocyten. Synthetisch CMAP27 peptide vertoonde krachtige bacteriedodende aktiviteit tegen veerafbrekende bacteriën en de veerafbrekende schimmel, *Chrysosporium keratinophilum*, waarvan bekend is dat ze voorkomen in het verenkleed van zowel wilde als gedomesticeerde vogels. Analyse van de CMAP27 promoter onthulde meerdere bindingsplaatsen voor factoren waarvan bekend is dat ze een sleutelpositie vervullen in de regulatie van vetsynthese. Hieruit volgt dat CMAP27 expressie zeer waarschijnlijk verbonden is met de productie van sebum. Tevens werden er mogelijke bindingsplaatsen gevonden die betrokken zijn in de regulatie van cathelicidine expressie in keratinocyten en myeloïde cellen. Deze bevindingen geven aan dat cathelicidines en mogelijk andere antimicrobiële peptiden, kunnen bijdragen aan de bescherming van de huid en het verenkleed van vogels.

In **hoofdstuk 7** worden mogelijke therapeutische toepassingen van AMP's in pluimvee besproken. Stimulatie van AMP expressie in zoogdier cellen is o.a. al

aangetoond voor bepaalde vetzuren, aminozuren, vitamine D_3 derivaten en probiotica. Met name het gebruik van probiotica lijkt veelbelovend ter bevordering van zowel diergezondheid als voedselveiligheid. Bijvoorbeeld bij pasgeboren kuikens zou het toedienen van kip-specifieke melkzuurbacteriën, naast competitie voor bindingsplaatsen met potentiële ziekteverwekkende microorganismen en bacteriocine productie, mogelijk ook locale AMP expressie kunnen stimuleren. De mechanismen betrokken bij de regulatie van AMP expressie zijn nog steeds grotendeels onbekend. Echter, de overvloedige vertegenwoordiging van AMP's in gewervelden en de veelbelovende resultaten bij modulatie van AMP expressie in zoogdieren doen vermoeden dat modulatie van endogene AMP expressie via voederadditieven een haalbare strategie kan zijn om de gezondheid en groei van pluivee te bevorderen.

Dankwoord

Ik had me er van af kunnen maken met een groot “BEDANKT IEDEREEN”, maar dat is niet mijn aard, dus hier gaan, figuurlijk gesproken, een hoop veren in achterwerken worden gestoken! Eerst en toch vooral wil mijzelf bedanken: Albert dank je wel!!!! Zo zie je maar weer, je hoeft niet gestoord te zijn om te kunnen promoveren, maar het helpt wel.

Henk, onze glorieuze leider en mijn promotor, wil ik vooral bedanken voor zijn vertrouwen in deze (extra) belegen “AIO”. Als geen ander k(h)an Henk meedogenloos, volgens eigen zeggen op de Mongoolse wijze (ja, alles wat je zegt kan tegen je worden gebruikt, Henk; Astrid is mijn getuige), AIO’s achter hun broek aan zitten met deadlines voor abstracts, posters en papers. Hier staat wel tegenover dat hij samen met zijn “betere helft” kerstborrels en zomerBBQ’s verzorgde waar menig cateringbedrijf een puntje aan kan zuigen. Edwinza, ik ben nog steeds blij dat jij mijn co-promotor bent geworden, ... wat 15 november (i.v.m. vervelende vragen) wellicht kan omslaan. Als geen ander was je in staat om in mijn manuscripten de “vinger op de zere plek te leggen”. Zeker opmerkingen als “nou, daar ben ik het niet helemaal mee eens” dwongen mij soms een extra slag om de arm te houden, maar zorgden uiteindelijk wel voor verbetering. Frans, je hebt mij als eerste de kansen gegeven me verder te ontplooien. De tijd als labcoördinator was zeer leerzaam en heeft mijn kijk op de mensheid in het algemeen en mijn collega’s in het bijzonder, ietwat doen bijstellen. Vooral de reacties op de BHV oefeningen zullen nog lang bijblijven. Assie en Vinnie, kamergenoten van het eerste uur. Assie, behalve gezellig “ouwehoeren” en lachen, konden we ook elkaar vaak helpen met biologische weetjes, labprotocollen en spatelen natuurlijk. Je verweet mij vaak dat ik behoorlijk langdradig kon zijn in mijn uitleg (is ook zo) en schroomde niet om, waar ik bij stond, mensen daar voor te waarschuwen! Na het tellen van de bladzijden in jouw proefschrift ben ik tot de conclusie gekomen dat deze eigenschap blijkbaar besmettelijk is. Vinnie, altijd was je behulpzaam met scheikundige en natuurkundige weetjes. Het was natuurlijk stinkende mazzel dat je ooit als hobby kippen had gehouden en bij mijn uitroep “uropygial gland, wat is dat in vredesnaam”, een tip gaf om deze klier in het onderzoek mee te nemen. Met een hoofdstuk 6 in dit proefschrift en een manuscript in de pijplijn als gevolg! Steffie, jammer dat je indertijd door geldgebrek ons clubje moest verlaten. Het bleef leuk hoe je geregeld op onnavolgbare wijze min of meer onschuldige uitspraken eruit wist te flappen die voor meervoudige, soms prikkelende, uitleg vatbaar waren. Het is toch wel frappant dat je nu bij Wieke op de kamer zit, wat een

ongelofelijk klein wereldje toch. Hanne, onvermoeibaar en met tomeloze inzet heb jij het merendeel van de vele antimicrobiële assays uitgevoerd. Helaas moest ik die vaak dan zelf tellen. Hoewel, het blijft altijd weer spannend of ze goed zijn gegroeid en vooral of ze goed zijn geremd. Het blijft me verbazen hoe je ondanks je deeltijdbaan het werk schijnbaar meestal zonder al te grote stress af kan krijgen. Monique, het duurde helaas lang om een goedwerkend anti-CMAP27 antilichaam te maken, maar eenmaal daar heb jij in een razend tempo beeldschone coupe's en kleuringen gemaakt die de omslag van vooraanstaande tijdschriften met dikke, vette impactfactoren, niet zouden ontsieren. Hoewel jouw agenda de laatste tijd gelukkig behoorlijk vol is, weet je mijn invallen toch altijd in je schema in te passen. PS: Peter (K), warme gevoelens voor kippen heb ik er niet aan overgehouden (over geiten laat ik me niet uit). Fons, jouw aanzienlijke kennis op dat vlak (en geduld) hebben mijn inwijding in het RNA en DNA wereldje soepel laten verlopen. Martin, achteraf gezien denk ik wel eens dat ik misschien beter een promotieonderwerp had moeten kiezen wat in het verlengde lag van mijn vorige onderzoeksveld. Karlijn en LeOOOOOnie, jullie waren vaak een bron van sappige verhalen tijdens de lunch. Jammer dat we nog maar zelden samen lunchen, misschien moeten we in het vervolg maar afwisselend in 't Donkervoet en Androclus gaan eten. Mijn IRAS/VPH collega's wil ik o.a. bedanken voor de praktische tips, het "uitlenen" van chemicaliën en het toesteken van een helpende hand bij het spatelen. Ook na de verhuizing blijven de liefhebbers van het laatste welkom.

De afgelopen jaren heb ik ook het voorrecht gehad een paar studenten te mogen begeleiden tijdens hun (afstudeer)stage. Het was de bedoeling dat ze daarna als analist aan de slag zouden gaan. Echter, René die al gek was op paarden, besloot na enige jaren als analist te hebben gewerkt, het pipetje erbij neer te leggen en is nu hoefsmid te Apeldoorn. Hoe anders is dat gelopen bij Canan (spreek uit: Djanan). Zij heeft al tijdens haar afstudeerstage een toelatingsexamen gedaan om geneeskunde te kunnen studeren en is nu huisarts in opleiding. Hoewel Martijn met succes en met goede cijfers zijn afstudeeropdracht bij mij had afgerond, miste hij echte leuke resultaten in zijn verslag. Dus heeft hij zijn afstudeerstage nog eens dunnetjes overgedaan met mosselen in Zierikzee, wat voor sommige mensen blijkbaar een smakelijker onderwerp is dan kippendarmschraapsels (sauf les Français, evidement). Nienke, die op haar verjaardag om vage redenen door niemand uit ons onderzoeksgroepje gekust wilde worden (zelfs niet door een jonge blonde god), timmert nog steeds met succes aan de weg als analiste.

Het belangrijkst komt altijd het laatste. Nathan & Nolan, de slapeloze nachten die jullie mij bezorgden hebben ervoor gezorgd dat ik van onderzoeksstrategie veranderde, 7 nieuwe kippen AMP's ontdekte en vele nieuwe spannende onderzoekslijntjes kon starten. Waar nachtelijke poepluiers en blèèèrsessies al niet goed voor zijn! Lieve Pa en Ma, bedankt voor alles, mede dankzij jullie steun in voor-

en tegenspoed ben ik geworden wie ik nu ben. Geregeld vroegen jullie me hoe ver ik nu was met de promotie. Het heeft misschien wat meer tijd en energie gekost om het boekje af te krijgen dan we hadden gehoopt, maar nu ligt het er toch. Valérie, lieve schat, om even terug te komen op jouw dankwoord uit 1995: toen bedankte je mij voor het zolang mogelijk uitstellen van mijn (terechte) klachten over jouw manier van leven en voor mijn geduld en tolerantie tijdens de laatste weken. Je beloofde dat het je laatste proefschrift zou zijn. Ha ha, maar niet de mijne. Dat van dat geduld klopt wel, 12 jaar heb ik gewacht om je dit koekje van eigen deeg te mogen presenteren. Alle gekheid op een stokje, zonder jouw steun was dit boekje er nooit gekomen. Zeker de laatste maanden waarin ik in een moordend tempo alle hoofdstukken thuis moest schrijven met twee rondrennende, jengelende bengeltjes was jij het die de vrede bewaakte. Een ding wat ik wel zal betreuren is dat ik je na 15 november niet meer met je titel kan plagen (maar er blijven genoeg dingen over waarmee ik dat wel kan doen).

Curriculum vitae

Albert van Dijk werd op 23 maart 1965 te Heerjansdam geboren. Na het doorlopen van de MAVO behaalde hij het diploma “Middelbaar Laboratorium Onderwijs, richting klinische chemie” in 1986 aan het van ’t Hoff instituut te Rotterdam. Na het vervullen van de militaire dienstplicht (1986/1987) werkte hij 2 jaar bij de Oranjeboom bierbrouwerij te Rotterdam. In december 1989 trad hij in dienst als analist bij de Vakgroep Voedingsmiddelen van Dierlijke Oorsprong (VVDO), Faculteit der Diergeneeskunde, Universiteit Utrecht. Daar participeerde hij in onderzoek naar sensorische vleeskwaliteit. In deeltijd studeerde hij aan de Hogeschool Rotterdam & Omstreken te Delft en behaalde in 1993 het diploma “Hoger Laboratorium onderwijs, richting biotechnologie”. Vanaf januari 2001 was hij werkzaam in onderzoek naar de biologische functies van antimicrobiële peptiden van de kip. Onder leiding van Prof. Dr. H. P. Haagsman en Dr. E. J. A. Veldhuizen werd het onderzoek dat in dit proefschrift beschreven staat deels uitgevoerd bij de hoofdafdeling Volksgezondheid & Voedselveiligheid (voorheen VVDO) en later bij het Departement Infectieziekten en Immunologie.