Antimicrobial peptides and proteins in innate immunity

Emphasis on isolation, characterization and gene regulation

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To my family
“Satisfaction of one's curiosity is one of the greatest sources of happiness in life.” - Linus Pauling
ABSTRACT

Antimicrobial peptides are endogenous antibiotics and effector molecules of innate immunity. These peptides are mainly localised to epithelial linings and circulating neutrophils. They kill microbes by disruption of their membranes. The two main families of mammalian antimicrobial peptides are defensins and cathelicidins. In this thesis, the presence of antimicrobial peptides and proteins in human colon mucosa and Vernix caseosa (vernix) has been demonstrated. In addition, the gene regulation of the human cathelicidin LL-37 has been investigated in a colon epithelial cell line. A rat model for cathelicidins has also been developed.

A peptide/protein extract was prepared from human colon mucosa and was found to exhibit antibacterial activity against both Gram-positive and Gram-negative bacteria, in addition to antifungal activity. Several antimicrobial peptides and proteins were identified in this extract. One of them was ubiquicidin, which has not previously been isolated from human tissue. The colon was thus shown to be protected by a complex mixture of antimicrobial peptides and proteins, which together exert potent antimicrobial activity.

Vernix is a creamy substance covering the skin of the foetus during the last trimester. Peptide/protein extracts of vernix displayed potent antimicrobial activity. A multitude of antimicrobial peptides, proteins and lipids were isolated and characterized in vernix. Interestingly, nearly all of the most abundant proteins in vernix are considered to be involved in innate immunity. Vernix thereby forms a potent surface defence barrier, protecting the foetus and the newborn against bacteria, fungi and parasites. In addition, vernix components also exhibit protease inhibition and opsonising features.

The rat cathelicidin rCRAMP has been isolated and characterized as a 43-residue peptide, which is processed differently than the mouse cathelicidin CRAMP, despite identical primary structure at the processing sites. rCRAMP was shown to have an antimicrobial activity and expression pattern similar to that of LL-37. Therefore, responses related to cathelicidin expression in health and disease can now be studied in the rat.

The gene regulation of LL-37 in a human colon epithelial cell line was investigated, utilising a luciferase reporter system. The promoter was found to contain at least one enhancer and two silencer elements. The enhancer element was demonstrated to be a functional Ets binding site and one of the silencers is probably regulated by Vitamin D. In addition, the second intron was found in our system to enhance the transcriptional activity in the presence of butyrate and in cooperation with the 3’ end of the promoter. Thereby the gene regulation of LL-37 is beginning to be unveiled.
LIST OF PUBLICATIONS

This thesis is based on the following papers, referred to by their Roman numerals:


V. Termén, S. Tollin, M. Agerberth, B. & Gudmundsson, G. H. Promoter elements controlling expression of the human gene encoding the antimicrobial peptide LL-37. Manuscript

* These authors contributed equally to this work.
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<td>AF</td>
<td>Amniotic fluid</td>
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<tr>
<td>B. megaterium</td>
<td><em>Bacillus megaterium</em></td>
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<td>BPI</td>
<td>Bactericidal/Permeability Increasing protein</td>
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<td>C. albicans</td>
<td><em>Candida albicans</em></td>
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<td>CAMP</td>
<td>Cathelicidin antimicrobial peptide</td>
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<td>CRAMP</td>
<td>Cathelicidin related antimicrobial peptide</td>
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<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<td>ECP</td>
<td>Eosinophil cationic protein</td>
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<td>EMSA</td>
<td>Electromobility shift assay</td>
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<td>GBS</td>
<td>Group B <em>Streptococcus</em></td>
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<td>GI</td>
<td>gastrointestinal</td>
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<td>HBD</td>
<td>human β-defensin</td>
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<td>HFBA</td>
<td>Heptafluoro butyric acid</td>
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<td>HNP</td>
<td>human neutrophil peptide</td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<td>IBD</td>
<td>inflammatory bowel disease</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>NK-cells</td>
<td>Natural killer cells</td>
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<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
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<td>PLA₂</td>
<td>Phospholipase A₂</td>
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<td>PRR</td>
<td>pattern recognition receptor</td>
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<td>PVDF</td>
<td>polyvinylidene difluoride</td>
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<td>rCRAMP</td>
<td>rat Cathelicidin related antimicrobial peptide</td>
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<td>SLPI</td>
<td>Secretory leucocyte protease inhibitor</td>
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<td>TBE</td>
<td>tris-borate-EDTA</td>
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<td>TCR</td>
<td>T-cell receptor</td>
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<td>TFA</td>
<td>Trifluoroacetic acid</td>
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<td>toll-like receptor</td>
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<td>Uteroglobin related protein-1</td>
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<td>VDR</td>
<td>vitamin D receptor</td>
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<td>VDRE</td>
<td>vitamin D responsive element</td>
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<td>Vernix</td>
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1 INTRODUCTION

1.1 Immunity

The human body is constantly exposed to a multitude of bacteria. However, because of our effective immune system we rarely get infected. In mammals, the immune system can be divided into two types; the innate and the adaptive immunity. The innate immunity is constantly acting and constitutes a first line of defence. If a pathogen can surpass innate immunity and invade our body, it will be recognised by cells of the adaptive immunity and an immune response is initiated. Cells of both the innate and adaptive immunity will then cooperate, with the ultimate goal of eliminating the invading microbe.

1.1.1 Adaptive immunity

The adaptive immune system is acquired and involves lymphocytes with receptors of unique specificities to foreign antigens. An immune response of adaptive immunity can be of either a humoral or cellular type. The humoral immunity is directed towards extracellular antigens, where B-cells with their surface immunoglobin receptors (antibodies) are crucial cells. When a B-cell recognises an antigen and/or is activated by dendritic cells, it differentiates into a plasma cell, which secretes antibodies with the same specificity as the surface receptor. The cellular immunity is directed towards intracellular antigens, e.g. antigens from virus infected cells or tumour cells. T-cells are mediators of cellular immunity and utilise the T-cell receptor (TCR) to recognise foreign peptide fragments presented on the surface of cells by the major histocompatibility complex (MHC). If a foreign antigen is presented and recognised by the T-cell, a subtype of T-cells is able to directly kill the cell. After activation, both B- and T-cells undergo a clonal expansion, thus increasing the response. The adaptive immunity is also capable of
generating a memory of encountered pathogens and at re-infection a rapid response is elicited, hindering disease progression. [1]

It takes four to five days for the adaptive immunity to gain the full force needed for microbe elimination, while bacteria can duplicate in 20 minutes. Uninhibited bacterial growth would theoretically result in the human cells being outnumbered by ten million to one after only one day, and after the five-day preparation of the adaptive response, the bacteria could outnumber the human cells by almost a googol \((10^{100})\). In conclusion, we would never survive relying only on the adaptive immunity.

1.1.2 Innate immunity

The innate immune system is constantly active and constitutes a first line of defence. An example of the efficiency of the innate immunity is that insects survive in microbe-rich environments such as soil or decomposing animal flesh, although they are solely dependent on innate immunity [2]. The cells of innate immunity are epithelial cells and phagocytes, which both produce microbicidal molecules. The epithelium is the tissue lining the body towards the outside world. It forms a strong mechanical barrier and produces effector molecules, leading to an efficient prevention of microbial entry into the body. A phagocyte of importance in innate immunity is the neutrophilic granulocyte, which is the first cell to be recruited to sites of infection, where the neutrophil engulfs invading microbes, and utilises antimicrobial molecules to kill the microbes. Some of the antimicrobial molecules are also secreted [3] and have the capacity to attract other immune cells [4, 5], and thus enhance the immune response.

The innate immune system is not as specific as the adaptive immunity and no clonal expansion of cells occurs. Instead an innate immune response is triggered by recognition of common structural patterns of microbes (pathogen-associated molecular patterns/PAMPs) [6]. Cells of innate immunity express pattern recognition receptors
(PRRs), which recognise these microbial structures. Among these are the toll-like receptors (TLR:s), which to date comprise a family of eleven members [7, 8]. These different TLR:s recognise PAMPs such as proteoglycan (TLR2) [9, 10], double stranded RNA (TLR3) [11], LPS (TLR4) [12], flagellin (TLR5) [13], single stranded RNA (TLR7) [14-16] and bacterial DNA (TLR9) [17]. If a microbe approaches a cell with an array of these receptors it will cause a signalling cascade in the cell involving MyD88 [7]. The resulting response is presumably specific for that microbe [18, 19] and in some cases involves induction of antimicrobial peptides [20, 21].

Commensal and pathogenic bacteria have similar structural features. How the immune system discriminates between them is not known in detail, but is an issue of extensive research. An example of such a discrimination has been demonstrated in the oral cavity, where commensal and pathogenic bacteria both upregulate antimicrobial peptides, however by different signalling pathways [22].

1.2 Antimicrobial peptides

Antimicrobial peptides have been found in almost all higher eukaryotic organisms [23]. However, they are also expressed in archaea, eubacteria, protists, plants and invertebrates, revealing that these peptides were present early in evolution [24]. In mammals, they are mainly expressed in the epithelia and in blood cells. Antimicrobial peptides are small polypeptides of less than 50 amino acid residues, with the ability to directly kill bacteria, fungi and enveloped viruses. The activity spectrum is unique for every peptide and single amino acid substitutions can affect the activity [25, 26]. The net charge of nearly all antimicrobial peptides is positive, and therefore they are often designated cationic antimicrobial peptides. The tertiary structures of the peptides are diverse. However, they have an amphipathic character in common. (Fig 1)
Fig 1. Edmundson wheel projection of the amphipathic α-helix in LL-37 (residue 11-28). Boxed letters indicate hydrophobic residues, while bold letters indicate hydrophilic residues.

1.2.1 Mechanism of action

Most antimicrobial peptides are membrane active and lyse the target cell by disrupting the integrity of the membrane. Many models explaining their mechanism of action have been proposed, of which the barrel-stave pore, thoroidal pore, and carpet models are well established [27]. (Fig 2) In the barrel-stave pore model the peptides transverse the membrane with the hydrophobic surfaces directed towards the lipid membrane and the hydrophilic surfaces inwards forming a pore which allows water and electrolyte leakage [28]. The toroidal pore model describes the killing mechanism in a similar manner as the barrel stave pore model. However, here the peptides are suggested to aggregate and induce the lipid monolayers to bend continuously through the pore, causing the lipid head groups to be directed towards the water core [27]. In the carpet model, the bacterial membrane surface is covered with the
peptides after an electrostatic interaction, and the membrane is disrupted in a detergent-like manner [28].

![Fig 2. Mechanism of action of antimicrobial peptides. A. Barrel stave pore model, B. Toroidal pore model and C. Carpet model. (Modified from [27])](image)

Targets of antimicrobial peptides are the bacterial membranes, which have been called “the Achilles heel of microbes” [29]. The membranes are so finely tuned that changes in these structures are difficult to achieve. Hence, bacterial resistance against antimicrobial peptides is rarely observed [30].

At higher concentrations than the bactericidal, antimicrobial peptides are cytotoxic [31, 32]. Due to differences in the microbe and mammalian membranes the peptides preferentially attack microorganisms. The cationic antimicrobial peptides are more attracted to the negatively charged membranes of bacteria than to the neutral mammalian membranes. This difference in membrane charge is due to a higher level of acidic phospholipids in the outer leaflet of the bacterial
membranes, while the eukaryotic membranes have more cholesterol and zwitterionic phospholipids facing the extracellular space, and the acidic phospholipids are positioned at the cytoplasmic side. (Reviewed in [33])

In addition to lysis, other mechanisms of action have been reported for antimicrobial peptides [27]. Proline-rich antimicrobial peptides have been shown to inhibit critical intracellular processes, such as protein synthesis [34, 35]. Histatins employ a complex antifungal mechanism, targeting the mitochondria and causing efflux of ATP [36].

1.2.2 Other functions of antimicrobial peptides

Apart from being able to directly kill or inhibit growth of microorganisms, antimicrobial peptides also have other functions in immunity. They have been shown to chemoattract cells of both the innate and adaptive immunity, i.e. polymorphonuclear leukocytes [37], monocytes [4], and T-cells [5, 37]. The peptides thereby form a link between innate and adaptive immunity. In addition, by using primary cultures or cell lines, it has been demonstrated that antimicrobial peptides exhibit specific immunomodulatory properties, such as modulating the differentiation and enhancing the endocytic capacity of dendritic cells [38]. Furthermore, upregulation of the expression of chemokines in epithelial cells and macrophages has been reported [39, 40], adding additional immunomodulatory functions. These spectra of immunomodulatory activities of antimicrobial peptides have lead some researchers to question whether the primary function of antimicrobial peptides really is to kill bacteria [41].

Many other activities have been attributed to these peptides, such as neutralising LPS [42], increasing phagocytosis [43], induction of mast cell degranulation [44], and participation in the regulation of the complement system [45, 46]. In addition, stimulation of angiogenesis [47] and re-epithelialisation in wounds [48], have also been shown, indicating that these peptides may be multifunctional in vivo.
1.2.3 Mammalian antimicrobial peptides

The major families of antimicrobial peptides expressed in mammals are the defensins and the cathelicidins. The defensins are characterized by three disulphide bonds [31], while the cathelicidin family is characterized by an evolutionarily conserved prepro-region [49].

The defensins are subdivided into the α-, β- and θ-defensins. The α- and β-defensins share a similar amphipathic β-sheet-rich structure (defensin fold) [50], but differ in primary structure and disulphide patterns. α-defensins are mainly expressed by neutrophils and Paneth cells of the small intestine. They are synthesised as preproproteins and are processed by proteolytic cleavage to mature peptides of ~30 residues. β-defensins are primarily expressed in epithelia and are produced as preproteins, hence the mature peptides of ~35-45 residues are only preceded by a signal peptide.

The θ-defensins are circular and are formed by ligation of two short peptides. The first θ-defensin discovered, was isolated from leukocytes of the rhesus macaque and characterized as an 18-residue circular peptide active towards both bacteria and fungi [51]. It was found that its circular property was important for its activity since an open chain analogue was much less active [51].

1.2.4 Human antimicrobial peptides

The α-defensin family in humans contains six members. These are the human neutrophil peptides 1-4 (HNP 1-4), which are located in circulating neutrophils [31], and human defensins 5 and 6 (HD5-6), which are expressed by Paneth cells of the small intestine [52, 53]. HNP 1-3 are stored in neutrophilic granules as mature peptides, and are fully active when these granules fuse with the phagolysosomes [50]. On the other hand, HD-5 has been shown to be stored and released as a proprotein and is processed extracellularly by Paneth cell trypsin [54].
Four human β-defensin peptides (HBD 1-4) have been characterized. They are mainly expressed by epithelial cells [55-58]. However, 28 β-defensin genes have been identified in the human genome [59]. Seven of these genes, HBD 5-6 and 25-29 have been verified to be transcribed in epididymis [60, 61].

Six θ-defensin genes have so far been detected in humans [62]. However, these genes contain a premature stop codon and thus represent pseudogenes [62]. Interestingly, mRNA encoding one of these genes has been detected in bone marrow and a synthetic replica of the potential protein product, retrocyclin, has been shown to protect cells from infection by HIV [63].

The only human member of the cathelicidin family is LL-37, which will be discussed in detail below.

There are other examples of antimicrobial peptides that have been isolated and characterised from human tissues. Hepcidin/LEAP-1 is a peptide mainly expressed in the liver, but was first isolated from human blood [64] and later from urine [65]. Dermicidin, a 47-residue peptide is specifically expressed by sweat glands [66], and histatins are histidine-rich antifungal peptides present in the saliva [36].

### 1.3 Cathelicidins

Cathelicidins are a diverse group of antimicrobial peptides, derived from preproproteins, sharing a conserved N-terminal proregion (cathelin) and a variable C-terminal antimicrobial domain [49]. In neutrophils cathelicidins are stored as proproteins and since the cathelin region has a net negative charge it has been suggested to fold over the positively charged C-terminal region and thus keep the peptide part inactive. The cathelicidins are released from the granules of neutrophils together with proteases, which cleave off the C-terminal peptide domain, thereby liberating the mature active antimicrobial peptide [67]. The active cathelicidin peptides exhibit a variety of
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structures. Several of the peptides are linear and $\alpha$-helical, such as human LL-37 (Fig 1), some of them form $\beta$-hairpins, such as pig protegrin-1 [68], and others have an extended helical structure due to an overrepresentation of proline residues i.e. the porcine PR-39 [69].

![Diagram of gene organisation of cathelicidins](image)

**Fig 3. The gene organisation of the cathelicidins**

The genes encoding cathelicidins consist of four exons of which the first three encode the signal peptide and the conserved cathelin region [70-72]. (Fig 3) The cathelin domain is similar to members of the cystatin superfamily of protease inhibitors [73] and in deed the cathelin protein has been shown to possess protease inhibitory activity [74]. The forth exon encodes the antimicrobial peptide domain and the processing site [70-72]. Interestingly, the cathelin protein has recently been demonstrated to also exert antimicrobial activity with a different spectra compared to the human cathelicidin peptide LL-37 [74]. This reveals that upon activation two antimicrobial polypeptides are released, enhancing the antimicrobial spectra of cathelicidins.

The fourth exon has a very high intraspecies and interspecies variation, suggesting that exon shuffling has occurred [75]. The promoter of the human cathelicidin has been characterised and a number of potential binding sites for transcription factors have been
identified. Functional studies have now been performed as will be discussed in paper V.

1.3.1 LL-37

The name of LL-37 originates from the N-terminal amino acids (leucine, leucine), and the length of the mature peptide (37aa). Before the processing site was determined the putative peptide was named FALL-39 [76], but after isolation and characterization of the mature peptide from neutrophils the name was corrected [71]. The name hCAP18 is often used when referring to the proform [42].

LL-37 is mainly localised in neutrophils [77, 78], but also other immune cells such as B-cells, γδ-T-cells, NK-cells, monocytes and alveolar macrophages express LL-37 [37, 79]. In contrast to the individual defensins, LL-37 is also expressed by epithelia of many different tissues, i.e. the gastrointestinal tract [80, 81], lung [82], eye [83], skin [84], and the reproductive tract [76, 80], revealing that LL-37 is involved in antibacterial defences of most surfaces of the body. In addition, secretory glands produce and release LL-37 into saliva [85], sweat [86], and milk [87]. Interestingly, hCAP18 is present in high levels in seminal plasma and is attached to the sperms [88], suggesting a role in conception.

It has been discussed whether LL-37 kill their target cell by the carpet model [89] or the toroidal pore model [90]. In physiological medium, LL-37 forms an amphipathic α-helical structure [32, 76] (Fig 1) and this structure is essential for its bactericidal activity [32]. The antimicrobial spectrum of LL-37 is broad with activity against an array of Gram-positive and Gram-negative bacteria [91]. In addition, it has been reported that LL-37 is able to kill some specific viruses such as herpes simplex virus [92] and vaccinia virus [93].

Proteinase-3 has been suggested to be the processing enzyme for the human cathelicidin. This enzyme cleaves hCAP18 extracellularly, and thus liberates the mature active LL-37 [94]. However, alternative
processing sites have been identified. In seminal plasma gastricin produces ALL-38 [95], and in sweat, specific enzymes generate the shorter peptides RK-31, KS-30 and KR-20 [96]. These variant forms of LL-37 exhibit different antimicrobial activities, thus further increasing the activity spectra from one gene product.

1.3.2 In disease

Studies that have demonstrated altered expression of antimicrobial peptides in disease emphasise their importance in vivo. For example, LL-37 is highly upregulated in psoriasis [97, 98], while a lower induction is observed in atopic dermatitis [98]. This could explain why psoriatic lesions rarely become infected compared to the eczema of atopic dermatitis patients. In contrast, the expression of LL-37 is downregulated in colonic epithelial cells during Shigella infection, as shown in biopsies from patients infected by Shigella [81]. This reduction of LL-37 at a surface barrier may facilitate the entry of the Shigella, and thus represent an immune escape mechanism.

Morbus Kostmann is a rare congenital disease characterised by severe neutropenia. Despite treatment with granulocyte colony stimulating factor, which restores the levels of neutrophils, these patients still have periodontal disease and recurrent infections. Diminished levels of LL-37, and reduced levels of α-defensins in the neutrophils of these patients have been suggested to be of importance for the susceptibility to the frequent infections in these patients [99].

To evaluate the importance of cathelicidins in vivo, a knock out mouse for the mouse cathelicidin CRAMP has been generated [100]. When these mice were challenged with group A streptococci larger infected wounds, which persisted longer, were observed and the amount of bacteria in the tissue was higher in comparison to normal littermates. In addition, infection of normal mice with a bacterial strain resistant to CRAMP showed the same phenotype as the CRAMP KO-mice. This
reveals that the mouse cathelicidin is of importance in the defence against group A streptococci [100].

1.4 Antimicrobial proteins

Apart from the antimicrobial peptides also proteins can display antimicrobial properties. In 1922 Alexander Fleming described the bactericidal activity of lysozyme [101] that was the first protein demonstrated to possess antimicrobial properties. Later, in the 40’s histones were shown to exhibit antibacterial activities [102]. This could be a residual effect of the high positive charge of histones, a property needed to bind negatively charged DNA. Recently however, studies have shown that histone H2B is not only located in the nucleus but also close to the cytoplasmic membrane [103]. Histone H2B is also located in the foetal membrane of the placenta from where it is actively released into the amniotic fluid [104]. It was also shown that histone H2B exhibits similar antibacterial potency as the frog peptide magainin, one of the first described mammalian antimicrobial peptides [104].

Bactericidal/permeability increasing protein (BPI) is a 55kDa antimicrobial protein located in neutrophils [105]. BPI is composed of two domains of which the N-terminal domain is antibacterial and binds endotoxins such as LPS, while the C-terminal domain exhibit opsonising properties.

Calprotectin is an antifungal and antibacterial complex consisting of a heterodimer of calgranulin A and B. Calprotectin constitutes over 60% of the protein content in the cytosol of neutrophils [106], indicating a role in innate immunity. Faecal calprotectin levels are highly increased in neoplasia, inflammatory bowel disease (IBD) and infections, and is thus a potential diagnostic marker in inflammation [107]. Calprotectin has been suggested to kill target micro-organisms by binding and depleting the environment of zinc, an essential metal ion
for microbes [108]. This mechanism of action is shared by psoriasin, which is a major *E. coli*-killing protein in human skin [109].

Granulysin is a 9kDa protein produced by Natural killer (NK-) cells and CD8 T-cells, and is included in the cytolytic machinery of these cells [110]. Granulysin kills Gram-positive and Gram-negative bacteria, as well as fungi and parasites [111]. Interestingly, granulysin can directly kill *Mycobacterium tuberculosis*, one of the most infectious killers in humans. With aid of perforin, another protein in the cytolytic vesicles of T- and NK-cells, granulysin can enter macrophages infected with *Mycobacterium tuberculosis* and kill the intracellular bacteria [111], thereby providing an endogenous defence mechanism towards a bacterium resistant to most commercial antibiotics.

Secretory leucocyte protease inhibitor (SLPI) is a 12 kDa serine protease inhibitor present in mucosal secretions [112]. The antimicrobial activity of SLPI appears to be independent of its inhibitory activities, and SLPI can kill Gram-positive and Gram-negative bacteria [113], fungi [114] and certain viruses, including HIV-1 [115]. Notably, inhibition of proteases may be involved in host defence by regulating the processing of antimicrobial peptides. For example, when the processing of the pig cathelicidin protegrin-1 was inhibited, the pig wound fluid exhibited reduced antimicrobial activity [116]. Hence, SLPI appears to have a dual role in host defence.

Additional human proteins, shown to exhibit antimicrobial activity are phospholipase A$_2$ (PLA$_2$) [117], eosinophil cationic protein (ECP) [118, 119], and lactoferrin [120].

### 1.5 Immunity of the colon

A small fraction of all microbial species are capable of colonising the skin and mucosal surfaces of the human. However, we harbour 10 times more bacterial cells than we have human cells in our body [121]. Many of the colonising microbes reside in the gastrointestinal (GI) tract,
representing around 400 different species. The most heavily colonised site in the GI tract is the colon lumen, where we harbour about 2 kg of bacteria with a concentration of around $10^{10-12}$ bacteria/ml [122].

The resident bacteria (the normal flora) are beneficial for the host. The normal flora produces vitamin K and vitamin $B_{12}$ [123] in addition to the essential amino acid lysine [124], and may be an important source for this essential amino acid at malnutrition. Bacteria in the colon also produce butyrate by fermenting dietary fibres [125], and butyrate is the main energy source for the colonic epithelial cells [125, 126]. In addition, the normal flora is important in the development of the immune system [127] and contributes to host defence by blocking pathogen colonisation such as alpha streptococci [128]. Furthermore, as much as 25% of the gut mass is represented by lymphoid tissue emphasising the close relationship of the gut and the immune system [122].

Fig 4. A cross section of the human colon. (Adapted from www.patientcenters.com)
The surface of the colon contains crypts of Lieberkuhn. (Fig 4) At the bottom of these crypts colonic epithelial stem cells are located. As the cells migrate towards the top of the crypt they differentiate, and when reaching the lumen they are fully differentiated colonic epithelial cells. However, their lifespan is short, and they are soon shed and replaced by new cells. In the small intestine it has been suggested that epithelial cells undergo apoptosis and secrete antimicrobial molecules [129]. Most likely this also occurs in the colon, adding this defence mechanism to the colon. The crypts are also lined by numerous goblet cells, which produce and secrete mucus, a viscous fluid composed of highly glycosylated proteins (mucins) suspended in a solution of electrolytes. The mucus serves many functions, including protection against shear stress and chemical damage.

The antimicrobial peptides known to be produced by the colonic epithelium are LL-37 [81, 130, 131] and the β-defensins HBD-1 and HBD-2 [132]. Both LL-37 and HBD-1 are constitutively expressed, while HBD-2 is induced in inflammation by proinflammatory stimuli that activate the transcription factor NF-κB [132]. The expression of LL-37 in colon is modulated by short chain fatty acids. The most potent inducer is butyrate, which upregulates the LL-37 expression in colonic epithelial cells, as shown by real time PCR [130, 131]. The GI tract is thus well protected against invasion of pathogens. However, pathogen entry still occurs and to facilitate invasion, pathogens have developed different immune escape strategies. Recently, downregulation of antimicrobial peptides has been suggested as such a strategy. For example, *Shigella* spp. downregulate LL-37 in human colon epithelia [81], while *Salmonella* downregulates Paneth cell defensins in mouse small intestine [133].
1.6 Immunity of the foetus

The foetus is residing in a sterile environment and is considered to be immunoprivileged, otherwise it would be recognised as non-self, rejected and destroyed by the adaptive immunity of the mother.

Intra-amniotic infections are however not uncommon and are believed to be a trigger for miscarriage and preterm labour [134]. In the foetus, the adaptive immunity and innate immunity are both immature [135]. For example, foetal granulocytes and monocytes have a reduced phagocytic capacity [136] and newborns have a diminished ability to produce neutrophils [135]. Furthermore, the neutrophils produced, are deficient in BPI [137]. Accordingly, newborns have a relatively high risk of invasive microbial infections [138].

How does the foetus defend itself if microbes invade the amniotic sac? One clue is that elevated levels of lactoferrin [139], HNP 1-3, BPI and calprotectin in amniotic fluid [140, 141] are associated with intra-amniotic infections in preterm labour. Furthermore, after 17 weeks of gestation maternal antibodies are transferred to the foetus, and at 33 weeks foetal IgG levels approximate the levels of the mother [135].

1.6.1 Amniotic fluid

The foetus floats in amniotic fluid (AF), which is mainly derived from maternal blood transported across the amnion membrane. The foetus constantly circulates the amniotic fluid by swallowing and inhaling, and replaces it through exhalation and urination. Amniotic fluid has many functions such as protecting the baby from outside injury by cushioning sudden blows or movements, allowing freedom of foetal movement, and permitting symmetrical musculoskeletal development and proper lung development. It also protects the foetus from heat loss by keeping the temperature constant.

It has been known since 1949 that amniotic fluid exhibits antimicrobial properties [142]. Since then, several investigators have confirmed this finding and have identified some of the components e.g.
spermine [142], calprotectin [141], histone H2A and H2B [104], phospholipase A2 [143], BPI [141], HNP 1-3 [140], and lactoferrin [144] as responsible for the antimicrobial activity. During pregnancy the antimicrobial potency of AF changes with maximal activity reached at 36-42 weeks of gestation [145]. This is consistent with the fact that the lactoferrin level in amniotic fluid increase with gestational age [139].

1.6.2 Vernix caseosa

A creamy white substance designated Vernix caseosa (Latin for “Cheese-like varnish”) covers the skin of the foetus during the last trimester. (Fig 5)
The composition of vernix is water (81%), lipids (9%), and proteins (10%) [146]. Research on vernix has mainly focused on the lipids [147-150], while the vernix proteins have not been thoroughly investigated.

The function of vernix has been debated. Early, many protective functions were proposed, such as heat insulation, moisturising the skin, and protection of the skin from macerating effects of the amniotic fluid. Also other functions have been suggested such as hormonal effects, anti-inflammatory effects, nutritive functions, and facilitation of passage through the birth canal. (reviewed in [151]) More recently, a skin cleansing function [152] has been proposed.

Studies on whether vernix possesses antimicrobial activities have yielded contradictory results [151, 153], most likely due to different antimicrobial assays. Members of the defensin and cathelicidin families have recently been detected in vernix, as will be discussed in paper II. Furthermore, vernix has been suggested to constitute a mechanical obstruction to bacterial passage [153]. In association with the discussion on the antimicrobial properties of vernix there has been a debate on whether vernix should be left on the skin after birth [154, 155]. Skin colonisation of neonates was compared after washing (leaving vernix on the skin) or bathing (removing vernix) the newborn. That study resulted in no difference in colonisation [155]. However, the bathing reduced heat loss, and made the newborns calmer, more quiet and comfortable than washed babies [155].

The amount of vernix on the skin correlates with gestational age [156]. Preterm infants lacking vernix have a higher rate of nosocomial and community acquired infections [157]. This suggests that vernix plays a role in the protection against infection of the foetus and the newborn.
2 AIMS

My aims in this thesis were

1. to study the presence of antimicrobial peptides and proteins in
   ♦ human colon mucosa
   ♦ vernix caseosa
   ♦ amniotic fluid

2. to study the cathelicidin family
   ♦ by developing a rat model
   ♦ by studying the gene regulation of the human cathelicidin LL-37.
3 METHODS

The main methods used in this thesis are as follows:

3.1 Extraction procedure

Tissues were homogenised in 60% acetonitrile in 1% trifluoroacetic acid (TFA) and extracted overnight. The homogenates were then centrifuged and the supernatants lyophilized. The material soluble in 0.1% TFA was then loaded onto activated OASIS™ HLB columns, which were equilibrated in 0.1% TFA. After washing with 0.1% TFA and 10% acetonitrile in 0.1% TFA, the bound material was eluted with 80% acetonitrile in 0.1% TFA and lyophilized.

3.2 Inhibition zone assay

Antimicrobial activity was analysed in an inhibition zone assay. Activity against four different microbes were analysed, i.e. the Gram-positive bacteria *Bacillus megaterium* (*B. megaterium*) strain Bm11, group B *streptococcus* (GBS) (clinical isolate), the Gram-negative bacterium *Escherichia coli* (*E. coli*) strain D21, and the fungus *Candida albicans* (*C. albicans*). Thin plates (1mm) of 1% agarose containing 6x10⁴ microbes/ml were prepared. LB-medium was used for the bacteria and LB or YM-medium was used for the fungus. In some experiments medium E [158] was added to the agarose, and in some experiments NaCl was omitted from the LB-medium. Wells of 3 mm in diameter were punched in the agarose and 3 µl of sample was loaded into each well. After an overnight incubation, the inhibition zones were measured.

3.3 High performance liquid chromatography (HPLC)

In this thesis different chromatographic methods have been utilised for separation of peptides/proteins. Reversed phase chromatography was employed, utilising C8 or C18 columns equilibrated in aqueous 0.1% TFA or 0.1% heptafluorobutyric acid.
(HFBA) and an eluting gradient of acetonitrile in 0.1% TFA or 0.1% HFBA.

### 3.4 Dot blot and Western blot analyses

In dot blot analyses, samples were spotted directly onto Hybond C Super membranes, and in Western blot analyses, peptides/proteins were separated by SDS/PAGE using 10–20% Tricine gels and further blotted onto polyvinylidene difluoride (PVDF) membranes. For HNP1–3 detections the peptides/proteins were fixed onto the membrane with 0.05% glutaraldehyde in PBS. The membranes were then blocked in 5% fat-free milk, incubated with specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. To visualise the results, chemiluminescence detection systems (ECL or ECL+) were utilised.

### 3.5 Promoter studies of the LL-37 gene

Plasmids were prepared by inserting different LL-37 promoter segments in front of the firefly luciferase reporter gene in pGL3-basic vectors by using standard molecular biology techniques.

HT-29 cells were seeded in 48-well plates and grown to 60-90% confluence. Cells were then transfected in triplicate utilising TransFast Transfection Reagent with 1 µg of the plasmid and 50 ng of the co-transfection plasmid pRL-TK (which had Renilla luciferase as a reporter) in 100 µl serum-free cell medium. After 1h incubation, cells were covered with 900 µl complete cell medium and in some wells 2 mM sodium butyrate were added. After 48 h of incubation, the cells were lysed. The firefly and Renilla luciferase activities in the lysates were measured utilising the Dual-Luciferase Reporter Assay System. Measured firefly luciferase values were related to plasmid size and to the Renilla luciferase values.
3.6 Electromobility shift assay (EMSA)

Nuclear extracts were prepared from HT-29 cells according to instructions described in [159]. The protein concentrations of the resulting extracts were determined utilising the Bradford assay [160].

Double-stranded DNA probes were end-labeled with $^{32}$P-ATP utilising T4 polynucleotide kinase. Binding reactions were performed at room temperature with 5 µg nuclear extract per reaction in a binding buffer (20 mM Hepes, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 100 mM NaCl, 10% glycerol, 0.5 mM DTT and 0.2 mM PMSF). After 10 min of preincubation with 100 ng Poly(dI-dC)·Poly(dI-dC), blocking non-specific DNA-binding proteins, 10 fmol of labeled probe was added, followed by 40 min of incubation at room temperature. The reactions were then separated on 6% non-denaturing polyacrylamide gels in 0.5 x tris-borate-EDTA (TBE) buffer. For competition experiments non-labeled probes were added before the labeled probe in an additional incubation period of 30 min. For supershift EMSA, the antibodies were added after the incubation of nuclear extract and probe, and incubated for 30 min at room temperature. Notably, a different binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA and 5% glycerol) was used for the supershift experiments.
4 RESULTS AND DISCUSSION

4.1 Antimicrobial polypeptides in human colon mucosa (Paper I)

In order to evaluate the contribution of antimicrobial peptides and proteins to the human colonic defence, a peptide/protein extract from normal human colon mucosa was prepared and analysed for antimicrobial activity. In the extract, activity against both Gram-positive and Gram-negative bacteria was detected, in addition to antifungal activity against C. albicans. Four antibacterial polypeptides were isolated and identified by N-terminal sequence analysis and mass spectrometry. These were ubiquicidin, ECP, Histone H2B and PLA2. Using immunodetection and mass spectrometry LL-37, HNP 1-3, and HBD-1 were also identified. The results recently reported by Bevins group are in agreement with ours [161].

Notably, this is the first time ubiquicidin has been isolated from human tissue. Ubiquicidin is an evolutionarily conserved C-terminal fragment of 40S ribosomal protein S30, and has previously been isolated from mouse macrophages [162]. Furthermore, ubiquicidin is upregulated by the normal flora in mouse small intestine [163], indicating a functional role in innate immunity. The colon mucosa contains many cell types, complicating determination of the origin of the ubiquicidin in our preparation. However, ubiquicidin has later been shown to be localised to epithelial cells of the human colon [161], indicating that the origin may be epithelial cells and/or resident macrophages.

Apart from killing bacteria, some of the antimicrobial peptides identified in the colon mucosa are chemoattractants for immune cells [4, 5, 37]. The peptides can thereby participate in orchestrating the total immune response. Combined, our results indicate that the colon
mucosa is protected by a complex mixture of polypeptides, able to kill invading microbes, working together as a barrier against bacterial invasion.

4.2 Vernix caseosa as an innate defence barrier
(Papers II and III)

Vernix caseosa has been suggested to participate in the host defence but the components involved had not been characterized. This was a motive for investigating whether vernix contains antimicrobial peptides and proteins. Peptides/proteins in 99 samples of vernix, 11 collected after elective caesarean sections (paper II), and 88 after uncomplicated vaginal deliveries (paper III) were extracted and analysed for antimicrobial activity. Proteins/peptides in all vernix extracts exhibited antibacterial activity against *B. megaterium*, and antifungal activity against *C. albicans*, while the activity against GBS, and *E. coli* varied between babies. In addition, six samples of amniotic fluid (AF) were analysed in a similar manner for activity against *B. megaterium, E. coli* and *C. albicans*. However, only activity against *B. megaterium* was detected. The difference in activity between vernix and AF, indicates that the antimicrobial components may differ in these two compartments, despite their close contact.

The presence of the α-defensins HNP 1-3 in vernix and AF samples was established with mass spectrometry and dot blot analysis. After separation of peptides/proteins in vernix extract with reversed phase HPLC, the fraction containing HNP 1-3 exhibited the most prominent antibacterial activity. In additional vernix fractions with antibacterial activity, lysozyme, ubiquitin, and psoriasin were identified by amino acid sequence analysis. Using Western blot analysis, LL-37 and an apparently extended form of the peptide were detected in vernix and AF samples. In a related study, expression of LL-37 was detected in both
Results and Discussion

Recent studies have identified various proteins in Vernix, including SLPI and lactroferrin [84, 164]. However, LL-37 was not detected in that study [164].

Moreover, in a proteomic approach, we identified 17 additional proteins in vernix by utilising N-terminal sequence analysis and peptide mass fingerprinting. The most abundant proteins in vernix were haemoglobin α-chain, cystatin A, calgranulin A, ubiquitin, and UGRP-1. Of these, all except haemoglobin have been implicated in innate immunity. Cystatin A is a protease inhibitor, which has been suggested to be a first line protector against cysteine proteases released from infectious micro-organisms and parasites [165], while uteroglobin related protein-1 (UGRP-1) is a recently described protein suggested to have opsonin-like properties [166]. Calgranulin B was also identified but in smaller amount. This reveals that both subunits (calgranulin A and B) of the heterodimer calprotectin are present in vernix, thereby indicating that the detected antifungal activity of vernix may originate from calprotectin. Furthermore, we identified calgranulin C, which has been reported to be released by activated neutrophils and thereby to attack and kill nematodes [167, 168]. Together these proteins add protective functions to vernix such as antifungal activity, opsonising features, protease inhibition, and parasite inactivation.

The composition of the lipids in vernix was also characterized, and among the lipids, the free fatty acids were found to exhibit antimicrobial activity. Interestingly, we have found that the lipids in vernix interact with LL-37, and hence increase the activity of LL-37 under our experimental conditions. LL-37 has previously been demonstrated to display synergy with HBD2 [98], lactoferrin and lysozyme [169, 170].

Furthermore, the colonisation of neonates was investigated and the main species colonising the neonates were Staphylococcus (coagulase negative) (86%), Bacillus sp. (27%), and E. coli (21%). Most babies were
colonised by one to three groups of bacteria, while none were colonised by more than four.

Our two studies demonstrate that *vernix caseosa* comprise a complex antimicrobial surface defence on the skin of neonates, thus protecting the immunologically immature foetus and newborn against invasion by bacteria, fungi and parasites.

4.3 The rat cathelicidin rCRAMP as a model for cathelicidins (Paper IV)

Since limited research on the effects of antimicrobial peptides has been performed *in vivo*, it is of importance to develop animal models for this purpose. A mouse model for cathelicidins has previously been utilised [100]. However, additional disease models are available in the rat. Notably, rat neutrophils contain defensins, while mouse neutrophils do not. In this respect, rat is more similar to human, and would in some cases be a more appropriate model for studies on antimicrobial peptides.

In a computer search, we identified a rat cDNA clone with homology to the human cathelicidin LL-37. This rat cathelicidin is named rCRAMP for rat-CRAMP, a name relating to the mouse cathelicidin cathelin related antimicrobial peptide (CRAMP). The relation of rCRAMP to other members of the cathelicidin family was established by performing a multiple sequence alignment of the cathelin regions, and a phylogenetic tree was constructed based on the sequence alignment. This revealed that rCRAMP is most closely related to mouse CRAMP with a sequence identity of 84%, but also fairly closely related to the human LL-37 with a sequence identity of 60%. Based on these analyses we suggest that the cathelicidin family has evolved by gene duplications that are species or lineage specific. Recently, several cathelicidins from trout [171], chicken [172], and hagfish [173] were
identified, and since hagfish lack adaptive immunity this indicates that cathelicidins are older than the adaptive immune system [173].

The expression pattern of rCRAMP was studied by RT-PCR and Western blot analysis and was found to essentially correspond to that of the mouse and human cathelicidins. Interestingly, germ-free rats were also found to express rCRAMP in lung and gastrointestinal tract, indicating that live bacteria are not required for the expression. However, the germ-free rats are still exposed to bacterial products in their sterilized food, such as LPS, which may induce the expression of rCRAMP. This is in agreement with the expression of LL-37 in the GI-tract, which is not dependent on bacteria [131].

The mature, active rCRAMP peptide was characterized after isolation from the granules of rat granulocytes, utilising reversed phase HPLC. By determination of the mass value and the N-terminal amino acid sequence of the isolated peptide, rCRAMP was identified as a 43-residue peptide. Interestingly, this reveals a discrepancy between the processing of rCRAMP and the reported processing of the mouse CRAMP, which results in a 34–residue peptide [174]. This is notable, since the primary structures are identical at the processing sites. This could be due to different processing enzymes in the two species. An alternative theory could be that the processing differs at different locations of the body as has been shown for LL-37 [95, 96]. However, the CRAMP peptide was isolated from mouse bone marrow [174] and the mature peptide probably originates from granulocytes, as was the case for the isolated rCRAMP. In addition, the size of the rCRAMP peptide observed in tissues, when analysed by Western blot analysis, was in agreement with a 43-residue peptide. This strongly indicates that rCRAMP is indeed processed differently than mouse CRAMP.

The secondary structure of the mature rCRAMP peptide was predicted to form two $\alpha$-helices connected by a hinge region. This is in agreement with the structure of a 38-residue version of mouse CRAMP.
An Edmundson wheel projection of the $\alpha$-helices revealed that both helices are amphipathic, a feature common to most antimicrobial peptides. Accordingly, the mature rCRAMP peptide was found to be antibacterial and active against both Gram-positive and Gram-negative bacteria, but not against fungi. Since salts have been demonstrated to stabilise the functional structure of LL-37 [32] we investigated if salt influences the activity of rCRAMP. With increasing salt concentrations the antibacterial activity of rCRAMP was enhanced, however not as strongly as the activity of LL-37.

In conclusion, we have demonstrated that rCRAMP is the rat homologue of human LL-37. We have also isolated and characterized the mature active peptide, and verified that its expression pattern and activity is similar to that of human LL-37. Our results thereby open the possibility to study responses related to cathelicidin expression in health and disease with the rat as a model system.

4.4 Functional studies on the LL-37 promoter (Paper V)

Potential binding sites for different transcription factors have been identified in the LL-37 promoter but to date only a vitamin D responsive element (VDRE) has been shown to be functional [176]. We have performed promoter studies on the gene encoding LL-37, with the name CAMP (cathelicidin antimicrobial peptide). In order to identify regulatory regions, i.e. enhancers and/or silencers in the CAMP promoter, we transfected the colonic epithelial cell line HT-29 with plasmids containing different promoter and intron segments of the CAMP gene in front of a luciferase reporter gene.

Butyrate has previously been demonstrated to stimulate the expression of LL-37 in colonic epithelial cell lines [130, 131] and
accordingly, butyrate increased the activity of the LL-37 promoter also in our system, as measured by luciferase activity.

By performing 5’ deletions of the promoter we could identify one enhancer element and two silencer elements. The enhancer element is crucial for both the constitutive and butyrate-induced expression of LL-37. Further analyses of the enhancer element in electromobility shift assays (EMSAs) revealed that most likely a transcription factor of the Ets family binds to the element. This was demonstrated by using competing probes with consensus binding sites for Ets-family members. This was further verified in a transfection experiment by mutation of the Ets-binding site within a longer sequence, which resulted in loss of promoter activity. There are to date 25 known human members of the Ets-family [177], which all bind similar sequences. We could not pinpoint which member of this family that binds to the identified enhancer element in the LL-37 promoter. However, members of the Ets family have previously been implicated in the regulation of the human defensins HNP-1 and HBD-2 [178, 179].

The VDRE previously reported in the LL-37 promoter was identified as an enhancer element [176]. However, it is located in one of the segments where we detected a silencing element. The appearance of a silencer in our study could be due to the lack of Vitamin D in our cell culture media. In the absence of Vitamin D, the vitamin D receptor (VDR) binds the VDRE as a heterodimer with the Retinoid X Receptor. This complex recruits a corepressor, hence reducing the transcription. However, when Vitamin D binds, the corepressor is replaced by a coactivator, and transcription is instead enhanced [180]. Moreover, treatment of colonic epithelial cells with butyrate upregulates VDR expression [181]. Therefore, Vitamin D and butyrate may synergize in gene activation.

The second intron in the CAMP gene is the most conserved of the three introns. Therefore, we hypothesized that this intron might be involved in the gene regulation of LL-37. By insertion of the second
intron downstream of the luciferase gene in promoter-luciferase constructs we investigated if there were any intronic enhancers or silencers present. An enhancing effect of the transcription was found, but only after stimulation with butyrate, indicating the presence of butyrate responsive elements in the intron. However, when using constructs lacking the 3’ end of the promoter the induction was lost, suggesting that the intron cooperates with the 3’ end of the promoter in the induction. Whether this reflects the in vivo situation is not known. Sp1 binding sites are known to be butyrate responsive elements [182] and several potential Sp1 binding sites are located in the second intron. These Sp1 binding sites are likely candidates for conveying the induction of the LL-37 expression by butyrate.

LPS does not affect the LL-37 expression in colonic epithelia [131], while in sinus epithelia LPS upregulates the expression of LL-37 [183]. This difference may have evolved to allow the normal flora to inhabit the colon. Thus, the regulation of LL-37 expression appears to vary between different tissues. To fully understand the gene regulation of LL-37, many cell systems have to be investigated. In this study we have developed tools that can be used also in other cell systems.

In conclusion, we have identified two enhancers and two silencers in the CAMP gene. One of the enhancers is a functional Ets binding site in the promoter, while the other is located in intron number two, which in our system exerts its effect only in the presence of butyrate and in cooperation with the 3’ end of the promoter. One of the identified silencer elements is most likely regulated by Vitamin D.

An objective in antimicrobial peptide research has been to use synthetic peptides as antibiotics and thereby limit the use of conventional antibiotics. A major problem is however the rapid degradation of orally administrated peptides. An interesting idea is to develop drugs that stimulate the endogenous production of antimicrobial peptides at specific sites. Thus, it is crucial to understand the gene regulation of antimicrobial peptides in different tissues.
5 CONCLUSIONS

- Human colon is protected by a complex mixture of antimicrobial peptides and proteins. These exert potent antimicrobial activity against both Gram-positive and Gram-negative bacteria in addition to antifungal activity.

- *Vernix caseosa* protects the foetus and the newborn by a complex mixture of antimicrobial peptides, proteins and lipids that together provide protection against bacteria, fungi and parasites. In addition, vernix components also exhibit protease inhibition and opsonising features.

- Rat cathelicidin rCRAMP has been isolated and characterized as a 43-residue peptide, which is processed differently than the mouse cathelicidin CRAMP, despite identical primary structures at the processing sites. rCRAMP was shown to have a similar expression pattern and antimicrobial spectra as LL-37. Thereby, responses related to cathelicidin expression in health and disease can now be studied in the rat.

- Gene regulation of the human cathelicidin LL-37 in the colon epithelium includes two enhancers and two silencers. One of the enhancer elements is a functional Ets binding site in the promoter, and the other is intron number two that exerts its effect in the presence of butyrate and in cooperation with the 3’ end of the promoter. One of the silencers is probably regulated by Vitamin D.
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7 REFERENCES


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