LUND UNIVERSITY

ANTIMICROBIAL ACTIVITIES OF HISTIDINE-RICH GLYCOPROTEIN AND CATIONIC PEPTIDES

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Front: Negative staining and electron microscopy analysis of bacteria subjected to HRGP, kindly provided by Dr. Matthias Mörgelin.

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Oskar, Erik och Carl

“Healing is a matter of time, but it is sometimes also a matter of opportunity”
Hippocrates - Greek physician (460 BC - 377 BC)

ABSTRACT

In an environment full of potential pathogens it is of importance for organisms to mount a fast and effective defence. Antimicrobial peptides are ancient and integral effector molecules of the innate immune system. They are found in all kinds of species from bacteria to plants and animals, indicating their importance during evolution. They possess a broad-spectrum antimicrobial activity and some peptides can also participate in wound healing and connect the innate and adaptive immune systems.
Results presented in this thesis show that structural motifs connected with heparin-binding may confer antimicrobial activity to a given peptide. Peptides from various heparin-binding endogenous proteins exerted antimicrobial activity against Gram-positive and Gram-negative bacteria and similar results were obtained with consensus sequences for heparin-binding. Furthermore, we demonstrated that replacement of lysine and arginine by histidine in the consensus motifs abrogated the antibacterial effects of these peptides. Antibacterial effects of the histidine-rich consensus peptides were restored by the addition of Zn^{2+} or low pH. Similar results were obtained with histidine-rich peptides derived from domain 5 of kininogen and histidine-rich glycoprotein (HRGP).

HRGP, an abundant heparin-binding plasma protein, exerted antimicrobial effects against Gram-positive and Gram-negative bacteria and fungi. The antibacterial activity of HRGP was dependent on Zn^{2+}-ions or low pH, and the antifungal activity was increased under low pH conditions.
Electron microscopy demonstrated that HRGP induced lysis of bacteria and fungi. Truncated HRGP, devoid of the heparin-binding and histidine-rich domain, was not antimicrobial. In addition, HRGP was found to have antifungal effects *ex vivo* when bound to fibrin clots.

CONTENTS

ABBREVIATIONS

AMP	antimicrobial peptide
cfu	colony forming units
CF	cystic fibrosis
CRAMP	cathelin related antimicrobial peptide
D5	domain 5
HD	human defensin
HMWK	high molecular weight kininogen
HNP	human neutrophil peptide
HRGP	histidine-rich glycoprotein
HRR	histidine-rich region
LPS	lipopolysaccharide
MAC	membrane attack complex
MRSA	methicillin-resistant *Staphylococcus aureus*
PAMP	pathogen-associated molecular pattern

ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I. Andersson E, **Rydengård V**, Sonesson A, Mörgelin M, Björck L, Schmidtchen A.
Antimicrobial activities of heparin-binding peptides
Eur J Biochem. 2004 Mar;271(6):1219-26

II. **Rydengård V**, Andersson Nordahl E, Schmidtchen A.
Zinc potentiates the antibacterial effects of histidine-rich peptides against *Enterococcus faecalis*
FEBS J. 2006 Jun;273(11):2399-406

III. Kacprzyk L, **Rydengård V**, Malmsten M, Schmidtchen A
Antimicrobial activity of histidine-rich peptides is dependent of acidic conditions
Manuscript (under consideration in *BBA Biomembranes*)

IV. **Rydengård V**, Olsson A-K, Mörgelin M, Schmidtchen A.
Histidine-rich glycoprotein exerts antibacterial activity
FEBS J. 2007 Jan;274(2):377-89

V. **Rydengård V**, Kacprzyk L, Olsson A-K, Mörgelin M, Malmsten M, Schmidtchen A.
Antifungal activity of histidine-rich glycoprotein
Manuscript

BACKGROUND

In the 1960s, Spitznagel and Zeya identified basic and antibacterial proteins in polymorphnuclear leukocytes [1-3]. These publications are the first reports describing the growing field of antimicrobial peptides (AMPs). Almost twenty years later, these proteins were characterized and named defensins [4,5]. Other milestones in this area include Hans G Boman´s discovery of antibacterial defences in Drosophila [6], and antibacterial peptides in the hemolymph of cecropian moth [7], as well as Michael Zasloff´s discovery of magainin in the African clawed frog [8].

To protect the body from invading pathogens, vertebrates have in general terms, two complimentary immune systems, the adaptive and the innate.
The adaptive immune system is based on an antibody dependent response that is antigen-specific. It gives a faster response when the host is re-infected with the same microbe, since a memory is developed [9].
In contrast, the innate immune system provides an instant defence against invading microbes, serving as a first line of defence. Innate immunity is a broad conception, meaning that it is something that we are born with. It covers among others, physical barriers like skin and mucosa [10], the complement system and also the subject of this thesis, the AMPs.

AMPs are an important part of the innate immune system, with the mission to serve as the first defence against invading microbes. They provide a rapid and instant protection against microbes, compared with the adaptive immune system that requires several days for efficient function. The killing caused by AMPs is non-specific and does not include a memory.
The peptides generally contain between 12 and 50 amino acids and in many cases, AMPs kill the microbes via a non-receptor mechanism that leads to lysis [11,12]. AMPs are active at μM concentration, corresponding to their biological concentrations at sites of infection [13], and they are either expressed constitively or expressed upon exposure of pathogen-associated molecular patterns (PAMPs) like polysaccharides and peptidoglycans. For example LL-37 is constitutively expressed in small amounts by epithelial keratinocytes [14] and by sweat ductal epithelial cells and therefore present in sweat [15], but the release of LL-37 from neutrophils is dependent on PAMPs.

ANATOMY

The skin is one of the biggest organs in the body [16]. It protects the host against physical and chemical agents, dehydration but also serves as a shelter to protect the body against invading microorganisms. Our skin is at the interface between the external and the internal environment, and can be divided into three different parts: epidermis, dermis and subcutis [17].

Epidermis

Normal skin is composed of different layers [18]. The outer part denoted epidermis is a thin coating (20-300 μm) consisting of ~95% keratinocytes at different differentiation stages. The keratinocytes migrate from the bottom layer towards the skin surface. This takes about seven weeks and during that journey the keratinocytes differentiate from square formed cells with a distinct nuclei to flat, stratified and dead cells without nuclei [17]. The keratinocytes in the *Stratum basale* are cubical, contain a nucleus and are anchored to the basale membrane. The cells in the *Stratum spinosum* are angular due to the desmosome interaction. Desmosomes are intercellular junctions that contribute to a tight network of keratinocytes [19]. *Stratum granulosum* is constructed of non-dividing cells rich in keratohyalin granules. Cells at the upper part of this layer have lost most of their organelles and nuclei.
The development and maintenance of an intact epidermis is extremely important to the function of skin as a protective barrier and a shelter against invading microbes.

Stratum corneum, the outer layer is a stratified epithelium and the keratinocytes, formed by terminal differentiation (keratinization) to corneocytes, are dead cells that have lost their nuclei and cytoplastic organelles. The purpose of the corneocytes is to protect the underlying viable layers. *Stratum corneum* is also rich in ceramides, cholesterol and free fatty acids which preserve a proper barrier function [20]. Bacterial infections are restrained by the constant shedding of corneocytes in the outermost layer.

Other cell types present in epidermis are the antigen-presenting Langerhans´ cells (found in all layers of epidermis) and the basal layer Merkel cells that are

associated with sensory nerve and melanocytes. The function of melanocytes is to produce melanin to protect, among others, the nuclei of the keratinocytes in the basal layer from damaging UV-radiation [18].

Dermis

The thickness of this layer is between 1-5 mm. It is composed of a papillary region, with a ridged structure to strengthen the connection between the epidermis and dermis. The major part of the dermis is the reticular region, composed of connective tissue [21]. Collagen, elastin and proteoglycans are secreted by the fibroblasts and contribute to building up a structure and a mechanical network [21]. Also found are roots of hair, sebaceous glands, sweat glands and blood vessels. The blood vessels are responsible for providing the blood supply to the epidermis.

Subcutis

Subcutis is the layer under cutis (epidermis and dermis). It is a layer composed of mostly adipose tissue for insulation and storage of energy in the form of fat.

WOUND HEALING

Wound healing can be divided into three different stages, which overlap in time.

Inflammatory phase The first phase of wound healing, the inflammatory phase, lasts for 2-5 days. A blood clot is formed when thrombocytes aggregate with fibrinogen that converts to fibrin, to physically protect the wounded area. Thrombocytes release growth factors like platelet-derived growth factor and transforming growth factor-β to attract inflammatory cells [22]. Other important growth factors are vascular endothelial growth factor, fibroblast growth factor, keratinocyte growth factor and the cytokine interleukin-1. The blood vessels in dermis become dilated to allow neutrophils, macrophages, thrombocytes and plasma proteins to infiltrate the wound [23]. Neutrophils and macrophages start to phagocyte microbes and debris from damaged cells and to give space for the coming construction of new tissue.

Proliferation (epithelialization and angiogenesis) After a couple of days the clot becomes a scab, with migrating cells forming a bridge under the scab. Fibroblasts migrate onto fibrin and produce collagen and keratinocytes migrate on laminin and fibronectin in the basal membrane to start the closure of the wound [24]. Angiogenesis is controlled by different growth factors in order to provide the wound with oxygen and nutrients [25].

Remodelling phase In this last phase that can last up to three years newly formed collagen is cross-linked to increase tensile strength in the wound.

INNATE DEFENCE

Compared with the adaptive immune system, the innate immune system is instant and not based on an antigen specific response. Since microbes surround the human body it is necessary to have a rapid and effective defence. The skin is covering our body, and prevents infiltration by microbes by desquamation of corneocytes and by the lipid layer that is found in the outer *Stratum corneum.* An additional barrier, in form of AMPs is found in the epithelial linings of the body [26].

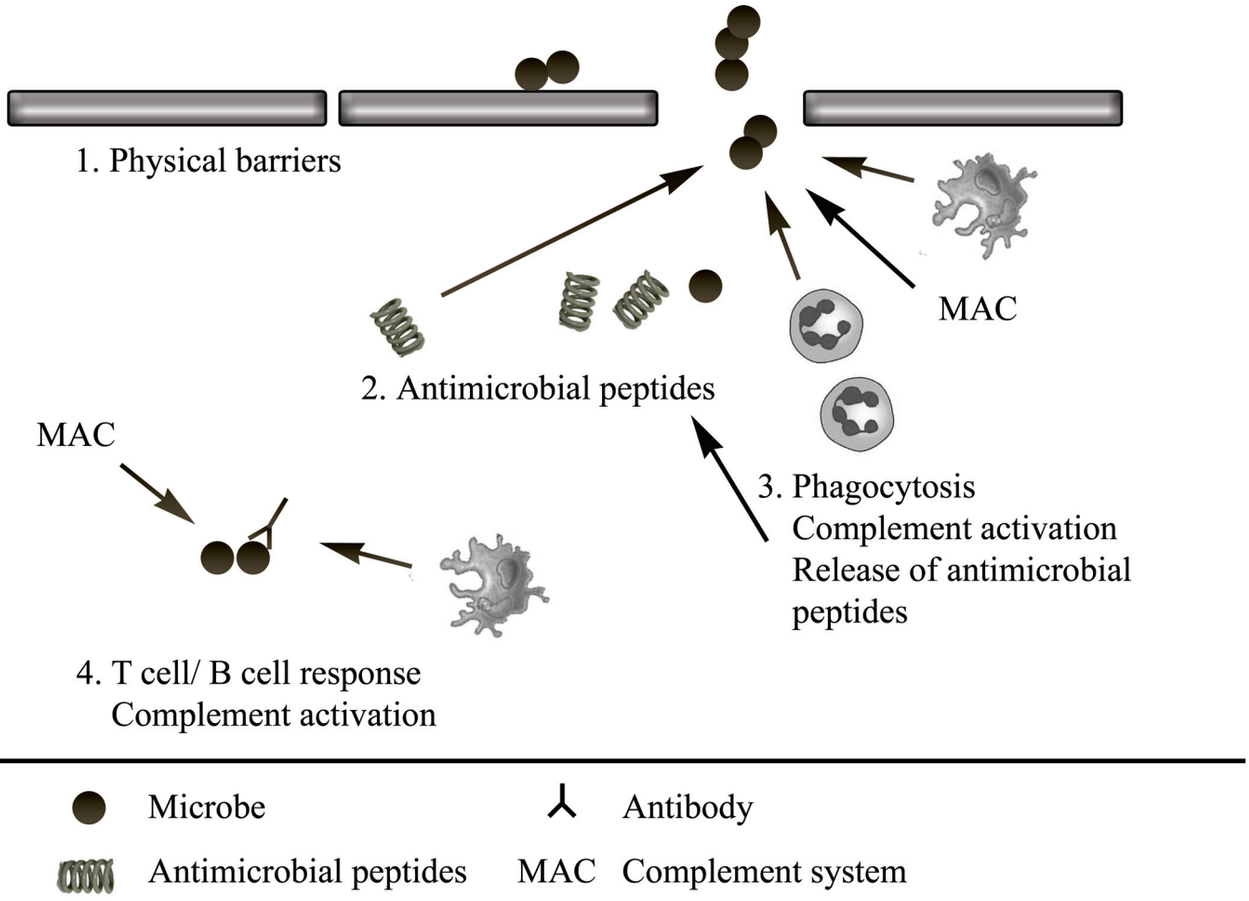

Figure 1. A schematic drawing over the host defence. 1) Physical barriers such as epithelia and mucosa prevent microbes from entering the body, followed by 2) killing by constitutively expressed antimicrobial peptides, already present at the site of injury. 3) Microbes are ingested by monocytes or macrophages. Complement is activated by PAMPs. Antimicrobial peptides are released by neutrophils, keratinocytes and thrombocytes upon stimulation. 4) The adaptive immune system is activated by T-cells and B-cells, and antibodies are produced. The complement cascade is activated by the antigen/antibody complex.

Most of these peptides are small, have a positive net charge, and kill the microbes via membrane perturbation or by intracellular action.
Innate immunity also covers the complement system composed of an enzymatic cascade of proteins that via the classical, alternative or lectin pathway forms the membrane attack complex (MAC), which causes cytolysis of mainly Gram-negative bacteria [27,28].

ANTIMICROBIAL PEPTIDES

Generally, AMPs are small and cationic [29], properties that facilitates interaction with biological membranes.
The majority of these peptides are amphipathic, meaning that they contain both hydrophilic and hydrophobic amino acids, organized into discrete sectors of the molecule (figure 2).

They are effector molecules of innate immunity, and have been confirmed as an important part of the host immunity [30]. They are found in all groups of organisms covering bacteria, fungi, plants and animals, and can be ordered into different groups due to their primary and secondary structure (table 1).

The major groups are peptides with α-helix, β-sheet or peptides with an over-representation of some amino acids. The linear peptides with an α-helical structure, are often unorganized in aqueous solution, and adopt an α-helical formation in hydrophobic environments [31]. β-sheet peptides, including among others the defensin family, contain intramolecular disulphide bridges.
In the next group are peptides constructed with a preponderance of one or more amino acids, often proline, histidine or tryptophane. In addition, there is a small group of cyclic peptides containing loop structures.

Table 1. Diverse AMPs divided in four groups dependent on their primary or secondary structure.

GROUP	PEPTIDE	SEQUENCE	ORIGIN	REFERENCE
α-helix	LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	*Homo sapiens* (human)	32
	Magainin-1	GIGKFLHSAGKFGKAFVGEIMKS	*Xenopus laevis* (frog)	8
β-sheet	HNP-1	ACYCRI PACIAGERRYGTCIYQGRLWAFCC	*Homo sapiens* (human)	5
	Protegrin-1	RGGRLCYCRRRFCVCVGR	*Sus scrofa* (pig)	33
Over-representation of some amino acids	Histatin-5	DSHAKRHHGYKRKFHEKHHSHRGPY	*Homo sapiens* (human)	34
	PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP	*Sus scrofa* (pig)	35
Cyclic/ Looped	θ-defensin-1	RCICTRGFCRCLCRRGVC	*Macaca mulatta* (monkey)	36
	Lactoferricin B	GRRRRSVQWCAVSQPEATKCFQWQRNMRKVRGPPVSCIKRDSPIQCIQA	*Homo sapiens* (human)	37

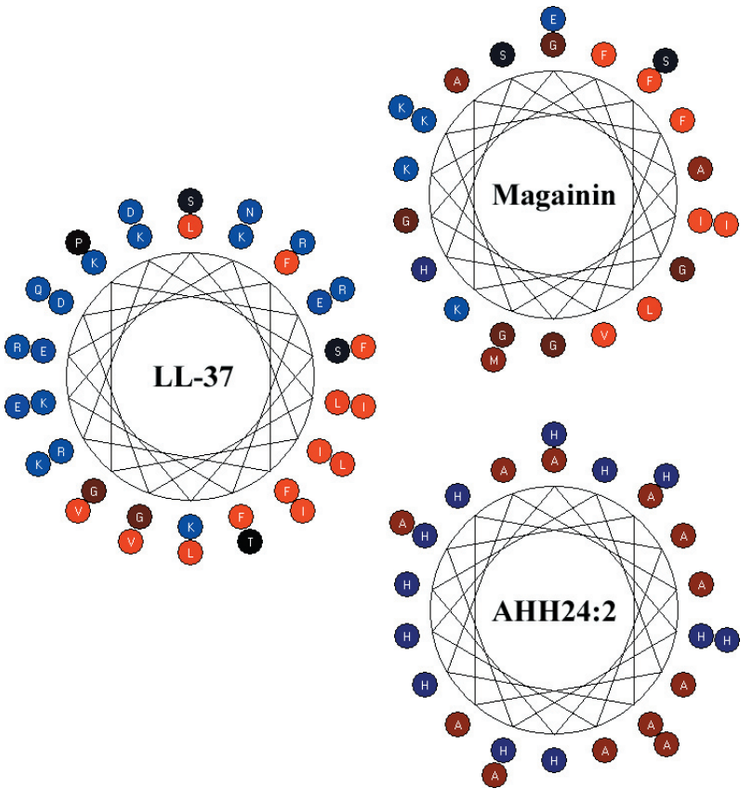

Figure 2. Amphipathic structure of Magainin-1, LL-37 and histidine-rich consensus motif AHH24:2, shown as a helical wheel projection. Hydrophobic residues are presented as red circles and charged amino acids as blue circles. Black circles represents amino acids that are neither charged or hydrophobic.

The majority of mammalian AMPs are gene-encoded and synthesized as prepropeptides. The active peptide can then be released by proteolytic cleavage [38]. An intact protein can also be antimicrobial like lactoferrin [39], HBP [40] and peptidoglycan recognition proteins [41], or peptides generated thereof [42,43].
Many AMPs shows broad-spectrum activity against bacteria, fungi and viruses, and in some case they can possess synergistic effects.
In addition, some of these peptides are multifunctional with other activities like neutralizing LPS, promoting wound healing and recruiting the adaptive immune system by chemotaxis of inflammatory cells.

Cathelicidins

This family is found only in mammals. The only human cathelicidin is denoted LL-37. It is localized in the specific granulae of neutrophils as the prepropeptide hCAP-18, released and cleaved to the active peptide LL-37 [44,45].
It has been reported that LL-37 can be expressed by epithelial linings, such as keratinocytes in the skin, it can be found in sweat [15], salivary glands [46] and seminal plasma [47], but also in "early life" in amniotic fluids and vernix caseosa [48]. LL-37 is also chemotactic for neutrophils, monocytes, mast cells and T cells [49].

Defensins

The defensins can be divided into α, β, or θ (a cyclic peptide, so far only found in neutrophils of the rhesus monkey), depending on a difference in the spacing of the disulfide bridges. They are structurally composed of three disulfide bridges that fold α and β-defensins into three β-strands and a β-hairpin loop [50]. HNP (human neutrophil peptide) 1-4 are stored as processed and mature peptides in the azurophilic granulae in neutrophils.
To generalize, most α-defensins are expressed by different blood cells like neutrophils, B-cells and natural killer cells and most β-defensins are expressed by epithelial linings like skin, salivary glands and tonsils. But there are exceptions like α-defensins, HD (human defensin) 5 and 6 that are expressed by Paneth cells in the small intestine [51] and β-defensin, hBD-2 which is expressed by neutrophils.

Histatins

Histatins are a group of histidine-rich antimicrobial peptides found in humans and in higher primates. They have some antibacterial activity, but the main activity is seen against fungal infections. Histatin 1 to 12 is found, but the major representatives are 1, 3 and 5.
Histatin-5 is an α-helical peptide, and the structure is known to be stabilized by the presence of Zn^{2+}-ions [52]. The antifungal killing is non-membrane active [53].

ANTIMICROBIAL POLYPEPTIDES AND PROTEINS

Besides these "classical" AMPs, a number of antimicrobial polypeptides and proteins have been identified. They are often larger then a classic AMP, but the active domain of the protein has usually a positive net charge and an amphipathic structure.

C3a

C3a, a 9 kDa anaphylatoxin and central effector molecule of the complement system was found to be antimicrobial against both bacteria and fungi [42,54,55]. The active domain of C3a is located in the C-terminal α-helical part of C3a. The holo-protein C3 is not antimicrobial.

Lactoferrin

Lactoferrin, is an antimicrobial protein of 80 kDa, found in mammalian milk, tears, saliva, seminal fluid and also in the secondary granules of the neutrophile [56]. Two different active antimicrobial parts of lactoferrin have been determined, the N-terminal derived lactoferricins [37] and the kaliocins derived from an interior sequence of the protein [57].

Bactericidal/permeability increasing protein (BPI)

BPI is a 50 kDa cationic protein isolated from polymorphonuclear leukocytes with the highest activity against Gram-negative bacteria [58,59]. An N-terminal 25 kDa fragment seems to carry the antimicrobial effect of the protein [60]. The structure of BPI contains one N-terminal barrel and one C-terminal barrel linked together with a central β-sheet [61]. It is known to neutralize LPS [62] and has antiangiogenic properties [63].

Heparin-binding protein (HBP)

HBP is a 37 kDa heparin-binding and antimicrobial protein also called azurocidin or CAP37 [40]. The antibacterial activity of HBP is mainly directed against Gram-negative bacteria, and the activity is increased at low pH [64]. The basic amino

acids within the molecule have been proposed to contribute to the antimicrobial activity, and interestingly Cardin and Weintraub heparin-binding motifs (XBBXBX and XBBBXXBX) were found in the sequence of HBP [65,66]. HBP is also chemotactic for monocytes and fibroblasts [40,67].

Histidine-rich glycoprotein (HRGP)

HRGP is a 67 kDa heparin-binding and histidine-rich glycoprotein [68,69], synthezised in the liver and found in high concentrations in plasma. The protein can also be released from the α-granules of activated thrombocytes [70]. Being the subject of this thesis, it was recently shown that HRGP is antibacterial [71].
The active domain is proposed to be the histidine-rich domain containing the heparin-binding motif GHHPH [71].

MODE OF ACTION

The bacterial cell wall contains peptidoglycan repeats (composed of N-acetylglucosamine and N-acetylmuramic acid), which are found only in bacteria and are responsible for cell wall integrity. In addition, the Gram-negative outer cell wall is covered with LPS (consisting of O-specific side chain, a core and lipid A) [72]. Polysaccharides in fungi are glucan, chitin and mainly mannoproteins [73]. The negative charge of the peptidoglycans and LPS facilitates interactions with positively charged AMPs [74].

The peptides kill microorganisms via a non-receptor mediated mechanism and the target is the cell wall of the microorganism [75], leading to permeabilization of the microbes and in many cases, internalization of the peptides. In many cases, the exact mode of action is not known.
Independent of the mode of action of the peptide, it must first attach to the lipid bilayer of the microbe.
Bacterial and eukaryotic cell membranes are differently composed, which give AMPs the opportunity to distinguish between different kinds of membranes. The eukaryotic membrane is constructed of mainly zwitterionic phospholipids, whereas the bacterial membrane is composed of negatively charged phospholipids. Furthermore, the plasma membranes of eukaryotic cells contains sterols, which are missing in prokaryotes [76]. The fungal membrane composition is similar to the eukaryotic cell, but with ergosterol instead of cholesterol [77].
All these subtle differences between microbial and eukaryotic membranes to able a certain specificity for AMPs vis-à-vis microbes. In many cases, however, the exact mechanisms determining the specificity of AMPs against certain types of microbes are not exactly known, and are currently the subject of many investigations.

Concerning AMP action on the bacterial membrane, the killing can be divided into membrane active (see figure 3) and non-membrane active [75].

Membrane active

The detergent-like model "*carpet mechanism*" describes peptide aggregation on the lipid bilayer of the bacteria, with the hydrophobic regions of the peptides, associated with the membrane [31]. At a given peptide concentration, micelles and pores are formed through the membrane.

In the "*barrel-stave*" and "*toroid-pore*" models, pores are formed which lead to a collapse of the membrane. In both models the peptides are inserted horizontally into the membrane, either as a cluster of peptides that form a pore (barrel-stave) or as single peptides integrating the membrane leading to the bilayer lining the pore (toroid-pore) [31,78].

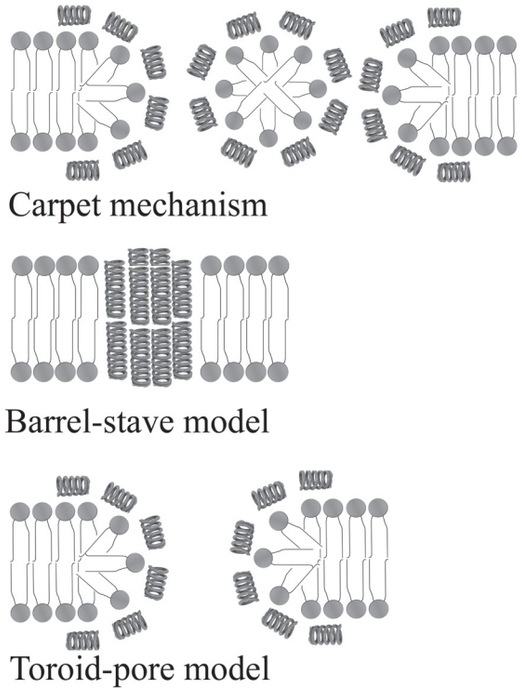

Figure 3. Illustration of the permeabilizing mechanisms of action. In the barrel-stave model and toroid-pore model, the peptides are inserted directly into the membrane, whereas the peptides are assembled first on the membrane, leading to a collapse of the membrane in the carpet model.

Non membrane active

Some peptides translocate the bacterial membrane without causing lysis, and enter the bacterial cytoplasm and may interfere with the synthesis of nucleic acids, proteins [79] or cell wall components or inhibit the enzymatic activity of the bacteria [80].

OTHER FUNCTIONS OF AMPs

Host defence peptides are a group of peptides that possess immunomodulatory effects. Many AMPs have immunomodulatory effects and the reverse is also true, many peptides with immunomodulatory effects have antimicrobial activity. The most studied host defence peptides are LL-37 and defensins. In addition to their membrane breaking activities they may also promote epithelialization and wound healing [81,82]. For example LL-37 is induced in keratinocytes during wound healing [83], and may be essential for epithelialization of a healing wound [84]. LL-37 can also be chemotactic for neutrophils, monocytes and for T-cells [49,85]. Furthermore, many chemokines are shown to have a defensin-like antimicrobial activity [86].

Several antimicrobial peptides have been connected with the regulation of angiogenesis. LL-37 is known to promote angiogenesis by signalling through the formyl peptide-like receptor-1 on endothelial cells [87]. Angiogenesis is regulated by both proangiogenic and antiangiogenic factors acting together. Other antimicrobial proteins are involved in the inhibition of angiogenesis. A histidine-rich fragment of HRGP is anti-angiogenic by inducing an arrest of endothelial cell motility [88]. Angiogenins was first associated with angiogenic activity, and later on found to exert antimicrobial activity [89].

INFECTION

The human body carries 1-2 kg microbes of about 5% are on outer surfaces such as the skin, so it is of importance to maintain an effective and rapid defence to retain the balance between the microbes and the host.
If a bacterium gets the opportunity to settle, multiply and invade this balance is broken, leading to infections. The increasing amount of bacteria can lead to delayed or impaired wound healing, local infections (e.g. chronic ulcers, atopic dermatitis or erysipelas) or in the worst cases sepsis, when bacteria reaches the blood stream [90].
The cause of progression from bacterial colonization to bacterial infection is dependent on both the bacterial count, multiple and complex virulence mechanisms as well as the efficiency of the host defence [91]. In other situations, a compromised skin barrier function is associated with a chronic colonization and repeated infections by various microbes.
For example, chronic ulcers are caused by both endogenous and exogenous factors (for example venous insufficiency and bacteria). This condition is characterized by increased levels of cytokines and proteases, leading to recruitment of inflammatory cells and matrix degradation [92]. The healing of the wound does not proceed into the proliferative phase, and can therefore not heal properly [93]. A bacterial count of 10^5 colony forming units (cfu) or more per gram of wound tissue is connected to infection or a delayed wound healing [94].

In all these infective conditions it is in the interest of the microorganism to circumvene the host defense for survival. To delay the innate AMP respons *P. aeruginosa* can for example degrade and inactivate LL-37 [95]. *P. aeruginosa, E. faecalis* and *S. pyogenes* can release proteases that cleaves off dermatan sulphate from human fibroblasts which in turn inactivate α-defensin [96]. Gram-negative *Salmonella typhimurium* can increase resistance to AMPs, like LL-37, by modification of LPS in the bacterial cell wall [97]. *Streptococcus pyogenes*, secrete a protein, SIC (streptococcal inhibitor of complement) which inactivates serveral AMPs, such as defensin and LL-37 [98].

ROLE IN DISEASES

It is accepted that antimicrobial peptides are an important part of the innate immunity, and many different studies are confirming the importance of these molecules.

In immunodeficiencies such as Chediak-Higashi syndrome, specific granule deficiency and in patients with morbus Kostmann, AMPs such as Cathepsin G, defensins and LL-37 are proposed to play a significant role [99,100].

It had been suggested that antimicrobial peptides like psoriasin and human β-defensin-2 are upregulated in psoriatic skin [101], which may explain the low prevalence of skin infections in psoriatic patients compared with for example atopic dermatitis patients, in which LL-37 is down-regulated [102].

Human β-defensin is inactivated by the high salt concentration in the lung of patients with cystic fibrosis (CF). The inactivation of antimicrobial peptides could be an explanation for the increased inflammation and bacterial colonization in the lungs of CF patients [103].

Mouse cathelin related antimicrobial peptide (CRAMP), a homologue to the human LL-37, was shown to protect against *Streptococcus pyogenes* infection in a CRAMP knockout mouse model [104].

By using a pig wound model it was shown that protease inhibitors could prevent activation of porcine cathelicidin, and thereby decrease the clearance of bacteria from the wound [105].

IMPORTANCE OF AMPs

There is an interest in creating new antibiotics, due to the growing resistance to conventional antibiotics [106], as examplified by methicillin-resistant *Staphylococcus aureus* (MRSA). Since AMPs are targeting the microbe cell membrane it is unlikely that resistance can develop against these molecules.

Some AMPs like polymyxin B and gramicidin S have been used as antibiotics for a long time [107]. Polymyxin B, an antibiotic cyclic cationic peptide from the Gram-positive bacteria *Bacillus polymyxa* [108] is used to treat Gram-negative urinary, meningeal and bloodstream infections. Gramicidin S, which also is bacteria-derived (from *Bacillus brevis*) is an amphipathic antibiotic AMP with a β-sheet structure, used for mild throat infections [109].
In the last years a couple of antimicrobial AMPs have gone through phase II and III trials, as either topical, oral or systemic treatments. Pexiganan, a modified version of magainin from the African clawed frog, was the first AMP that completed a phase III trial. It was administred topically the for treatment of infected diabetic foot ulcers and lead to cure or improvement in 90% of the patients. This was a comparable effect to the control, an orally administred conventional antibiotic [110].
Omiganan is a 12 amino acid analog of bovine neutrophil indolicidin [111], that is licensed for the prevention of wound, burn and device-related infections. In a phase III study it showed a 49% reduction of catheter-related infections.

PRESENT INVESTIGATION

Paper I: Antimicrobial activities of heparin-binding peptides

In two previous papers our group showed that α-defensin and LL-37 binds to glycosaminoglycans like heparin and dermatan sulphate [95,96]. In this work, we used a series of cationic peptides derived from laminin, fibronectin, von Willebrand factor, protein C inhibitor, vitronectin and complement factor C3. The heparin-binding capacity of these peptides were either previously known or determined using radiolabelled heparin in a slot binding assay. In addition to these heparin-binding peptides, we selected a few that did not bind heparin as negative controls. Next, the antimicrobial effects of the peptides were tested by two different methods, viable count analysis (killing) or radial diffusion assay (inhibition of growth). The test organisms used were Gram-positive *Enterococcus faecalis* (*E. faecalis*) and *Proteus mirabilis* (*P. mirabilis*), Gram-negative *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) and the fungus *Candida albicans* (*C. albicans*).
The results showed a good correlation, such that peptides that were heparin-binding were also antimicrobial. In addition, we used peptides that were specially designed to be heparin binding, containing the Cardin and Weintraub heparin-binding motifs. We could show that the antimicrobial activity of these peptides was dependent on the number of repeats of the motif.
The motifs were constructed of sequences of XBBXBX or XBBBXXBX, were X represented a hydrophobic or uncharged amino acid (alanine) and B represented a basic amino acid (lysine or arginine).
Electron microscopy showed that local perturbations and breaks were introduced in the *P. aeruginosa* bacteria after treatment with some of the peptides.
Thus, we demonstrate that heparin-binding Cardin and Weintraub as well as heparin-binding motifs of endogenous proteins exhibit antimicrobial activity.

Paper II: Zinc potentiates the antibacterial effects of histidine-rich peptides against *Enterococcus faecalis*

The starting point for this study was the observation that histidine-rich peptides, such as those derived from domain 5 of high molecular weight kininogen (HMWK) require Zn^{2+} for interaction with heparin. We re-designed the Cardin

and Weintraub motifs used in *Paper I*. The uncharged amino acid used was still alanine, but we substituted the basic lysine for histidine. Histidine is uncharged at neutral pH, but basic at low pH.
Both the heparin-binding and antimicrobial activity of the peptides was abolished at neutral pH, but then restored on addition of Zn^{2+}-ions. Gram-positive *E. faecalis* bacteria was used. Other cations, such Ca^{2+} and Mg^{2+} did not activate the histidine-rich AMPs. By flourecsence microscopy, we could demonstrate that the binding of the histidine-rich consensus motifs was increased by Zn^{2+}-ions, and that the interaction was totally blocked by an excess of heparin.
The same effects could be seen for histidine-rich peptides derived from the histidine-rich domain of HMWK and for histatin 5. The results from *paper II* suggest that Zn^{2+} may play an important role in the regulation of the antimicrobial activities of histidine-rich peptides.

Paper III: Antimicrobial activity of histidine-rich peptides is dependent of acidic conditions

Zn^{2+} imposes a positive charge on histidine-rich peptide sequences, leading to enhanced antimicrobial effects. We decided to examine whether low pH, leading to protonation of histidines, thus generating positively charged peptides, could affect the antibacterial properties of histidine-rich peptide sequences.
Similar to *paper II*, we used the histidine-rich Cardin and Weintraub motifs, and the histidine-rich peptides from HMWK and HRGP. In this paper, pH was lowered to 5.5 to restore the activity of these peptides. Similar results were obtained for histidine-rich peptides derived from HRGP and HMWK. Radial diffusion assay was performed to determine the antimicrobial avtivity against Gram-positive *Staphylococcus aureus* (*S. aureus*) and *Bacillus subtilis* (*B. subtilis)*, Gram-negative *E. coli* and *P. aeruginosa* and the fungus *C. albicans*. By using synthetic liposomes we could demonstrate that the peptides gave rise to breaks in membranes at low pH but not in neutral pH. By using flow cytometry, we could demonstrate that the binding of the histidine-rich consensus peptides to the cell wall of *E. coli* and the fungus *C. albicans* was pH dependent. Concludingly, we show that the antimicrobial activities of histidine-rich Cardin and Weintraub motifs as well as other endogenous histidine-rich peptides are induced at low pH.

Paper IV: Histidine-rich glycoprotein exerts antibacterial activity

The biological function of histidine-rich protein (HRGP) is still unknown, although new activities of this molecule are revealed continuously. With the exception of the function the protein is rather well characterized. Among other features HRGP is known to have a histidine-rich and heparin-binding domain. The heparin-binding activity requires zinc ions or presence of low pH.
In this paper we could demonstrate that the heparin-binding capacity of both HRGP as well as a peptide from the histidine-rich domain of HRGP was increased by low pH or Zn^{2+}-ions. HRGP was able to bind to bacterial membranes, and this binding was inhibited by an excess of heparin.
We showed that HRGP was antibacterial in a dose and time dependent manner against both Gram-negative and Gram-positive bacteria in low pH, or in the presence of zinc ions, plasma or serum.
Thus, we proposed that the histidine-rich domain was responsible for the antibacterial effect since a peptide derived from that region was zinc or low pH dependent in the same manner as the holo protein. Furthermore, to verify this, truncated HRGP, devoid of the histidine-rich domain was not antibacterial. Electron microscopy showed that HRGP induced breaks in the bacterial membrane that were low pH or zinc dependent.

Paper V: Antifungal activity of histidine-rich glycoprotein

In this work, we continued to investigate the antimicrobial properties of HRGP. We showed, using viable count assays, that HRGP was antifungal against various strains of *Candida* in a viable count analysis. The activity was enhanced at low pH. HRGP was able to bind to the fungal cell wall, and we could also show that HRGP induced local breaks in the membrane, leading to lysis of the fungi. In addition to this, HRGP lysed ergosterol containing liposomes.
The antifungal domain of HRGP was investigated both by using overlapping 20mer peptides from HRGP as well as using truncated recombinant HRGP devoid of the HRR. The results showed that HRR of HRGP was mainly responsible for the antifungal activity of the protein.
We could, *in vitro*, demonstrate that plasma clots lacking HRGP were more prone to *Candida* infection compared with normal clots containing HRGP.

MAIN CONCLUSIONS

¤ Many heparin-binding peptides of endogenous origin are antimicrobial.
¤ Consensus sequences for heparin-binding (Cardin and Weintraub motifs) are antimicrobial.
¤ Various histidine-rich peptides show an induction of antimicrobial activity in presence of Zn^{2+} or at low pH.
¤ Histidine-rich glycoprotein is antibacterial against Gram-positive and Gram-negative bacteria in the presence of Zn^{2+} or at low pH.
¤ Histidine-rich glycoprotein is antifungal, and the activity is increased in low pH.
¤ HRGP exerts an antifungal activity in fibrin clots *ex vivo*.

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Denna avhandlingen är en så kallad sammanläggningsavhandling, där delarbete I, II och IV är publicerade i vetenskapliga artiklar. Delarbete III är inskickat för bedömning i en vetenskaplig tidskrift och delarbete V är i manuskriptform.

Både i och på vår kropp har vi mikroorganismer, en del tillhör vår normalflora och en del kan orsaka sjukdomar. Att vi inte blir sjuka beror på att vi lever i en jämvikt med våra mikrober och att dessa inte tillåts växa ohämmat. När jämvikten rubbas eller om vårt försvar inte klarar att skydda oss mot invaderande mikroorganismer så kan vi bli sjuka.
Försvaret är uppdelat i två olika delar, en förvärvad del och en del som man föds med.
Det förvärvade immunsystemet är väldigt effektivt, men tar flera dagar för att komma igång. Därför börjar alltid kroppen att försöka försvara sig med hjälp av det medfödda försvaret och om inte det räcker så tar det förvärvade över. Dessutom kan dom två olika systemen samverka i många fall.
Den förvärvande delen baseras på celler i blodet som kan känna igen främmande ämnen i kroppen, som i sin tur talar om för andra celler att börja tillverka antikroppar. Antikropparna kan sedan sätta sej på det främmande ämnet så att det kan bli uppätet av andra celler.
Det medfödda systemet består av många olika delar. Här ingår de fysiska barriärerna som skyddar kroppen, tex huden och slemhinnorna i mun, mage och tarm. Det finns också speciella celler i blodet som kan äta upp sådant som kroppen inte känner igen och ett system, komplementsystemet, som kan döda mikroorganismer med hjälp av en kedja av proteiner som aktiverar varandra för att slutligen kunna sticka hål på och döda mikroorganismen. Dessutom finns det delar av proteiner, som kallas peptider, som kan döda mikroorganismer.

Avhandlingen handlar om dessa *antimikrobiella peptider*.

Antimikrobiella peptider är en stor grupp som kan se ut och fungera på många olika sätt. Peptiderna är uppbyggda av byggstenar, aminosyror, som ofta är hydrofoba (som inte gillar vatten). De hydrofila aminosyrorna (som gillar vatten) kan dessutom vara positivt laddade. Peptiderna kan vecka sej på olika sätt,

antingen som en korkskruv eller som ett veckat lakan. När dessa peptider formar sej så organiserar de sej så att en del gillar vatten och andra delen inte gillar vatten. Det gör att peptiden kan binda till en bakterie eller jästcell, eftersom deras cellväggar är negativt laddade och fettrika. När den väl fäst sej så kan den ta död på mikroorganismen på olika sätt. Antingen så tar den hål på mikroorganismens cellvägg vilket dödar den direkt, eller så kan den påverka mikroorganismen så att den tex slutar att tillverka proteiner som är livsnödvändiga.

Många av dom antimikrobiella peptiderna liknar varandra på olika sätt. Dom flesta är små, består av en vatten-gillande och en fett-gillande del och dom flesta har en positiv laddning. I *delarbete I* tittar vi på på förmågan hos peptiderna att binda till ett slags sockerkedja som heter heparin. Sedan tidigare visste vi att många antimikrobiella peptider kan binda till heparin, antagligen inte för att det är en funktionell egenskap, utan snarare att peptidernas egenskaper gör att dom gärna binder negativt laddade sockerkedjor. I detta arbetet har vi valt ut en mängd olika peptider vars antimikrobiella förmåga inte tidigare var undersökt. Vi upptäckte att det fanns ”gömda” antimikrobiella delar i många olika proteiner.

I *delarbete II och III* tittar vi på peptider som innehåller mycket av en byggsten som heter histidin. Det visar sej att dessa peptider inte alls är heparinbindande under neutrala förhållanden, dvs i normalt pH. För att peptiderna ska bli heparinbindande tillsätter vi zinkjoner eller sänker pH, vilket gör att peptiderna blir mer positivt laddade.
Detsamma gäller för den antimikrobiella funktionen, peptiderna har ingen aktivitet alls i neutrala förhållanden, mot bakterier och avampar. Men däremot i närvaro av zinkjoner eller i surt pH så blir dom aktiva. I kroppen finns det lågt pH i hud och slemhinnor och vid sårläkning är det gott om zinkjoner som trombocyterna avger. Man kan tänka sej att detta är vid förhållanden som dessa som den antimikrobiella aktiviteten hos de histidin-rika peptiderna kan bli aktiverad.

I *delarbete IV* så undersöker vi ett protein som heter histidinrikt glykoprotein. Proteinet finns i ganska stora mängder i blodet, och finns också i en sorts blodceller som heter trombocyter. Funktionen hos detta protein är okänt, men man vet i att det kan binda heparin i närvaro av zinkjoner eller i surt pH. Med tanke på våra tre föregående arbete så funderade vi på om det kunde vara så att detta proteinet var antimikrobiellt. Vi fann att histidinrikt glykoprotein var antimikrobiellt mot bakterier i närvaro av zinkjoner och i lågt pH. Vi kunde också visa att det verkade som om proteinet orsakade hål i bakteriernas cellvägg, och att det var på det sättet dom blev dödade.

I nästa arbete, *delarbete V*, så undersökte vi histidinrikt glykoproteins aktivitet mot svamp. Lite förvånande så upptäckte vi att proteinet var aktivt även under neutrala förhållanden, och att aktiviteten ökade i surt pH. Zinkjoner verkade inte påverka aktiviteten alls. Vi kunde visa att proteinet verkade göra hål i cellväggen hos svamparna. För att kunna avgöra i vilken del av proteinet som den antimikrobiella egenskaperna fanns i, så tillverkade vi mindre bitar av proteinet som man kunde testa för aktivitet mot svamp. Då kunde vi se att den delen av proteinet som var mest antimikrobiellt var den delen som innehöll många histidiner.
Vi tillverkade fibrinkoagel av plasma med eller utan HRGP i, och kunde sedan se att koagel utan HRGP lättare blev infekterat av svamp jämfört med koagel som innehöll HRGP.

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REFERENCES

1. Zeya, H.I. & Spitznagel, J.K. Cationic proteins of polymorphonuclear leukocyte lysosomes. II. Composition, properties, and mechanism of antibacterial action. *J Bacteriol* **91**, 755-62 (1966).
2. Zeya, H.I. & Spitznagel, J.K. Cationic proteins of polymorphonuclear leukocyte lysosomes. I. Resolution of antibacterial and enzymatic activities. *J Bacteriol* **91**, 750-4 (1966).
3. Zeya, H.I. & Spitznagel, J.K. Antibacterial and Enzymic Basic Proteins from Leukocyte Lysosomes: Separation and Identification. *Science* **142**, 1085-7 (1963).
4. Selsted, M.E., Szklarek, D. & Lehrer, R.I. Purification and antibacterial activity of antimicrobial peptides of rabbit granulocytes. *Infect Immun* **45**, 150-4 (1984).
5. Ganz, T. et al. Defensins. Natural peptide antibiotics of human neutrophils. *J Clin Invest* **76**, 1427-35 (1985).
6. Boman, H.G., Nilsson, I. & Rasmuson, B. Inducible antibacterial defence system in Drosophila. *Nature* **237**, 232-5 (1972).
7. Hultmark, D., Steiner, H., Rasmuson, T. & Boman, H.G. Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of Hyalophora cecropia. *Eur J Biochem* **106**, 7-16 (1980).
8. Zasloff, M. Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc Natl Acad Sci U S A* **84**, 5449-53 (1987).
9. Chaplin, D.D. 1. Overview of the human immune response. *J Allergy Clin Immunol* **117**, S430-5 (2006).
10. Basset, C., Holton, J., O'Mahony, R. & Roitt, I. Innate immunity and pathogen-host interaction. *Vaccine* **21 Suppl 2**, S12-23 (2003).
11. Jenssen, H., Hamill, P. & Hancock, R.E. Peptide antimicrobial agents. *Clin Microbiol Rev* **19**, 491-511 (2006).
12. Sitaram, N. & Nagaraj, R. Host-defense antimicrobial peptides: importance of structure for activity. *Curr Pharm Des* **8**, 727-42 (2002).
13. Breukink, E. et al. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. *Science* **286**, 2361-4 (1999).

14. Conner, K., Nern, K., Rudisill, J., O'Grady, T. & Gallo, R.L. The antimicrobial peptide LL-37 is expressed by keratinocytes in condyloma acuminatum and verruca vulgaris. *J Am Acad Dermatol* **47**, 347-50 (2002).
15. Murakami, M. et al. Cathelicidin anti-microbial peptide expression in sweat, an innate defense system for the skin. *J Invest Dermatol* **119**, 1090-5 (2002).
16. Goldsmith, L.A. My organ is bigger than your organ. *Arch Dermatol* **126**, 301-2 (1990).
17. Burns, V., Breathnach, S., Cox, N. & Griffiths, C. Rook´s textbook of dermatology. *Blackwell publishing, Oxford* (2004).
18. Wickett, R.R. & Visscher, M.O. Structure and function of the epidermal barrier. *Am J Infect Control* **34**, S98-S110 (2006).
19. Skerrow, C.J. Intercellular adhesion and its role in epidermal differentiation. *Invest*
Cell Pathol **1**, 23-37 (1978).
20. Choi, M.J. & Maibach, H.I. Role of ceramides in barrier function of healthy and diseased skin. *Am J Clin Dermatol* **6**, 215-23 (2005).
21. Sorrell, J.M. & Caplan, A.I. Fibroblast heterogeneity: more than skin deep. *J Cell Sci* **117**, 667-75 (2004).
22. Martin, P. & Leibovich, S.J. Inflammatory cells during wound repair: the good, the bad and the ugly. *Trends Cell Biol* **15**, 599-607 (2005).
23. Springer, T.A. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* **76**, 301-14 (1994).
24. Ryan, M.C. et al. The functions of laminins: lessons from in vivo studies. *Matrix Biol* **15**, 369-81 (1996).
25. Li, J., Zhang, Y.P. & Kirsner, R.S. Angiogenesis in wound repair: angiogenic growth factors and the extracellular matrix. *Microsc Res Tech* **60**, 107-14 (2003).
26. Schröder, J.M. & Harder, J. Antimicrobial skin peptides and proteins. *Cell Mol Life Sci* **63**, 469-86 (2006).
27. Seelen, M.A., Roos, A. & Daha, M.R. Role of complement in innate and autoimmunity. *J Nephrol* **18**, 642-53 (2005).
28. Taylor, P.W. Bactericidal and bacteriolytic activity of serum against gram-negative bacteria. *Microbiol Rev* **47**, 46-83 (1983).
29. De Smet, K. & Contreras, R. Human antimicrobial peptides: defensins, cathelicidins and histatins. *Biotechnol Lett* **27**, 1337-47 (2005).

30. Bulet, P., Stocklin, R. & Menin, L. Anti-microbial peptides: from invertebrates to vertebrates. *Immunol Rev* **198**, 169-84 (2004).
31. Oren, Z. & Shai, Y. Mode of action of linear amphipathic alpha-helical antimicrobial peptides. *Biopolymers* **47**, 451-63 (1998).
32. Agerberth, B. et al. FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis. *Proc Natl Acad Sci U S A* **92**, 195-9 (1995).
33. Zhao, C., Liu, L. & Lehrer, R.I. Identification of a new member of the protegrin family by cDNA cloning. *FEBS Lett* **346**, 285-8 (1994).
34. Oppenheim, F.G. et al. Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on Candida albicans. *J Biol Chem* **263**, 7472-7 (1988).
35. Agerberth, B. et al. Amino acid sequence of PR-39. Isolation from pig intestine of a new member of the family of proline-arginine-rich antibacterial peptides. *Eur J Biochem* **202**, 849-54 (1991).
36. Tang, Y.Q. et al. A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. *Science* **286**, 498-502 (1999).
37. Gifford, J.L., Hunter, H.N. & Vogel, H.J. Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. *Cell Mol Life Sci* **62**, 2588-98 (2005).
38. Sørensen, O.E. et al. Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. *Blood* **97**, 3951-9 (2001).
39. Kutila, T., Pyorala, S., Saloniemi, H. & Kaartinen, L. Antibacterial effect of bovine lactoferrin against udder pathogens. *Acta Vet Scand* **44**, 35-42 (2003).
40. Flodgaard, H. et al. Covalent structure of two novel neutrophile leucocyte-derived proteins of porcine and human origin. Neutrophile elastase homologues with strong monocyte and fibroblast chemotactic activities. *Eur J Biochem* **197**, 535-47 (1991).
41. Lu, X. et al. Peptidoglycan recognition proteins are a new class of human bactericidal proteins. *J Biol Chem* **281**, 5895-907 (2006).
42. Nordahl, E.A. et al. Activation of the complement system generates antibacterial peptides. *Proc Natl Acad Sci U S A* **101**, 16879-84 (2004).

43. Tomita, M. et al. Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. *J Dairy Sci* **74**, 4137-42 (1991).
44. Sørensen, O.E. et al. Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. *Blood* **97**, 3951-9 (2001).
45. Zanetti, M., Gennaro, R. & Romeo, D. Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. *FEBS Lett* **374**, 1-5 (1995).
46. Murakami, M., Ohtake, T., Dorschner, R.A. & Gallo, R.L. Cathelicidin antimicrobial peptides are expressed in salivary glands and saliva. *J Dent Res* **81**, 845-50 (2002).
47. Malm, J. et al. The human cationic antimicrobial protein (hCAP-18) is expressed in the epithelium of human epididymis, is present in seminal plasma at high concentrations, and is attached to spermatozoa. *Infect Immun* **68**, 4297-302 (2000).
48. Yoshio, H. et al. Antimicrobial polypeptides of human vernix caseosa and amniotic fluid: implications for newborn innate defense. *Pediatr Res* **53**, 211-6 (2003).
49. De, Y. et al. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med* **192**, 1069-74 (2000).
50. Lehrer, R.I. & Ganz, T. Defensins of vertebrate animals. *Curr Opin Immunol* **14**, 96-102 (2002).
51. Ouellette, A.J. & Selsted, M.E. Paneth cell defensins: endogenous peptide components of intestinal host defense. *Faseb J* **10**, 1280-9 (1996).
52. Melino, S. et al. Zn(2+) ions selectively induce antimicrobial salivary peptide histatin-5 to fuse negatively charged vesicles. Identification and characterization of a zinc-binding motif present in the functional domain. *Biochemistry* **38**, 9626-33 (1999).
53. Kavanagh, K. & Dowd, S. Histatins: antimicrobial peptides with therapeutic potential. *J Pharm Pharmacol* **56**, 285-9 (2004).
54. Pasupuleti, M. et al. Preservation of antimicrobial properties of complement peptide C3a, from invertebrates to humans. *J Biol Chem* **282**, 2520-8 (2007).

55. Sonesson, A. et al. Antifungal activity of C3a and C3a-derived peptides against Candida. *Biochim Biophys Acta* (2006).
56. Weinberg, E.D. Human lactoferrin: a novel therapeutic with broad spectrum potential. *J Pharm Pharmacol* **53**, 1303-10 (2001).
57. Viejo-Diaz, M., Andres, M.T. & Fierro, J.F. Different anti-Candida activities of two human lactoferrin-derived peptides, Lfpep and kaliocin-1. *Antimicrob Agents Chemother* **49**, 2583-8 (2005).
58. Elsbach, P. et al. Separation and purification of a potent bactericidal/permeability-increasing protein and a closely associated phospholipase A2 from rabbit polymorphonuclear leukocytes. Observations on their relationship. *J Biol Chem* **254**, 11000-9 (1979).
59. Elsbach, P. & Weiss, J. Prospects for use of recombinant BPI in the treatment of gram-negative bacterial infections. *Infect Agents Dis* **4**, 102-9 (1995).
60. Ooi, C.E., Weiss, J., Elsbach, P., Frangione, B. & Mannion, B. A 25-kDa NH2-terminal fragment carries all the antibacterial activities of the human neutrophil 60-kDa bactericidal/permeability-increasing protein. *J Biol Chem* **262**, 14891-4 (1987).
61. Beamer, L.J. Structure of human BPI (bactericidal/permeability-increasing protein) and implications for related proteins. *Biochem Soc Trans* **31**, 791-4 (2003).
62. Marra, M.N. et al. The role of bactericidal/permeability-increasing protein as a natural inhibitor of bacterial endotoxin. *J Immunol* **148**, 532-7 (1992).
63. van der Schaft, D.W., Toebes, E.A., Haseman, J.R., Mayo, K.H. & Griffioen, A.W. Bactericidal/permeability-increasing protein (BPI) inhibits angiogenesis via induction of apoptosis in vascular endothelial cells. *Blood* **96**, 176-81 (2000).
64. Shafer, W.M., Martin, L.E. & Spitznagel, J.K. Late intraphagosomal hydrogen ion concentration favors the in vitro antimicrobial capacity of a 37-kilodalton cationic granule protein of human neutrophil granulocytes. *Infect Immun* **53**, 651-5 (1986).
65. McCabe, D., Cukierman, T. & Gabay, J.E. Basic residues in azurocidin/HBP contribute to both heparin binding and antimicrobial activity. *J Biol Chem* **277**, 27477-88 (2002).
66. Cardin, A.D. & Weintraub, H.J. Molecular modeling of protein-glycosaminoglycan interactions. *Arteriosclerosis* **9**, 21-32 (1989).

67. Chertov, O. et al. Identification of human neutrophil-derived cathepsin G and azurocidin/CAP37 as chemoattractants for mononuclear cells and neutrophils. *J Exp Med* **186**, 739-47 (1997).
68. Haupt, H. & Heimburger, N. [Human serum proteins with high affinity for carboxymethylcellulose. I. Isolation of lysozyme, C1q and 2 hitherto unknown -globulins]. *Hoppe Seylers Z Physiol Chem* **353**, 1125-32 (1972).
69. Heimburger, N., Haupt, H., Kranz, T. & Baudner, S. [Human serum proteins with high affinity to carboxymethylcellulose. II. Physico-chemical and immunological characterization of a histidine-rich 3,8S- 2 -glycoportein (CM-protein I)]. *Hoppe Seylers Z Physiol Chem* **353**, 1133-40 (1972).
70. Leung, L.L., Harpel, P.C., Nachman, R.L. & Rabellino, E.M. Histidine-rich glycoprotein is present in human platelets and is released following thrombin stimulation. *Blood* **62**, 1016-21 (1983).
71. Rydengård, V., Olsson, A.K., Mörgelin, M. & Schmidtchen, A. Histidine-rich glycoprotein exerts antibacterial activity. *Febs J* **274**, 377-89 (2007).
72. Trent, M.S., Stead, C.M., Tran, A.X. & Hankins, J.V. Diversity of endotoxin and its impact on pathogenesis. *J Endotoxin Res* **12**, 205-23 (2006).
73. Calderone, R.A. Recognition between Candida albicans and host cells. *Trends Microbiol* **1**, 55-8 (1993).
74. Yount, N.Y., Bayer, A.S., Xiong, Y.Q. & Yeaman, M.R. Advances in antimicrobial peptide immunobiology. *Biopolymers* **84**, 435-58 (2006).
75. Shai, Y. Mode of action of membrane active antimicrobial peptides. *Biopolymers* **66**, 236-48 (2002).
76. Opekarova, M. & Tanner, W. Specific lipid requirements of membrane proteins--a putative bottleneck in heterologous expression. *Biochim Biophys Acta* **1610**, 11-22 (2003).
77. Arora, A., Raghuraman, H. & Chattopadhyay, A. Influence of cholesterol and ergosterol on membrane dynamics: a fluorescence approach. *Biochem Biophys Res Commun* **318**, 920-6 (2004).
78. Matsuzaki, K., Murase, O., Fujii, N. & Miyajima, K. An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation. *Biochemistry* **35**, 11361-8 (1996).
79. Boman, H.G., Agerberth, B. & Boman, A. Mechanisms of action on Escherichia coli of cecropin P1 and PR-39, two antibacterial peptides from pig intestine. *Infect Immun* **61**, 2978-84 (1993).

80. Brogden, K.A. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol* **3**, 238-50 (2005).
81. Tokumaru, S. et al. Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37. *J Immunol* **175**, 4662-8 (2005).
82. Niyonsaba, F. et al. Antimicrobial peptides human beta-defensins stimulate epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines. *J Invest Dermatol* **127**, 594-604 (2007).
83. Dorschner, R.A. et al. Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against group A Streptococcus. *J Invest Dermatol* **117**, 91-7 (2001).
84. Heilborn, J.D. et al. The cathelicidin anti-microbial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium. *J Invest Dermatol* **120**, 379-89 (2003).
85. Agerberth, B. et al. The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. *Blood* **96**, 3086-93 (2000).
86. Cole, A.M. et al. Cutting edge: IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity. *J Immunol* **167**, 623-7 (2001).
87. Koczulla, R. et al. An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *J Clin Invest* **111**, 1665-72 (2003).
88. Dixelius, J. et al. Minimal active domain and mechanism of action of the angiogenesis inhibitor histidine-rich glycoprotein. *Cancer Res* **66**, 2089-97 (2006).
89. Hooper, L.V., Stappenbeck, T.S., Hong, C.V. & Gordon, J.I. Angiogenins: a new class of microbicidal proteins involved in innate immunity. *Nat Immunol* **4**, 269-73 (2003).
90. Bowler, P.G. Wound pathophysiology, infection and therapeutic options. *Ann Med* **34**, 419-27 (2002).
91. Wysocki, A.B. Evaluating and managing open skin wounds: colonization versus infection. *AACN Clin Issues* **13**, 382-97 (2002).
92. Schmidtchen, A. Degradation of antiproteinases, complement and fibronectin in chronic leg ulcers. *Acta Derm Venereol* **80**, 179-184 (2000).

93. Loots, M.A. et al. Differences in cellular infiltrate and extracellular matrix of chronic diabetic and venous ulcers versus acute wounds. *J Invest Dermatol* **111**, 850-7 (1998).
94. Bendy, R.H., Jr. et al. Relationship of Quantitative Wound Bacterial Counts to Healing of Decubiti: Effect of Topical Gentamicin. *Antimicrobial Agents Chemother (Bethesda)* **10**, 147-55 (1964).
95. Schmidtchen, A., Frick, I.M., Andersson, E., Tapper, H. & Björck, L. Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. *Mol Microbiol* **46**, 157-68 (2002).
96. Schmidtchen, A., Frick, I.M. & Björck, L. Dermatan sulphate is released by proteinases of common pathogenic bacteria and inactivates antibacterial alpha-defensin. *Mol Microbiol* **39**, 708-13 (2001).
97. Ernst, R.K., Guina, T. & Miller, S.I. How intracellular bacteria survive: surface modifications that promote resistance to host innate immune responses. *J Infect Dis* **179 Suppl 2**, S326-30 (1999).
98. Frick, I.M., Åkesson, P., Rasmussen, M., Schmidtchen, A. & Björck,L. SIC, a secreted protein of Streptococcus pyogenes that inactivates antibacterial peptides. *J Biol Chem* **278**, 16561-6 (2003).
99. Putsep, K., Carlsson, G., Boman, H.G. & Andersson, M. Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. *Lancet* **360**, 1144-9 (2002).
100. Ganz, T., Metcalf, J.A., Gallin, J.I., Boxer, L.A. & Lehrer, R.I. Microbicidal/cytotoxic proteins of neutrophils are deficient in two disorders: Chediak-Higashi syndrome and "specific" granule deficiency. *J Clin Invest* **82**, 552-6 (1988).
101. Harder, J. & Schröder, J.M. Psoriatic scales: a promising source for the isolation of human skin-derived antimicrobial proteins. *J Leukoc Biol* (2005).
102. Howell, M.D. et al. Interleukin-10 downregulates anti-microbial peptide expression in atopic dermatitis. *J Invest Dermatol* **125**, 738-45 (2005).
103. Goldman, M.J. et al. Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell* **88**, 553-60 (1997).
104. Nizet, V. et al. Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* **414**, 454-7 (2001).

105. Cole, A.M. et al. Inhibition of neutrophil elastase prevents cathelicidin activation and impairs clearance of bacteria from wounds. *Blood* **97**, 297-304 (2001).
106. Bonomo, R.A. Multiple antibiotic-resistant bacteria in long-term-care facilities: An emerging problem in the practice of infectious diseases. *Clin Infect Dis* **31**, 1414-22 (2000).
107. Hancock, R.E. & Sahl, H.G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol* **24**, 1551-7 (2006).
108. Regna, P.P., Solomons, I.A., Forscher, B.K. & Timreck, A.E. Chemical Studies on Polymyxin B. *J Clin Invest* **28**, 1022-7 (1949).
109. Prenner, E.J., Lewis, R.N. & McElhaney, R.N. The interaction of the antimicrobial peptide gramicidin S with lipid bilayer model and biological membranes. *Biochim Biophys Acta* **1462**, 201-21 (1999).
110. Dutton, C.J., Haxell, M.A., McArtur, H.A.I. & Wax, R.G. Peptide Antibiotics, Discovery, Mode of Action and Applications. *Marcel Dekker, Inc., New York* (2002).
111. Sader, H.S., Fedler, K.A., Rennie, R.P., Stevens, S. & Jones, R.N. Omiganan pentahydrochloride (MBI 226), a topical 12-amino-acid cationic peptide: spectrum of antimicrobial activity and measurements of bactericidal activity. *Antimicrob Agents Chemother* **48**, 3112-8 (2004).