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Antimicrobial Peptides and Innate Immunity

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Preface

Antimicrobial peptides have been the subject of intense research in the past decades and are now considered as an essential part of the defense system in bacteria, plants, animals, and humans. Whereas lysozyme was identified in the 1920s, research on the smaller antimicrobial peptides started later. Pioneering work in, e.g., insects provided evidence for the central role that these so-called endogenous antibiotics play in host defense against infection. This is further supported by the observation that these peptides have been conserved throughout evolution and that they are present in vertebrates and invertebrates, plants, and microorganisms. Studies on antimicrobial peptides in cystic fibrosis that were performed in the 1990s prompted a range of research efforts that were aimed to define their role in disease development and progression. This increase in research on antimicrobial peptides also led to the conclusion that they contribute to host defense against infection not only through a direct and broad-spectrum antimicrobial activity but also through a variety of other mechanisms. This explains why the name host defense peptides is an appropriate alternative that is widely used. The aim of this book is to provide an update on these effector molecules of the innate immune system both for researchers that are already actively involved in the area and for those with a general interest in the topic.

The first three chapters of this volume provide an overview of the evolution of cysteine-containing antimicrobial peptides (including defensins) and the role of these peptides in host defense in plants and microorganisms. The realization that antimicrobial peptides also display functions distinct from their direct antimicrobial action is the focus of the next five chapters and puts these peptides center stage in immunity and wound repair. The remarkable increase in structure–function studies has provided new insights into how the peptides fulfill their various activities. The next block of chapters discusses the role of antimicrobial peptides in disease, by providing an overview of mechanisms in bacterial resistance to antimicrobial peptides and a discussion of their role in inflammatory bowel disease, cystic fibrosis lung disease, and chronic obstructive pulmonary disease. Although bacteria do not develop resistance against antimicrobial peptides as easily as they do to conventional antibiotics, bacteria do use resistance mechanisms to defend themselves

against antimicrobial peptide attacks by the host. Studies on these interactions provide insight into the host–microbe interaction during infection. Our insight in the role of antimicrobial peptides in disease has also improved considerably in recent years through studies that focus on, e.g., genetic and epigenetic regulation and studies that explore the activity of these peptides in complex environments that are changing as a result of the underlying disease. The final two chapters describe how knowledge of the function of antimicrobial peptides and their regulation can be used to design new therapies for inflammatory and infectious disorders. This is a very important area of research, in particular because of the increase in resistance of microorganisms to conventional antibiotics. Therefore, the use of synthetic or recombinant peptides, or agents that stimulate the endogenous production of antimicrobial peptides, provides an attractive alternative for conventional antibiotics.

Each chapter in this book was written by experts in the field of antimicrobial/host defense peptide research and provides a state-of-the-art summary of their area of research. The time and expertise of these experts were essential, and we would like to thank them for their excellent contributions.

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Evolution of Antimicrobial Peptides: A View from the Cystine Chapel

Robert I. Lehrer

Abstract An animal's environment contains smaller entities that may attack it and cause illness or death. The immune system evolved to protect against such threats. It has two branches, one innate and the other adaptive. The former relies on field-tested molecules that have been selected over eons. Since they are gene-encoded, these innate molecules are deployed with little or no delay. The adaptive immune system consists of molecular and cellular machinery that produces custom-tailored molecules. Its handiwork is relatively slow, and many clients in need of its products would be lost if their innate systems did not also exist. This chapter focuses on cysteine-containing antimicrobial peptides that contain one or more internal disulfide bonds. Special emphasis is placed on the evolution of two superfamilies of defensins: small, usually cationic and amphipathic host defense molecules with three or four intramolecular disulfide bonds. The ancient roots of both defensin groups predate the advent of adaptive immunity by hundreds of millions of years. One superfamily includes the α-, β-, γ-, and θ-defensins of vertebrates, and the "big defensins" found in cephalochordates, mollusks, and crustaceans. The other superfamily of defensins is expressed in arthropods, mollusks, and fungi and may have arisen much earlier. Like defensins, the evolution of other families of cysteine-containing AMPs can be traced to the predawn of vertebrate existence. Collectively and individually, antimicrobial peptides provide a broad range of protective effects. Yet, despite their essential contributions to animal existence, and perhaps because specificity ranks higher than efficacy in the view of most immunologists, AMPs have often been undervalued. Ironically, it is precisely because AMPs lack specificity that these broadly efficacious molecules have been conserved and refined for more than one billion years.

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Abbreviation

AMP(s) Antimicrobial peptide(s)

1 Introduction

Antimicrobial peptides (AMPs) are central components of the innate network of gene-encoded proteins and peptides that protects animals from microbial, viral, or cellular intruders. Because they are gene encoded, some AMPs are pre-deployed at barrier sites, including the skin or at places that are vulnerable to invasion in the respiratory, gastrointestinal, and genitourinary tracts. Other AMPs provide reinforcements that are delivered rapidly by mobile convoys of neutrophils or produced locally in response to various molecular alarm signals.

Adaptive immune responses are more specific, but their precision comes at considerable cost because it requires the relatively slow clonal expansion of effector cells. Under optimal in vitro growth conditions, pathogenic bacteria may double every 20–30 min. In theory, a single exponentially growing bacterium with a 30-min generation time could produce over 10^{14} ($>2^{48}$) progeny in 24 h *if* its environment met its nutritional needs and removed wastes and growth-limiting signals. Unfettered microbial growth does not occur in vivo, in large part because innate defenses such as barriers, fever, phagocytosis, nutrient and iron limitation, and antimicrobial peptides (AMPs) prevent it. Invertebrates cannot mount adaptive immune responses, yet they are the most numerous animals and species on earth. Some invertebrates have life spans that exceed 100 years, including certain marine tubeworms (Bergquist et al. 2000), bivalve mollusks, red sea urchins, and deep sea corals (Ebert 2008).

Hans G. Boman (1924–2008), a pioneer in the field of animal AMPs, divided AMPs into five structural groups: (i) linear, mostly helical, peptides without cysteines, with or without a hinge; (ii) linear peptides without cysteine and with a high proportion of certain residues; (iii) peptides with one disulfide bond; (iv) peptides with two or more S–S bonds giving mainly or only β-sheet structures; and (v) antibacterial peptides derived from larger polypeptides with other known functions (Boman 1995). Rather than attempting to review the evolution of all five, we will focus on groups (iii) and (iv): AMPs that contain cysteine and form one or more disulfide bonds.

Piecing together AMP evolution and assembling a jigsaw puzzle are similar exercises but have some notable differences. Both can be time consuming and vexing. However, when the jigsaw puzzle comes in a box with a picture of the completed puzzle on its cover, and it contains all of the pieces without extraneous ones mixed in, success can be anticipated—or at least recognized. In contrast, our AMP puzzle has no cover picture, its pieces are scattered with many still missing, and similar pieces from extraneous puzzles are mixed in. Limiting our scope to

AMPs that contain cysteine is helpful, because cysteines carry information relevant to secondary and tertiary structure, and their placement and pairing motifs can provide recognizable hallmarks. However, just as an art historian would examine the ears and fingernails of subjects in a painting when attempting to identify its creator (Roskill 1989), we will also consider ancillary features such as the structure and layout of AMP precursors and genes and the presence of short "signature" sequence motifs. In appraising real estate, location is of primary importance. Similarly, expression in a "professional phagocyte," especially a granulocyte or a cell whose name includes the word "killer" (e.g., NK-cells), will also be noted.

Multigenerational human families may contain "black sheep." Similarly, some relatives of AMPs may deal in drugs (endorphins), deliver lethal weapons (toxins), engage in molecular texting (signaling), or do molecular tailoring (immunomodulation). Because such activities are peripheral to any homily about AMPs based on the Book of Genes, we will overlook them in this chapter. Now let us enter the cystine chapel.

2 AMPs with One Cysteine

Relatively few of these AMPs have been described. Distinctin, a 5.4-kDa heterodimeric AMP, was isolated from the skin of a tree frog, *Phyllomedusa distincta* (Batista et al. 2001). Each monomer had a net charge of +4, but their sequences were different. One monomer contained 22 residues and its C-terminus ended with cys-lys-ile-ile. The other monomer had 24 residues and its C-terminus ended with cys-lys-val. An intermolecular disulfide bond between these cysteines created a four-helical bundle that protected distinctin from degradation by proteases (Raimondo et al. 2005). A homodimeric AMP, di-(ILQKAVLDCLKAAGSSLSKAAITAIYNKIT), which was called dicynthaurin, was isolated from the hemocytes of a protochordate, the tunicate *Halocynthia aurantium*. Its monomers were C-terminally amidated and covalently linked by a disulfide bond (Lee et al. 2001). The hemocytes of this tunicate also contained a 3.4-kDa heterodimeric AMP called halocidin. The halocidin subunits contained eighteen (WLNALLHHGLNCAKGVLA) and fifteen (ALLHHGLNCAKGVLA) amino acids and were linked covalently by a single cystine disulfide bond (Jang et al. 2002).

3 AMPs with Two Cysteines

3.1 Frog Skin Peptides

Frogs of the widely distributed *Rana* genus express many AMPs that contain two cysteines and one intramolecular disulfide bond. These AMPs, which evidently arose via gene duplication events, are stored in specialized skin structures called

granular glands or poison glands (Conlon et al. 2004). Typically the peptides are hydrophobic and cationic and form an amphipathic α-helix in membrane-mimetic solvents. Their names (e.g., brevinins, esculentins, gaegurins, ranalexins) often derive from the genus or species name of the frog. Granular glands contain additional bioactive peptides with other functions (Chen et al. 2006). Recently, J. Michael Conlon, a prolific contributor to this literature, voiced skepticism about the contribution of these peptides to ranid host defense by pointing out that some anurans have skin that does not synthesize AMPs and that many frog skin AMPs show low potency in vitro (Conlon 2011a, b). Furthermore, although some frog skin AMPs inhibit the chytrid fungus, *Batrachochytrium dendrobatidis*, which is widely held responsible for worldwide anuran population declines, the ability of these AMPs to protect frogs is not clearly correlated with resistance to fatal chytridiomycosis in the wild (Conlon 2011a, b). We can consider only a few frog skin peptides here.

Ranalexin is a 20 amino acid peptide from the bullfrog *Rana catesbeiana* (Clark et al. 1994). The two cysteines in its sequence (FLGGLIKIVPAMICAVTKKC) form a disulfide bond that creates a heptapeptide loop containing two positively charged lysine residues. The ranalexin propiece has a net charge of −5, and its C-terminal AMP domain has a net charge of +3. A truncated ranalexin analog that lacked the carboxyl-terminal cysteine had markedly reduced antimicrobial activity, suggesting that the 7-membered loop contributed to this function (Clark et al. 1994). In addition to AMPs such as dermaseptins (Amiche and Galanth 2011), the skin of frogs in the genus *Phyllomedusa* expresses various hormones and neuropeptides (Vouille et al. 1997). The signal sequences of these very different molecules are encoded by nucleotides homologous to those in the first coding exon of dermaseptin genes (Vouille et al. 1997). The mammalian cathelicidin gene family (discussed in Sect. 4) also contains unrelated exons that are linked to exons encoding identical or substantially similar signal peptides and propieces.

Some frog skin AMPs exhibit potent trypsin-inhibitory activity, which is imparted by a loop region (Yan et al. 2011). Skin secretions from the Chinese Bamboo odorous frog, *Huia versabilis*, contain an octadecapeptide (SVIGCWTKSIPPRPCFVK-amide) that potently inhibits trypsin but lacks antimicrobial activity. Its 11-member loop resembles those in Bowman-Birk peptide protease inhibitors (Li et al. 2007). Based on their similar precursor structures, frog skin peptides with antimicrobial and/or trypsin-inhibitory activity probably evolved from a common ancestor. An ability to inhibit proteases is useful, since microbial proteases can promote virulence by degrading the tissues and antimicrobial molecules of the infected host (Orth et al. 2010; Dubin 2002).

Basir and Conlon purified 26 peptides from skin secretions of *Rana palustris*, the North American pickerel frog. Half of the peptides contained two cysteines, and half were cysteine-free. Six of the former had 12-residue loops, two had 11-residue loops, and five had 7-residue loops (Basir and Conlon 2003). As not a single one of these peptides inhibited the growth of *Escherichia coli* or *Staphylococcus aureus*, their functions are not yet known (Rinaldi 2002). Comparing signal sequence motifs in AMP precursors from higher (neobatrachian) and archaic (archaeobatrachian) frogs

suggested that convergent evolution of AMP genes took place in at least three different lineages (Koenig and Bininda-Emonds 2011). It is not known if transdermal absorption of frog skin AMPs occurs allowing them to afford systemic protection, as well as protecting the skin.

3.2 Bactenecin Dodecapeptides

Two mammalian AMPs, whose 12 residues include a pair of cysteines, were purified from the neutrophils of cattle, *Bos taurus* (Romeo et al. 1988), and sheep, *Ovis aries* (Huttner et al. 1998). The precursors of both contained a conserved, 114-residue targeting domain called "cathelin" (Romeo et al. 1988; Storici et al. 1992; Bagella et al. 1995). Cathelin is an abbreviation of "cathepsin L inhibitor" (Ritonja et al. 1989), and any AMP whose precursor has a cathelin domain is classified as a cathelicidin (Tomasinsig and Zanetti 2005; Zanetti 2004, 2005; Zanetti et al. 1995). The sequences of bovine (RLCRIVVIRVCR) and ovine (RICRIIFLRVCR) dodecapeptides are almost identical. Cathelin is discussed in Sect. 4.1, and other cathelicidins are described in Sects. 4.5 and 4.7.

3.3 Arenicin

Certain invertebrates have leukocytes (hemocytes) that contain AMPs with a single disulfide bond. Coelomic cells of the marine polychaete worm, *Arenicola marina*, contain a pair of 21-residue AMPs (Ovchinnikova et al. 2004) named arenicins 1 and 2. Each has a net charge of +6 and both kill bacteria and fungi. Their sequences, RWC(V/I)YAYVRVRGVLVRYRRCW, are almost identical, and both have a disulfide bond linking Cys3 to Cys20 (Ovchinnikova et al. 2004). In aqueous solution, the arenicins have a β-hairpin structure formed by antiparallel β-strands with a right-handed twist (Ovchinnikova et al. 2007). Arenicin analogs lacking the disulfide bond show reduced activity against a polymyxin B-resistant *Proteus mirabilis* (Andra et al. 2009).

4 AMPs with Four Cysteines

4.1 Cathelin

Although it is not an AMP and may not even inhibit cathepsin L (Zhu et al. 2008), were an "Oscar" to be given for best supporting role in an AMP production, cathelin would almost certainly win. Its first known appearance in a supporting role took

place in *Myxine glutinosa*, the Atlantic hagfish—a primitive fish without jaws, vertebrae, or the usual accoutrements of adaptive immunity, i.e., discrete thymus tissue and immunoglobulin genes (Basanez et al. 2002; Bajoghli et al. 2011). Cathelin domains typically contain 99–114 residues (Zanetti 2005), including four conserved cysteines that form two intramolecular disulfide bonds (Zhu 2008a). These cysteines are also conserved in cystatins, an even older superfamily of cysteine protease inhibitors. Shunyi Zhu reviewed the relationships between cystatins and cathelin and concluded that the emergence of cathelicidins may have taken place after the gain of a 3′ intron in a duplicated copy of an ancestral cystatin (Zhu 2008a, c). Cathelicidins developed into a multigene family in Cetartiodactyla, a clade that includes whales, dolphins, and even-toed ungulates. Their expansion resulted from gene duplications and changes in the structure of antimicrobial domains secondary to exon shuffling, gene duplication, and post-duplication sequence remodeling (Zhu and Gao 2009).

4.2 Cathelicidins and Vitamin D

The specific granules of human neutrophils contain hCAP18, the 18-kDa, cathelin-containing precursor of LL-37, an α-helical AMP (Agerberth et al. 1995; Cowland et al. 1995). The roles of LL-37 in inflammation and immunity are described in chapter "LL-37: An Immunomodulatory Antimicrobial Host Defence Peptide". Since LL-37 lacks cysteine, its evolution would not be described in this chapter except for an event that brought its expression under the control of vitamin D (Gombart et al. 2005). This resulted from the insertion of a vitamin D response element into its promoter by a primate-specific, short interspersed element (SINE) (Gombart et al. 2009). α-Defensins (Ogata et al. 1992; Miyakawa et al. 1996) and LL-37 both exert in vitro activity against *M. tuberculosis*, and 1α,25 dihydroxy-vitamin D enhances the ability of human macrophages to inhibit intracellular growth of the tubercle bacillus in an LL-37 dependent manner (Sonawane et al. 2011). These findings have re-awakened interest in vitamin D therapy for tuberculosis (Selvaraj 2011). Ironically, good empirical medical practice in the nineteenth century included giving cod-liver oil to patients with tuberculosis (Williams 1849) and exposing them to sunlight (Solis-Cohen 1901)—both excellent ways to provide vitamin D. Mice also have a single 37-residue, α-helical cathelicidin peptide called "CRAMP" (Gallo et al. 1997). However, because the murine gene lacks a vitamin D response element, giving this vitamin does not induce or enhance CRAMP production (Gombart et al. 2009).

4.3 LEAP-2

The liver produces a pair of cysteine-containing molecules called *l*iver-*e*xpressed *a*ntimicrobial *p*eptides (LEAPs)-1 and -2. LEAP-1, which is better known as hepcidin, contains eight cysteines and is discussed in Sect. 6.1. LEAP-2 (net charge, +4)

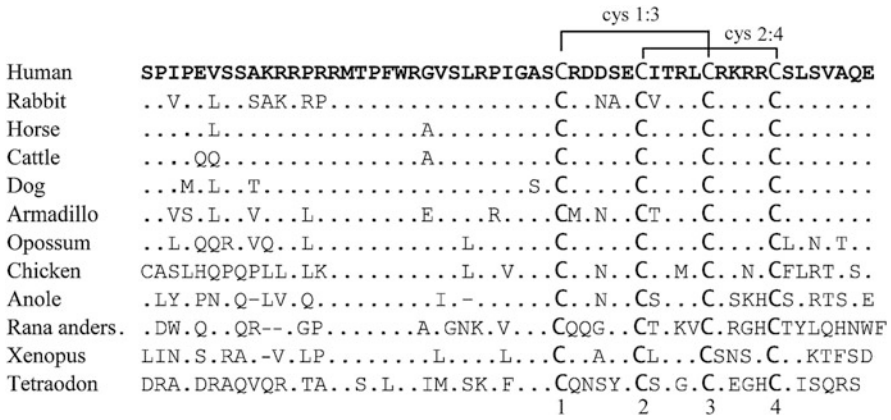

Fig. 1 Liver-expressed antimicrobial peptide-2 (LEAP-2). The sequence of human LEAP-2 is shown in its entirety. Residues that are identical to those in human LEAP-2 in the other peptides are represented by *dots*. Cysteine residues are numbered at the *bottom*, and their connectivity is shown at the *top*. *Tetraodon nigroviridis* is the green puffer fish, *Rana andersoni* is a Vietnamese frog, *Xenopus* is an African clawed frog, and the green anole, *Anolis carolinensis*, is an arboreal lizard

was isolated from human blood plasma ultrafiltrates and has homologs in all vertebrate classes (Fig. 1). The sequence of LEAP-2 is unusually well conserved in mammals and marsupials, suggesting that an endogenous binding partner may exist. The 77-residue precursor of LEAP-2 is synthesized mainly in the liver, an organ unique to vertebrates and a counterpart to the fat body of insects (Arrese and Soulages 2010). The largest native LEAP-2 molecules in plasma contain 40 amino acid residues and are accompanied by shorter forms with N-terminal and C-terminal truncations (Krause et al. 2003). LEAP-2 has features associated with classic peptide hormones (Krause et al. 2003). The likelihood that it has important functions other than antimicrobial activity is reinforced by studies showing that its disulfide bonds are not required for its antimicrobial effects but are essential to maintain the shape of its central core, which contains a short 3_{10}-helix from Asp_{20} to Glu_{22}, a type I β-turn from residues Cys_{23} to Arg_{26}, and a β-hairpin from Cys_{28} to Cys_{33} with a type I' β-turn (Henriques et al. 2010).

4.4 Lactoferricins

It was shown in 1946 that transferrin, which was then called siderophilin, imparted candidastatic properties to serum (Schade and Caroline 1946). Structurally related proteins called lactoferrins (lactotransferrins) and conalbumins (ovotransferrins) were later discovered. These glycoproteins contain 670–690 amino acid residues, show 50–70 % sequence identity, and bind reversibly and with high affinity to iron (Baker et al. 2002). Lactoferrin is expressed widely, and large amounts are present

Fig. 2 Antimicrobial peptides with two disulfides. The top two peptides were isolated from the leukocytes of horseshoe crabs: tachyplesin from *Tachypleus tridentatus* and polyphemusin from *Limulus polyphemus*. Gomesin was purified from the leukocytes of a tarantula spider, *Acanthoscurria gomesiana*, and androctonin was purified from the leukocytes of the scorpion, *Androctonus australis*

in the secondary (specific) cytoplasmic granules of neutrophils, and in glandular secretions, including milk and tears. The N-terminal domain of lactoferrin is highly cationic and peptides released from it by proteases are called lactoferricins. Many lactoferricins are bactericidal in vitro, including lactoferricin B (Bellamy et al. 1992) from bovine lactoferrin (Gifford et al. 2005; Tomita et al. 1994). The sequence of lactoferricin B (net charge, +7) is FKCRRWEWRMKKLGAP-SITCVRRAF, and its two cysteines form a disulfide bond. Although this bond is not required for antimicrobial activity (Hoek et al. 1997), it allowed lactoferricins to enter this cystine chapel as the sole representative of Boman's group V, AMPs derived from larger proteins with other functions.

4.5 Porcine Protegrins

Eleven different cathelicidins are expressed in porcine neutrophils. They include PR-39, a proline (P) and arginine (R)-rich peptide with 39 amino acid residues, two prophenins (PFs 1 and 2) with many proline (P) and phenylalanine (F) residues, five protegrins (PGs) including the three shown in Fig. 2, and three linear peptides (PMAPs) with 23, 36, and 37 residues. The porcine cathelicidin genes are clustered on chromosome 13 and contain four exons and three introns (Zhao et al. 1995; Sang and Blecha 2009). Exons 1–3 encode the signal peptide and the cathelin domain. Exon 4 primarily encodes the various mature AMPs, which range from the 16–18-residue protegrins to the 78-residue prophenins. The proline-rich porcine cathelicidins have type II poly-L-proline helical structures, PMAPs-23,-36, and -37 have largely α-helical structures, and protegrins have β-hairpin configurations.

4.6 *Protegrin Analogs in Invertebrates*

AMPs that resemble protegrins in their size, structure, and potency exist in several invertebrates. They include the tachyplesins and polyphemusins of horseshoe crabs (Miyata et al. 1989); gomesin, an 18-residue AMP from hemocytes of the spider, *Acanthoscurria gomesiana* (Silva et al. 2000); and androctonin, a 25-residue peptide from leukocytes of the scorpion, *Androctonus australis* (Ehret-Sabatier et al. 1996). Leukocytes of the spider crab, *Hyas araneus*, contain a chimeric, proline-arginine-rich AMP whose C-terminal residues include four cysteines that form two disulfide bonds (Stensvag et al. 2008). There is no evidence indicating a common ancestry of protegrins and any of the invertebrate AMPs shown in Fig. 2. Structural and antimicrobial properties of protegrins (Kokryakov et al. 1993; Harwig et al. 1996; Steinberg et al. 1997; Aumelas et al. 1996), tachyplesins (Matsuzaki et al. 1993; Tamamura et al. 1993; Ohta et al. 1992; Nakamura et al. 1988), and gomesin (Fazio et al. 2006) are described elsewhere.

4.7 *Bovine Cathelicidins*

In addition to 13 β-defensins (Selsted et al. 1993), cattle neutrophils contain six cathelicidin AMPs (Scocchi et al. 1997), of which only the cyclic dodecapeptide described in Sect. 3.2 contains cysteine. The other bovine cathelicidins include Bac5, which is composed largely of X-P-P-Y repeats; PR59, a proline and arginine-rich peptide; indolicidin (LPWKWPWWPWRRG), a 13-residue tryptophan-rich peptide; and BMAP28 and BMAP34, which are α-helical. Bovine neutrophils store their cathelicidins as inactive propeptides in large cytoplasmic granules that are more numerous than and compositionally distinct from the azurophil and specific granules of human neutrophils (Zanetti et al. 1990; Gennaro et al. 1983). Phagocytic and soluble stimuli trigger concomitant proteolytic activation and secretion of these cathelicidins.

5 AMPs with Six Cysteines

5.1 *Introducing Defensins*

Many of the AMPs described in this section are called defensins. They are small (2–5 kDa) antimicrobial and/or antiviral peptides whose six or eight conserved cysteines form three or four intramolecular disulfide bonds. They have a largely β-sheet structure that may include an N-terminal α-helical domain, whose presence results in a cysteine-stabilized alpha-beta (CSαβ) structure. Animal defensins comprise two large superfamilies. The first superfamily considered below contains five subfamilies, four expressed in vertebrates and one expressed in invertebrates.

The vertebrate peptides are called alpha (α), beta (β), gamma (γ), and theta (θ) defensins and are descendants of invertebrate AMPs called "big defensins." The word "defensin" has itself evolved and expanded since its introduction to describe three peptides from human neutrophils that are now classified as α-defensins (Ganz et al. 1985; Selsted et al. 1985). β-defensins, the oldest subfamily expressed in vertebrates, occur in fish (Zou et al. 2007), reptiles (Alibardi et al. 2012), birds (van Dijk et al. 2008), and mammals (Scheetz et al. 2002). Defensins have not yet been described in any amphibian. β-defensins have given rise to two identifiable offspring, α-defensins and γ-defensins. Their parentage of α-defensins makes β-defensins the grandparents of primate θ-defensins. Section 5.6 explains our conclusion that β-defensin genes are descendants of an exon expressed in the "big defensin" family of invertebrate AMPs.

5.2 α- and β-Defensins

Using hidden Markov model profile searching, Lynn and Bradley found α-defensins in the genomes of basal mammals, including elephants, lesser hedgehogs, and armadillos (Lynn and Bradley 2007). Their identification of an α-defensin gene in the short-tailed opossum suggests that α-defensins evolved before placental mammals and marsupials diverged, some 130 million years ago (Lynn and Bradley 2007). Although α-defensins are expressed in mice, rats, guinea pigs, hamsters, rabbits, elephants, and primates, the horse is the only Laurasiatherian—a clade that includes whales, most hoofed mammals, carnivores, and others (Hou et al. 2009)—now known to express α-defensins (Bruhn et al. 2009).

In 2004, the genome of chickens (*Gallus gallus*) was reported to contain 13 avian β-defensin genes (Xiao et al. 2004), including the three initially purified from chicken neutrophils and called gallinacins (Harwig et al. 1994). Xiao et al. divided these peptides into two subgroups, based on their sites of expression. β-defensins 1–7 were expressed mainly in the respiratory tract and bone marrow, and β-defensins 8–13 were expressed primarily in the urogenital tract and liver. Chicken β-defensin genes were clustered, consistent with evolution via the duplication and diversification of an ancestral gene.

Studies of human and rodent α-defensin (DEFA) and β-defensin (DEFB) genes also show clustering and reduplication. The five human α-defensin genes, which include two (DEFA1 and DEFA3) that are themselves reduplicated, all reside on human chromosome 8p23 within 450 kb of DEFB1, the gene for human β-defensin-1 (Linzmeier et al. 1999). Based on the relative placements of the DEFA and DEFB1 genes, it was proposed that myeloid α-defensin genes (DEFA1, DEFA3, and DEFA4) evolved from DEFA genes encoding HD5&6, α-defensins expressed by human small intestinal Paneth cells (Linzmeier et al. 1999). Gene copy number polymorphism and strain-dependent variability in mouse DEFA genes (Linzmeier and Ganz 2005; Amid et al. 2009) make the defensin segment of their respective genomes very formidable puzzles to complete.

5.3 θ-Defensins

Theta-defensin genes arose in Old World monkeys via the mutation of a pre-existing α-defensin gene (Nguyen et al. 2003). Mature θ-defensin peptides contain only 18 residues, which include the requisite six cysteines and three disulfide bonds. A peptide-bond connects their amino- and carboxy-terminal residues, making θ-defensins the only known peptides of animal origin with a cyclic backbone. θ-defensin peptides have been isolated from the leukocytes or bone marrow of rhesus macaques (Leonova et al. 2001; Tang et al. 1999) and baboons (Garcia et al. 2008; Stegemann et al. 2010). However, they are absent from human leukocytes and are unlikely to exist in the leukocytes of chimpanzees, bonobos, and gorillas (Nguyen et al. 2003). Like humans, these apes have θ-defensin (DEFT) pseudogenes that contain a stop codon mutation within the signal sequence domain. The human DEFT gene is transcribed; however, the resulting mRNA is not translated because of the premature stop codon.

Family reunions can be confusing, so we will summarize what has already been said about defensin evolution. So far, we have introduced three generations of vertebrate defensins. The grandparents (β-defensins) have existed for ~250 million years. Their α-defensin offspring arose ~125 million years ago, and their θ-defensin grandchildren were born around 35–50 million years ago (Nguyen et al. 2003). θ-defensins are not further described in this chapter. Interested readers can consult other publications to learn about their unusual mode of assembly (Tang et al. 1999), antiviral properties (Venkataraman et al. 2009; Cole et al. 2002; Wang et al. 2003), and antimicrobial activities (Tongaonkar et al. 2011; Welkos et al. 2011; Tran et al. 2002).

5.4 Gamma (γ)-Defensins

γ-Defensins, which are also called "ovodefensins" (Gong et al. 2010), have so far been found only in the eggs of reptiles and birds. Although humans tend to view eggs primarily from a culinary perspective, they are incubators that contain sufficient nutrients and minerals to support embryonic growth and development and provide physical and chemical barriers to prevent infection. The chemical barriers in egg white include lysozyme, ovotransferrin, and perhaps ovalbumin itself, since this member of the serpin family contains multiple oligopeptide antimicrobial domains (Pellegrini et al. 2004). They also include defensins, such as the *Caretta caretta* γ-defensin shown in Fig. 3, which was purified from the egg white of a marine sea turtle. The *Caretta* peptide is cationic (net charge, +6), has six cysteines and three intramolecular disulfide bonds, exerts strong antibacterial activity against *Escherichia coli* and *Salmonella typhimurium*, and has impressive antiviral properties (Chattopadhyay et al. 2006). A similar peptide exists in eggs of an Indian tortoise, *Geomyda trijuga trijuga* (Chakrabarti et al. 1988). Based on its properties and structure, the *Caretta* peptide qualifies to be called a defensin, but to which

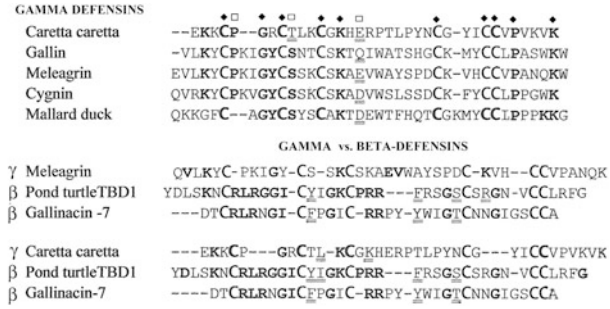

Fig. 3 Gamma defensins. Series I shows sequences of five γ-defensins, isolated from the white of various eggs. *Caretta caretta* is a turtle. Gallin, meleagrin, and cygnin came respectively from the eggs of chickens (*Gallus gallus*), turkeys (Meleagris gallopavo), and black swans (*Cygnus atratus*). Series II and III compares the sequences of meleagrin and the Caretta caretta peptide to b-defensins obtained from the leukocytes of a turtle and the chicken

subfamily should it be assigned? Its cysteines were reported to pair in a 1–6; 2–5; 3–4 manner (Chattopadhyay et al. 2006). This differs from the cysteine pairing in a β-defensin purified from leukocytes of the European pond turtle, *Emys orbicularis*, which manifests the cys 2–4 disulfide bond found in α- and β-defensins. The backbone fold of the *Caretta* peptide differs from the fold of α- and β-defensins (Chattopadhyay et al. 2006). For these reasons, we agree that the Caretta peptide is a charter member of the γ-defensin (ovodefensin) subfamily.

There were three main reasons for suggesting the term γ-defensins: consistency, orderliness, and whimsy. Gamma (γ) is consistent with the α, β, and θ nomenclature used to classify other vertebrate defensin subfamilies. Gamma is also orderly, since γ follows α and β in the Greek alphabet. Finally, gamma is somewhat whimsical since it follows α and β in αβγo, a Greek word for egg. For readers who prefer their eggs and peptides prepared Latin style, ovodefensin is a suitable alternative—at least until a γ-defensin is found outside the confines of an egg. Figure 3 shows additional avian γ-defensins, including gallin (Gong et al. 2010), expressed in the chicken oviduct; meleagrin (Odani et al. 1989) from the turkey, *Meleagris gallopavo*; cygnin (Simpson and Morgan 1983) from the black swan, *Cygnus atratus*; and BPS1 (Naknukool et al. 2008) from the mallard duck, *Anas platyrhynchos*. The cysteine-connectivity of these avian γ-defensins remains to be determined, so the figure shows the disulfide pairing of the *Caretta* peptide. Another mallard duck peptide, BPS2, has an identical sequence to that of cygnin, and related peptides exist in the zebra finch (Gong et al. 2010). To allow comparison, Fig. 3 also shows three β-defensins: TBD1, from turtle leukocytes; gallinacin-7, from chicken leukocytes; and HBD-126, a human epididymal β-defensin.

5.5 NK-Lysin and Granulysin

Certain AMPs that contain six cysteines have not evolved from a defensin lineage. This is exemplified by the AMP family that includes porcine NK-lysin (Andersson et al. 1995), human granulysin (Krensky 2000), and amoebapore (Bruhn et al. 2003) from the pathogenic protozoan, *Entamoeba histolytica*. NK-lysin, purified from porcine small intestinal tissue, was the first member of this group to be characterized (Andersson et al. 1995, 1996). It contained 78 residues, was cationic, had six cysteines and three disulfides, and had impressive cytolytic and antimicrobial activity, including an ability to kill *Mycobacterium tuberculosis* (Andreu et al. 1999). However, its four α-helix bundle structure (Dandekar and Leippe 1997) differs substantially from the β-sheet or CSαβ-structures of defensins, and its family allegiances are elsewhere. NK-lysin, granulysin, and amoebapore belong to the SAPLIP (*sap*osin-*li*ke *p*rotein) family, whose evolutionary history is described elsewhere (Bruhn 2005; Rorman et al. 1992; Zhai and Saier 2000; Leippe and Herbst 2004).

5.6 Big Defensins

For help in tracing the origins of vertebrate β-defensins, we could have no better guide than Aphrodite, who—according to Greek mythology—arose from the sea in an oyster shell. However, it would be the oyster and not the Goddess who could lead us back to our destination. Oysters and other mollusks express many varieties of AMPs, including several that are called defensins. Because the giant Pacific oyster, *Crassostrea gigas*, is an important commercial species, it has become a subject of detailed scientific study. Like primates, mollusks express three families of defensins, including "big defensins"—the likely ancestor of β-defensins.

Big defensins were discovered in the hemocytes of a horseshoe crab, *Tachypleus tridentatus* (Saito et al. 1995; Iwanaga et al. 1998). This big defensin contained 79 amino acids, organized in two tandem domains, each of which could exert antimicrobial activity by itself. The N-terminal domain was amphipathic, cysteine-free, and non-cationic. However, the C-terminal domain contained 37 amino acids, was cationic, and its six cysteines paired in exactly the same manner (cys1–5, 2–4, 3–6) as they pair in vertebrate β-defensins. Recent studies indicate that the C-terminal domain of the *C. gigas* big defensin is encoded by a separate exon (Rosa et al. 2011).

Figure 4 aligns the sequences of ten big defensins and β-defensins from fish, reptiles, birds, and mammals. Considering that β-defensins often show only 30–40 % homology with other β-defensins, the homology between big defensins and β-defensins is impressive. Because a peptide with six cysteine residues could join them pairwise in 15 different ways, their identical pairing in big defensins and β-defensins is not trivial. Additional support for the ancestry of big defensins to β-defensins comes from the recently reported presence of big defensins in the amphioxus, *Branchiostoma* (Teng et al. 2012). The existence of big defensins

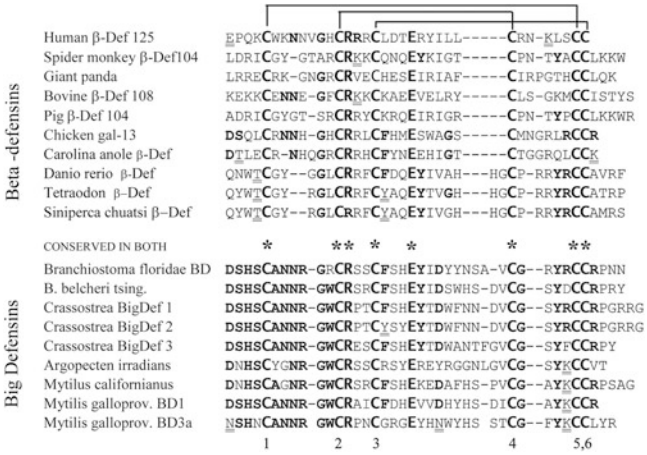

Fig. 4 Big defensins and beta-defensins. The upper ten sequences are of β-defensins from three fish (*Siniperca chuatsi*, *Tetraodon nigriviridis*, *Danio rerio*), a reptile (*Anolis californianus*), the chicken, (*Gallus gallus*), and five mammals. Residues (6 cys, 1 arg, 1 glu) shown in a larger font t are conserved in {beta}-defensins and big defensins. The nine big defensin sequences include 7 from mollusks and two from lancelets (*Branchiostoma floridae* and *Branchiostoma belcheri*). *Crassostrea* is an oyster, *Argopecten* (the bay scallop) is a saltwater clam, and *Mytilus* species are mussels. Identical residues are *bolded*, and conservative substitutions are *double underlined*. The cysteines are numbered at the *bottom*, and their connectivity is shown at the *top*. *Dashes* represent gaps that were introduced to maximize the alignment. *Stars* indicate residues that are highly conserved in both big defensins and β-defensins

in both protostomes (horseshoe crabs and mollusks) and deuterostomes (*Branchiostoma*) is noteworthy, especially since these phyla diverged ~670 million years ago (Ayala et al. 1998).

5.7 *Sapecin-Like Insect Defensins*

In addition to the peptides discussed above, there is a second AMP superfamily whose members are called defensins. In 1988, Matsuyama and Natori purified three AMPs (sapecins) from an embryonic cell line of *Sarcophaga peregrina*, a flesh fly. The peptides called "sapecins" were cloned the following year. The sapecin fold contained a flexible loop (residues 4–12), followed by a short helix (residues 15–23), and two extended strands that were formed by residues 24–31 and 34–40 (Hanzawa et al. 1990). In 1989, Lambert et al. isolated two antimicrobial peptides from immune blood of the dipteran *Phormia terranovae* (Lambert et al. 1989). The peptides ("phormicins") were positively charged, contained 40 residues, and had three intramolecular disulfide bridges. Their sequences differed by only a single amino acid. Because they generally resembled α-defensins, they proposed calling them "insect defensins" (Lambert et al. 1989). The sequences of sapecin, phormicin,

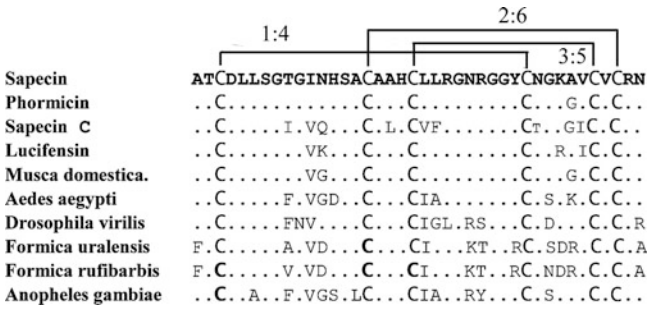

Fig. 5 Insect defensins. These sequences come from ants (*Formica* spp.), mosquitoes (*Aedes* and *Anopheles*), and flies (*Musca domestica*, the common house fly, and *Drosophila virilis*, a fruit fly). Sapecin was purified from a cell line derived from the flesh fly, *Sarcophaga peregrina*. Phormicin was isolated from larvae of the blue bottle fly *Phormia terranovae* and lucifensin from maggots of the blowfly, *Lucilia sericata*

and eight other defensins expressed by flies, mosquitoes, and ants can be seen in Fig. 5, which also shows that their cysteines pair in a 1:4, 2:6, 3:5 manner to create a cysteine-stabilized αβ-structure (Dassanayake et al. 2007). The sequences are highly conserved, even though flies and mosquitoes belong to the order Diptera, and ants belong to the order Hymenoptera. The evolution of insect defensins has been reviewed elsewhere (Dassanayake et al. 2007; Wong et al. 2007).

5.8 Older Defensins

The sequences in Fig. 6 are divided into four groups. Dashes were introduced into the group II and III sequences to align them with group IV. Group I contains defensins from a fly, a mosquito, and an ant. They resemble sequences shown in Fig. 5 except that several motifs have been boxed. Group II shows defensin sequences from more ancient invertebrates, including a dragonfly (*Aeschna cyanea*) and five arachnids: a scorpion, *Leiurus*, and four ixodid ticks. The sequences in group II show extensive homology within the group and with the molluskan defensins in group III. Residues conserved in groups II and III are indicated by their bold font and by vertical lines in the space between the groups. In addition to this conservation across the arthropod/mollusk boundary, the sequences in groups II and III are homologous to the fungal defensins in group IV. Since fungi and animals diverged from their last common ancestor more than one billion years ago, the ancestral gene for these defensins is extremely ancient. Also note that the defensins in groups II and IV contain six cysteines, and those in group III contain eight. If entry into the defensin confraternity is governed by descent, then the definition of a defensin should encompass peptides with six or eight cysteines. While this notion might furrow a few human brows, it would be logical to a tick, whose nymphal and larval forms have six legs and whose adult form has eight.

Fig. 6 Older invertebrate defensins. Four sets of sequences are shown. Set I shows three insect defensins homologous to those in Fig. 5; set II shows sequences from more primitive arthropods, including a dragonfly (*Aeschna cyanea*), four ticks (*Amblyomma maculatum*, *Argas monolakensis*, *Ixodes scapularis*, and *Ornithodorus moubata*), and a scorpion (*Leiurus quinqefasciatis hebraicum*). Set III shows defensins from the oyster, *Crassostrea gigas*, and the Mediterranean mussel, *Mytilus galloprovincialis*. Set IV shows sequences of defensins expressed by three fungi: *Pseudoplectania nigrella* (plectasin), *Ajellomyces dermatitidis*, and *Ajellomyces capsulatus*. The last two fungi may be more familiar under their older names of *Blastomyces dermatitidis* and *Histoplasma capsulatum*. Conserved residues in sets II, III, and IV appear in a *bold* font, and conservative replacements are *doubly underlined*. *Vertical lines* identify residues conserved between sets II and IV. Please note that the mollusk defensins in set III contained eight cysteines and the defensins in sets I, II, and IV contained six cysteines. In set I, the *boxed* residues show motifs that also appear in sets II–IV, perhaps coincidentally or perhaps not

5.9 For Extra Credit

Because defensins and defensin-like peptides also exist in plants (Broekaert et al. 1995; Zhang and Kato 2003; Thomma et al. 2002) and in prokaryotes (Zhu 2007), their evolutionary history might be traced even further back. Readers interested in this exercise can consult recent reports on this subject (Zhu 2007, 2008b). The AMPs of plants are described in chapter "Innate Immunity in Plants: The Role of Antimicrobial Peptides" and various antimicrobials produced by prokaryotes are described in chapter "Antimicrobial Peptides Produced by Microorganisms".

5.10 The Importance of Being Nonspecific

Defensins and other AMPs had already existed for over 750 million years before the adaptive immune system of vertebrates came into existence. This is important to recall when evaluating suggestions that defensins and other AMPS function *primarily* as molecular alarms that summon the adaptive immune system into action. If AMPs were independent contractors before there was an adaptive immune system, they undoubtedly remain so now—especially given the kinetic features of innate and adaptive immunity discussed in the introduction.

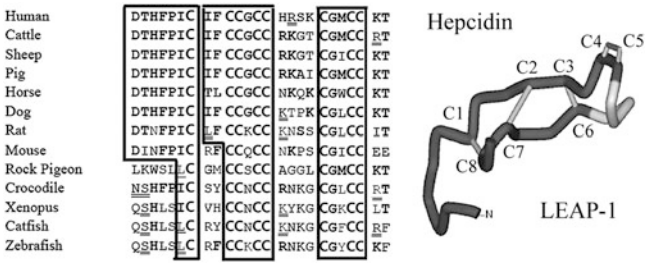

Fig. 7 Hepcidin (LEAP-1). This 25-residue iron-regulatory peptide is exceptionally well conserved among mammals but somewhat less so among the other vertebrate classes. Highly conserved motifs are *boxed*, identical conserved residues are *bolded*, and conservative substitutions are *underlined*. The disulfide pairing motif is illustrated in the structural diagram

6 AMPs with Eight or More Cysteines

6.1 Hepcidin

LEAP-1, more commonly called hepcidin, is a 25-residue cationic peptide with eight cysteines that is produced by the liver. Its sequence is unusually well conserved for an AMP, especially among mammals (Fig. 7). Although hepcidin has antimicrobial properties, its major physiological role is that of a central hormonal regulator of iron metabolism. Deficiencies of hepcidin cause disorders characterized by tissue iron overload, and excessive hepcidin is associated with anemia of inflammation, chronic renal disease, and other disorders (Ganz and Nemeth 2011).

6.2 eNAP-2 and Related WAP/FDC Peptides

eNAP-2, a 6.5-kDa AMP that is relatively rich in proline, glycine, and cysteine residues, was purified from equine neutrophils, which contain it in considerable amounts (Couto et al. 1992b). In addition to its antimicrobial activity, eNAP-2 inhibited two serine proteases of microbial origin, subtilisin A and proteinase K, by binding to the active site of these enzymes (Couto et al. 1993). This serine protease inhibition was selective in that eNAP-2 did not inactivate elastase or cathepsin G from human neutrophils or bovine pancreatic trypsin, all of which are mammalian serine proteases. Based on the sequence of its first 46 residues, eNAP-2 belongs to the large and diverse whey acidic protein/4-disulfide core (WAP/FDC) family. Other members of this family are also protease inhibitors, including secretory leukocyte protease inhibitor (SLPI), which also has antimicrobial, antiviral, and immunomodulatory properties (Hiemstra et al. 1996; Tomee et al. 1998; Williams et al. 2006; Scott et al. 2011). Figure 8 shows that homologs of

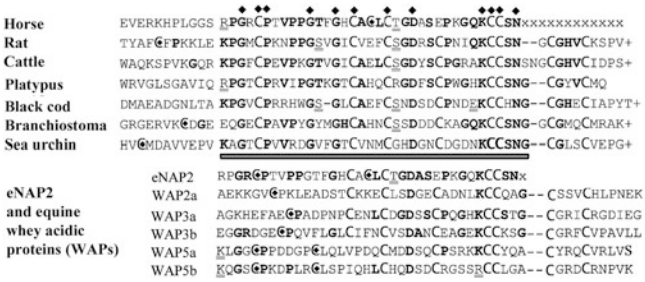

Fig. 8 eNAP-2 belongs to the "whey acidic protein/four disulfide core" (WAP/FDC) family. Two sets of sequences appear in the figure. The upper set shows eNAP-2 (first sequence) and WAP/FCD peptides from other animals. As eNAP-2 was not fully sequenced (Couto et al. 1992), its unknown residues are represented by xxx. The *long narrow rectangle* beneath the top set indicates the 35-residue region of greatest conservation. The twelve large *black diamond symbols* identify the twelve most highly conserved residues in this 35-residue region. Identical residues are in *bold* font, and conservative substitutions are *underlined*. The bottom set compare eNAP2 to other WAP/FDC peptides in the equine genome. Cysteine residues outside the highly conserved region have a small *black diamond symbol* within the letter C. Note that eNAP2 uniquely contains an additional cysteine within the highly conserved region

eNAP-2 exist not only in other vertebrates but also in the amphioxus-like cephalochordate, *Branchiostoma floridae*, and in an echinoderm, the purple sea urchin *Strongylocentrotus purpuratus*. The bottom part of this figure compares eNAP-2 to other equine WAP/FDC peptides and shows that eNAP2 is not alone in having a cysteine in other than its canonical position.

6.3 A Curious Brew

If we made a laboratory bouillabaisse with oysters, mussels, scorpions, and spiders and threw in a few primitive arachnids and insects for spice, the broth would contain multiple AMPs with six, eight, or ten cysteines. They would include MGDs (*Mytilus galloprovincialis* defensins), which have eight cysteines (Hubert et al. 1996; Mitta et al. 1999; Gerdol et al. 2011) and the cysteine-stabilized α-β (CSαβ) structure also found in scorpion toxins (Yang et al. 2000). *Mytilus* has three other AMPs that contain eight cysteines: mytilins, myticins, and mytimacins (Gerdol et al. 2011). All of these are stored as processed, active forms within leukocyte granules, able to act locally in phagosomes or systemically after entering the hemolymph. Unlike α-defensin propeptides, wherein the cationic antimicrobial domain follows an anionic propiece, the defensin domain in an MGD precursor precedes a 21-residue anionic post-piece (Mitta et al. 1999). The eight cysteine residues of MGD-1 pair in a [Cys 1–5; 2–6; 3–7; 4–8]-manner (Yang et al. 2000).

Mytimacins have 85–101 residues, including 8–10 conserved cysteines and four or five intramolecular disulfide bridges (Gerdol et al. 2011). They are homologous

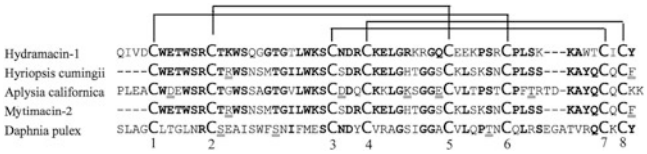

Fig. 9 How to recognize a macin. Hydramacin-1 is from the primitive coelenterate, *Hydra magnipapillata* (GI: 213424017). *Hyriopsis cunningii* is the triangle shell pearl mussel, and the peptide shown in this figure is called theromacin (AEC50045.1). *Aplysia californica* is also called a California sea hare, and the illustrated peptide is currently called "neuromacin-like" (NP_001 191629). Mytimacin-2 (CCC15016.1) comes from the Mediterranean mussel, *Mytilus galloprovincialis*. The final peptide is expressed by the tiny crustacean, *Daphnia pulex*, which is commonly called a "water flea" is. The cysteine connectivity was established in hydramacin-1

to hydramacin, an antimicrobial peptide expressed by the ancient cnidarian and basal metazoan, *Hydra magnipapillata* (Jung et al. 2009). Molluskan "macins" and hydramacins both likely possess a CSαβ-structure (Gerdol et al. 2011). The macin group is larger and more complex than the single *Mytilus* representative shown in Fig. 9. A peptide from *Daphnia pulex*, the "water flea," also appears in this figure. This microscopic crustacean played a crucial role in Eli Metchnikoff's discovery of phagocytosis. His experiments with Daphnia, which are described in his 1893 monograph "Lectures on the Comparative Pathology of Infection," remain worth reading.

6.4 Is eNAP-1 an AMP?

When equine neutrophils were examined two decades ago (Couto et al. 1992) instead of finding any defensins, two other cysteine-rich peptides with antimicrobial activity were discovered and named equine neutrophil antimicrobial peptides (eNAPs) 1 and 2. eNAP-2, which is described in Sect. 6.2, existed in relatively large amounts to allow it to function as an AMP. However, eNAP-1 was present in much smaller amounts, making it unlikely that its direct antimicrobial effects are functionally significant in vivo. The sequence of eNAP-1 and its homology to other members of the granulin family are shown in Fig. 10. The biological activities of granulins were recently reviewed (Bateman and Bennett 2009) and are distinct from direct antimicrobial activity. For these reasons, we would answer the rhetorical question introducing this paragraph with a simple "No."

6.5 Drosomycin

It is fitting to end this discussion with an AMP from *Drosophila*, a tiny fly whose contributions to genetics have been immense. Drosomycin, a 44-residue peptide, is produced after puncturing larval or adult *Drosophila* with a bacteria-soaked needle

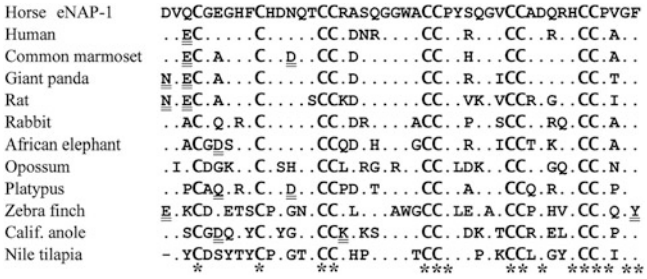

Fig. 10 eNAP-1 and granulins. Only the first 46 residues of eNAP-1 were sequenced. They are shown, along with the corresponding 46 residues of homologous peptides from other animals. Residues identical to those in eNAP1 are shown as *dots*, and conservative substitutions are *underlined*. The 16 *asterisks* beneath the sequences identify the most highly conserved residues. Full-length granulins have twelve cysteines and six disulfide bonds. Human granulin contains twelve additional C-terminal residues (including two cysteines that do not appear in the figure)

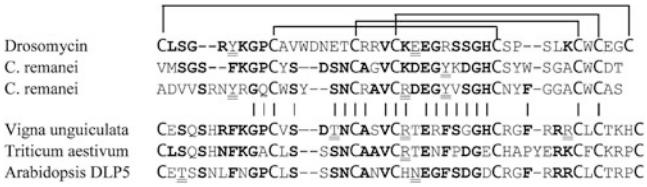

Fig. 11 Drosomycin. Drosomycin, from the fruit fly (*Drosophila melanogaster*), has homologs in the nematode, *Caenorhabditis remanei*, and it also has them in plants, including the cowpea (*Vina unguiculata*), wheat (*Triticum aestivum*), and mouse-ear cress (*Arabidopsis thaliana*). The disulfide bonds of drosomycin are indicated above its sequence. *Vertical lines* traversing the space between animal and plant sequences show conserved sites. Identical residues found on both sides of the central plant/animal divide are in *bold* font

(Fehlbaum et al. 1994). Synthesized in the fat body, it contains eight cysteines and four intramolecular disulfide bonds and is primarily antifungal. In their description, the authors recognized its structural resemblance and sequence homology to antifungal peptides produced by plants (Fig. 11) and wrote, "It is tempting to speculate that drosomycin and plant defensins have evolved from a common ancestor molecule that predated the separation of plants and animals." Additional pieces of the puzzle that have since been found, including the nematode (*Caenorhabditis remanei*) sequences in Fig. 10, leave little doubt that their initial speculation is correct.

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Innate Immunity in Plants: The Role of Antimicrobial Peptides

H.U. Stotz, F. Waller, and K. Wang

Abstract Antimicrobial peptides (AMPs) are part of innate immunity, establishing a first line of defense against pathogens. All plant organs express AMPs constitutively or in response to microbial challenges. Plant AMPs are structurally and functionally diverse. Five classes of AMPs are considered in this review, the thionins, defensins, lipid transfer proteins (LTPs), snakins, and a group of related knottins, cyclotides and hevein-like AMPs. Besides targeting fungal, bacterial, and oomycete pathogens, certain AMPs can be directed against other organisms, like herbivorous insects. The biological activity of plant AMPs primarily depends on interactions with membrane lipids, but other modes of action exist as in the case of defensins with α-amylase activity or a defensin-like peptide that interacts with a receptor kinase. Limited information exists on the regulated expression of plant AMPs, their processing, and posttranslational modification. Conclusive data on the role of certain AMPs in plant defense have only recently become available. This review can therefore only be considered as a snapshot of the progress in this field of research.

1 Introductory Remark

Protection of plants and animals against infectious microorganisms depends on both constitutive and induced defense mechanisms. Antimicrobial peptides (AMPs) are an important component of constitutive and induced epithelial defenses, contributing to the first line of defense in animals (Schröder 1999). Plants produce

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AMPs in all organs either constitutively or in response to microbial infection, but information about the expression of AMPs in epidermal cells is limited. As plants also biosynthesize protective secondary metabolites, AMPs may not be as crucial for the first line of defense as in animals. In light of the induction of specific AMPs, a brief review of the plant immune system will be given first.

2 Innate Immunity in Plants

The innate immune system of plants consists of two branches (Jones and Dangl 2006). Initial recognition of microbes by host plants is analogous to the animal system in that pattern recognition receptors (PRRs) on the surface of the host cell detect the presence of pathogen-associated molecular patterns (PAMPs), representing small motifs of larger molecules that are essential for microbial survival (Janeway 1989). PAMP-triggered immunity (PTI) activates a myriad of processes, including mitogen-activated protein kinase (MAPK) cascades, production of reactive oxygen species (ROS), hormone signaling, and gene expression (Schwessinger and Zipfel 2008). Successful pathogens suppress PTI by delivering virulence effector proteins to the host. In turn the second branch of plant immunity, which is defined by another set of largely intracellular plant receptors, the resistance (*R*) gene products, is activated. These receptors recognize specific effector proteins directly or indirectly to activate effector-triggered immunity (ETI), a stronger plant defense response that can culminate in programmed cell death (Jones and Dangl 2006).

PTI and ETI stimulate distinct hormone biosynthesis and signaling pathways. ETI triggers salicylic acid (SA) biosynthesis and signaling, leading to local and systemic acquired resistance (SAR) against biotrophic pathogens (Metraux et al. 1990; Delaney et al. 1994). SAR is the induction of broad-spectrum disease resistance in uninfected distal tissues activated by local pathogen infection that results in tissue necrosis, also known as the hypersensitive response (Ward et al. 1991). Conversely, PTI stimulates ethylene (ET) biosynthesis (Felix et al. 1999). The cross talk between PTI and ET biosynthesis and signaling has recently been reviewed (Trujillo and Shirasu 2010). ET and jasmonic acid (JA) synergistically activate plant defenses against necrotrophic pathogens (Thomma et al. 1998). Moreover, antagonistic interaction exists between JA/ET and SA signaling (Niki et al. 1998). Both pathways induce the expression of AMPs in different ways, as will be discussed later.

3 General Characteristics of Plant AMPs

Like AMPs of animal origin, the molecular diversity of AMPs from plants is striking (Padovan et al. 2010). Plant AMPs were assigned to different classes according to their tertiary structures (Fig. 1). The most common classes are

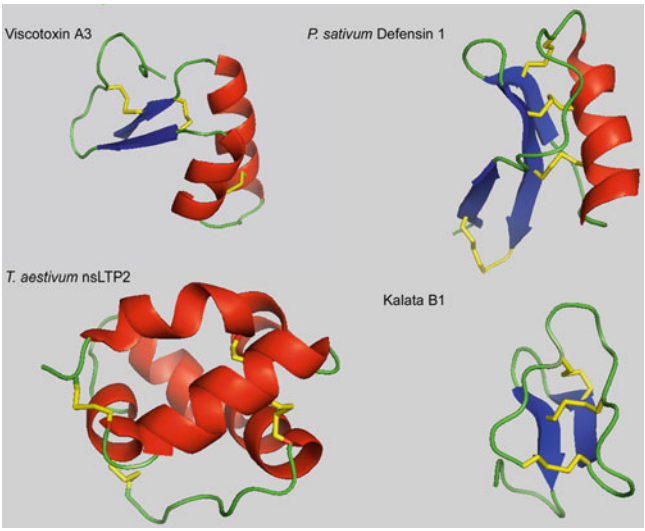

Fig. 1 *3D structures of representative antimicrobial peptides* (*AMPs*) *belonging to different classes of AMPs from plants*. Viscotoxin A3 (PDB entry 1ED0), a thionin from mistletoe (*Viscum album*) is a representative of thionins. Defensins are represented by defensin 1 (PDB entry 1JKZ) from pea (*Pisum sativum*). An example of lipid transfer proteins (LTPs) is the non-specific nsLTP2 (PDB entry 1TUK) from wheat (*Triticum aestivum*). Kalata B1 (PDB entry 1NB1) is a cyclotide from the perennial herb *Oldenlandia affinis*. Disulfide bridges are highlighted in *yellow*; α-helices and β-sheets are color-coded in *red* and *blue*, respectively

thionins, defensins, and lipid transfer proteins. Plant AMPs share the following important features: They are small cationic peptides with molecular masses of 2–10 kDa. The structures of these small peptides are stabilized through formation of 2–6 disulfide bridges. The activities of plant AMPs are primarily directed against fungal, oomycete, and bacterial microorganisms, but certain members of a class can be directed against other targets, including herbivorous insects.

Different classes of plant AMPs will be discussed next. Special attention will be given to their structures and functions, their regulated expression, and their modes of action.

4 Thionins

The first AMP isolated from plants was a thionin from the endosperm of wheat (Balls et al. 1942). The protein moiety of a proteolipid was later shown to be a mixture of two forms, purothionins α and β (Nimmo et al. 1968). Additional thionins were isolated, including α- and β-hordothionins from barley endosperm, viscotoxins and phoratoxins from mistletoe species, and crambin from the cruciferous plant *Crambe abyssinica* (Bohlmann and Apel 1991). Thionins from cereals

and *Pyrularia pubera* have four disulfide bonds. Other dicotyledonous thionins have three disulfide bonds. The structural feature common to all thionins is the Γ (gamma) fold consisting of two antiparallel α-helices that form a stem and antiparallel β-sheets that form an arm (Padovan et al. 2010). A groove exists between the helical and β-sheet segments. Thionins are cationic peptides with amphipathic properties with the exception of crambin, which is hydrophobic and carries no net charge.

Both purothionins differentially inhibit the growth of and kill several bacterial plant pathogens but not mycelial fungi (Fernandez de Caleya et al. 1972). Thionins from the endosperm and leaf of barley inhibit the fungi *Drechslera teres*, a pathogen of barley, and *Thielaviopsis paradoxa*, a pathogen of sugar cane (Bohlmann et al. 1988). The thionin from *P. pubera* has antifungal as well as antibacterial activities (Vila-Perello et al. 2005). Thionins also affect organisms other than plant pathogens. Purothionins are toxic to small mammals when injected intravenously or intraperitoneally but not when administered orally (Coulson et al. 1942). The reason behind these broad biological activities lies in the thionin structure.

Thionin exerts its primary effect on the membrane through binding of phospholipids (Stec et al. 2004). Amino acid residues 1 and 2 as well as residues 9–14 are highly conserved. Specifically, Lys1 and Arg10 contribute to phosphate binding. Ser2 and Tyr13 form the glycerol-binding site. Modeling of thionin interactions with phospholipids implied that the acyl chain of the phospholipid fits into the groove of the toxin (Stec et al. 2004). In the absence of phospholipids, thionins form dimers that bind inorganic phosphate and are stabilized by Asn11 and Asn14. Upon association with membranes, monomers are formed that insert into the membrane and segregate phospholipids. This action destabilizes the membrane, leading to ion leakage and eventually to lysis.

Mature thionins are derived from preproproteins that contain an N-terminal signal peptide for targeting to the endoplasmic reticulum (ER) and a C-terminal acidic peptide that is thought to neutralize the activity of the cationic thionin. Thionins are targeted to the vacuole (Romero et al. 1997). The function of vacuolar-targeted thionins is probably analogous to the action of basic chitinase and β-1,3-glucanase (Mauch and Staehelin 1989). These enzymes accumulate in the vacuole and are released during host cell lysis caused by pathogen attack. The sudden release of high concentrations of antimicrobial peptides is thought to overwhelm invading pathogens without time to adapt to the challenge.

The genome of the model plant *Arabidopsis thaliana* also encodes thionins. The *Thi2.1* and *Thi2.2* genes are regulated differently (Epple et al. 1995). *Thi2.1* is constitutively expressed at high levels in flowers and siliques and inducible in seedlings in response to methyl jasmonate and inoculation with the fungal pathogen *Fusarium oxysporum*. *Thi2.2* is constitutively expressed in seedlings but not inducible. The octadecanoid pathway, which is analogous to the inflammatory response pathway in animals that leads to the production of prostaglandins from arachidonic acid, culminates in JA biosynthesis and induction of *Thi2.1* expression (Bergey et al. 1996; Bohlmann et al. 1998). This was proven with the help of JA-insensitive

and JA-deficient mutants, which were no longer able to induce *Thi2.1* expression in response to stimuli like wounding, leading to JA accumulation and signaling (Bohlmann et al. 1998). Overexpression of *Thi2.1* in transgenic *A. thaliana* plants decreased susceptibility to *F. oxysporum* (Epple et al. 1997). Hyphae had more growth anomalies, including hyperbranching, when the fungal pathogen was grown on cotyledons of *Thi2.1*-overexpressing lines than on cotyledons of untransformed plants (Epple et al. 1997). A phenotypically similar disruption of fungal growth was observed when *F. oxysporum* was exposed to thionins in vitro (Vila-Perello et al. 2005). To address the endogenous function of thionins, a loss-of-function approach is needed. For this purpose, lines with T-DNA insertions in exon regions of both *Thi2.1* and *Thi2.2* genes are available from The Arabidopsis Information Resource (TAIR). However, their analysis still needs to be performed. As will become clear from the next chapter, knockout or knockdown mutants are useful for determining the in vivo function of AMPs.

5 Defensins

Defensins were originally grouped with the thionins and defined as γ-thionins. However, structural comparisons to insect defensins required a reclassification of this group of AMPs as defensins (Bruix et al. 1993). Characteristically, defensins consist of a well-defined triple-stranded antiparallel β-sheet and a single α-helix that lies parallel to the β-sheet. The α-helix is connected to the β-sheet with the help of two disulfide bridges, forming a characteristic Cys-stabilized α-helix β-sheet (CSαβ) motif (Cornet et al. 1995). Typical plant defensins form two additional disulfide bonds. Plant and arthropod defensins consist of a βαββ pattern, whereas mammalian β-defensins contain an N-terminal α-helix and an overall αβββ-fold. Plant defensins are small 45–54 amino acids long cationic peptides. Defensins are widely distributed among dicots and monocots. The genome of *A. thaliana* alone was shown to encode more than 300 defensin-like (DEFL) peptides, 78 % of which have a CSαβ motif (Silverstein et al. 2005). An even larger diversity of DEFL genes is present in legume species (Graham et al. 2008).

Unlike animal defensins, few plant defensins are active against bacteria (Franco et al. 2006; Yokoyama et al. 2008). Instead, the most common activity of these peptides is directed against diverse fungi (Osborn et al. 1995). Besides these antimicrobial activities, specific defensins have been reported to inhibit protein synthesis (Mendez et al. 1990), protease trypsin (Wijaya et al. 2000), or α-amylase activity (Bloch and Richardson 1991; Lin et al. 2007; Pelegrini et al. 2008). The α-amylase inhibitors are insecticidal. The defensins VrD1 from *Vigna radiata* and VuD1 from *Vigna unguiculata* use the L3 loop and the N-terminus, respectively, to inhibit α-amylase activity (Lin et al. 2007; Pelegrini et al. 2008).

Plant defensins also influence plant growth and development. *Medicago* spp. do not express MsDef1 and MtDef2 in roots, but when roots of *A. thaliana* are exogenously treated with MsDef1, MtDef2, or RsAFP2, their growth is retarded

and root hair elongation is inhibited (Allan et al. 2008). According to the authors, constitutive expression of MsDef1 in *A. thaliana* did not alter root or root hair growth. Altered expression of the tomato defensin DEF2 in transgenic tomato plants reduced pollen viability and seed production (Stotz et al. 2009). Constitutive expression of this defensin had pleiotropic effects on plant development. In contrast to the above examples, the following developmental process has been studied at the mechanistic level. The S-locus protein 11 (SP11), also known as S-locus Cys-rich (SCR) protein, is a DEFL peptide that interacts with the S-locus receptor kinase (SRK) to trigger self-incompatibility, a response that prevents self-fertilization via inhibition of pollen tube growth and that favors outcrossing (Nasrallah 2002). The L3 loop connecting β2 and β3 and the α-helical region are important for recognition of SP11 by SRK (Sato et al. 2004).

A group of DEFL peptides with four to six Cys residues was recently shown to control symbiotic interactions. Nodule-specific Cys-rich (NCR) peptides govern terminal differentiation of nitrogen-fixing endosymbiotic *Rhizobium* bacteria in root nodules of leguminous host plants (Van de Velde et al. 2010). NCR peptides are delivered to the bacterial symbiont via a nodule-specific secretory pathway (Wang et al. 2010). These peptides cross the bacterial membrane and accumulate inside the bacteria to inhibit cytokinesis and to stimulate DNA synthesis and cell enlargement (Van de Velde et al. 2010). These examples demonstrate that defensins and DEFL peptides are functionally diverse, probably reflecting their evolutionary diversification.

The defensins DmAMP1 from *Dahlia merckii* and RsAFP2 from *Raphanus sativus* induce rapid potassium efflux and calcium influx in the hyphae of the fungus *Neurospora crassa* in combination with medium alkalinization (Thevissen et al. 1996). Inhibition of fungal growth in response to both defensins was associated with cation-resistant membrane permeabilization (Thevissen et al. 1999). Using radiolabeled HsAFP1, a defensin from *Heuchera sanguinea*, specific high-affinity binding sites were identified on the plasma membrane of *N. crassa* (Thevissen et al. 1997). A genetic approach was used to identify these binding sites as complex lipids (Thevissen et al. 2000). Mutation in the *IPT1* gene, encoding an enzyme that catalyzes the last step in biosynthesis of the sphingolipid mannose-(inositol-phosphate)$_2$-ceramide, conferred DmAMP1 resistance to *Saccharomyces cerevisiae*. Sensitivity of the yeasts *Candida albicans* and *Pichia pastoris* to RsAFP2 was a function of the gene *GCS*, encoding UDP-glucose:ceramide glucosyltransferase (Thevissen et al. 2004). RsAFP2 was shown to interact with membrane components of lipid rafts to elicit the production of reactive oxygen species, leading to fungal cell death (Aerts et al. 2007). NaD1, a defensin from *Nicotiana alata*, also causes permeabilization of the fungal membrane, but it enters fungal cells via a cell wall-dependent mechanism possibly reaching intracellular targets (van der Weerden et al. 2008, 2010). Thus, even with respect to antifungal activity, different modes of defensin action exist.

Different defensins are expressed throughout the plant. Defensins were first isolated from seeds of monocot and dicot species. Importantly, the defensins from radish seeds RsAFP1 and RsAFP2 are released during seed germination after

disruption of the seed coat (Terras et al. 1995). The amount of released peptides was shown to be sufficient for inhibition of fungal growth around the germinating seedlings. RsAFP peptides are expressed in surface cell layers and in spaces between different organs of the seed. Moreover, RsAFP peptides are secreted into the middle lamella region of plant cell walls, which are important for cellular adhesion. Expression of RsAFPs is induced after pathogen challenge, and constitutive expression of RsAFP2 in transgenic tobacco resulted in increased resistance against the foliar pathogen *Alternaria longipes* (Terras et al. 1995). Different defensins are constitutively expressed in every organ of *A. thaliana* (Thomma and Broekaert 1998). *PDF1.2* has been established as an important marker gene to study the activation of the JA/ET signaling pathway (Manners et al. 1998; Mitter et al. 1998; Brown et al. 2003; Nandi et al. 2003; Ndamukong et al. 2007; Zander et al. 2009). *PDF1.2* is regulated by an amplification loop that involves recognition of the endogenous peptide elicitors AtPEP1-6 by the receptors AtPEPR1 and AtPEPR2 (Huffaker et al. 2006; Huffaker and Ryan 2007; Pearce et al. 2008; Yamaguchi et al. 2010). *AtPEPR1* and *AtPEP1-3* are induced after inoculation of *A. thaliana* with the necrotrophic ascomycete *Sclerotinia sclerotiorum*, which may be responsible for the dramatic induction of *PDF1.2* in response to pathogen infection (Stotz et al. unpublished). To determine the endogenous function of PDF1.1 and PDF1.2, knockout and knockdown lines were generated (De Coninck et al. 2010). However, no difference in pathogen susceptibility was observed between the knockout lines and wild-type plants, probably because of the functional redundancy of multiple *AtPDF* genes. Still, overexpression of *PDF1.1* resulted in reduced susceptibility to the necrotrophic fungus *Cercospora beticola* (De Coninck et al. 2010). More conclusive results were obtained in tobacco. Silencing of the tobacco defensin PR-13 resulted in increased susceptibility of *Nicotiana attenuata* to *Pseudomonas syringae* pv. *tomato* DC3000 under glasshouse conditions and increased susceptibility to opportunistic *Pseudomonas* spp. and mortality in the native habitat (Rayapuram et al. 2008). PR-13 is closely related to the defensin peptide NaD1 and more distantly related to thionins (Fig. 2). Based on these data, defensins are clearly important for plant defense, and their evolutionary diversification is the basis for various ecological functions.

Most defensins consist of a signal sequence that targets the peptides to the ER, followed by the mature peptide. Solanaceous floral defensins are an exception because they also contain an acidic C-terminal extension, which perhaps prevents inappropriate activation of mature cationic peptides (Stotz et al. 2009). This difference in proteolytic processing adds to the complexity of plant defensins. It is to be expected that additional information on the impact of defensins on membrane lipids and proteinaceous receptors will soon become available. To appreciate the function of plant defensins, this is the most urgent problem that needs to be addressed.

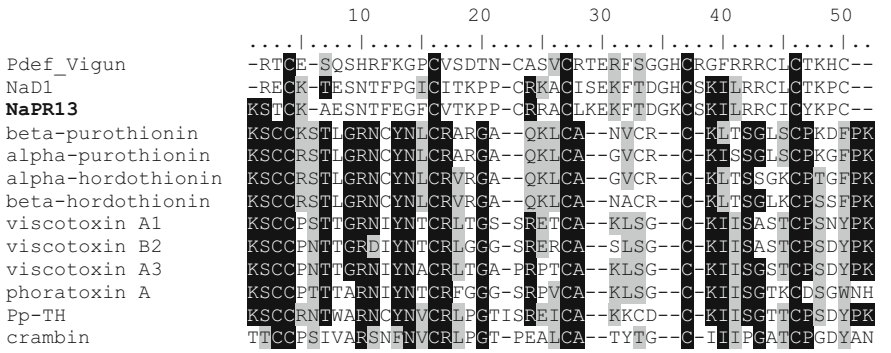

Fig. 2 *Alignment of defensin and thionin amino acid sequences*. The predicted mature peptide sequence of PR-13 from *Nicotiana attenuata* NaPR13 (GenBank AY456268) is highlighted in bold and compared to the related defensins NaD1 from *Nicotiana alata* (GenBank Q8GTM0) and Pdef_Vigun from *Vigna unguiculata* (GenBank ACN93800). Note that all eight Cys, two Gly, and one Glu residue are highly conserved and shared among these three peptides. Less closely related to NaPR13 are the thionins: β-purothionin from *Triticum urartu* (GenBank 218767043), α-purothionin from *Triticum aestivum* (GenBank 4007850), α- and β-hordothionins from *Hordeum vulgare* (GenBank 19110 and 225008, respectively), viscotoxins A1 (GenBank 190613619), B2 (GenBank 190613410) and A3 (GenBank 7245963) from *Viscum album*, phoratoxin A from California mistletoe (GenBank 135797), thionin from *Pyrularia pubera* (GenBank 135798), and crambin from *Crambe abyssinica* (GenBank 6226577). *Boxes* indicate identical (*black*) and similar amino acids (*gray*)

6 Lipid Transfer Proteins

Lipid transfer proteins (LTPs) were named based on their ability to facilitate transfer of phospholipids between a donor and an acceptor membrane in vitro (Bloj and Zilversmit 1977; Kader et al. 1984). As these LTPs have broad substrate specificity, they are also referred to as nonspecific LTPs (nsLTPs) (Kader 1996). Genome-wide analysis of *nsLTP* gene families resulted in detection of 52 and 49 members in rice and *A. thaliana*, respectively (Bureau et al. 1996).

Plant lipid transfer proteins are quite abundant and comprise of two families, LTP1 and LTP2. Members of the plant LTP1 family are about 10 kDa in size, consist of 90–95 amino acids, and are basic, with isoelectric points between 9 and 10. These LTPs have eight Cys residues conserved at similar positions in their primary structure, which form four disulfide bridges stabilizing the tertiary structure (Kader 1996). The LTP2 family members share the properties of the LTP1 family but are only about 7 kDa in size, possessing about 70 amino acids on average. LTPs contain a signal peptide at the amino terminal end, which is cleaved and targets the mature peptide to the cellular secretory pathway resulting in export to the apoplast.

The extracellular localization was confirmed for LTPs from a variety of plants (Sterk et al. 1991; Terras et al. 1992; Molina and Garcia-Olmedo 1993; Segura et al. 1993). Expression studies showed that LTP transcripts are abundant in epidermal

and peripheral cell layers (Sossountzov et al. 1991; Sterk et al. 1991; Fleming et al. 1992; Molina and Garcia-Olmedo 1993; Thoma et al. 1994). In broccoli, an LTP was found to be the main protein of the wax layer (Pyee et al. 1994).

Structural data were obtained for LTP1 proteins from wheat (Gincel et al. 1994; Charvolin et al. 1999), maize (Shin et al. 1995; Gomar et al. 1996), barley (Heinemann et al. 1996; Lerche and Poulsen 1998), and rice (Lee et al. 1998). LTP2 proteins from wheat and rice were also analyzed by solution NMR and X-ray diffraction (Samuel et al. 2002; Hoh et al. 2005). Most LTP proteins have a globular structure consisting of a bundle of four α-helices linked by flexible loops and contain a large central hydrophobic cavity. The size of this cavity differs between LTP1 and LTP2, the latter being more spacious, enabling binding of a planar sterol (Samuel et al. 2002). The cavity of LTP1 is variable and can adapt its volume to bind one or two mono- or diacylated lipids or other hydrophobic molecules. LTP1 proteins cannot load sterols or molecules with a rigid backbone (Douliez et al. 2000, 2001; Pato et al. 2002), suggesting that plasticity of the cavity and flexibility of the hydrophobic molecules limit the "nonspecificity" of the nsLTPs.

Antifungal and antibacterial activities were among the first functions shown for LTPs from barley, maize, spinach, and *A. thaliana* (Terras et al. 1992; Molina et al. 1993; Segura et al. 1993). Relative activities of different plant LTPs vary among pathogens, indicating some degree of specificity (Molina et al. 1993; Sun et al. 2008). Also, synergistic activity of an LTP with a thionin against the bacterial pathogen *Clavibacter michiganensis* ssp. *sepedonicus* was observed in vitro, while only additive effects were observed for a fungal pathogen (Molina et al. 1993; Garcia-Olmedo et al. 1995). Overexpression of a barley LTP2 in tobacco as well as in *A. thaliana* plants reduced disease symptoms after infection of leaves with the bacterial pathogen *P. syringae* (Molina and Garcia-Olmedo 1997), and overexpression of an onion LTP in wheat plants decreased growth of the fungal pathogen *Blumeria graminis* f.sp. *tritici* (Roy-Barman et al. 2006).

The mechanism of the toxicity observed for plant LTPs toward fungi and bacteria still remains to be elucidated. It is likely that lipid-binding properties and antimicrobial activity are independent of each other. Unlike related cereal LTPs, Ace-AMP1, an LTP from onion with antifungal activity, was not able to bind diacylphospholipids (Cammue et al. 1995; Tassin et al. 1998). The toxicity and lipid-binding activity of several wheat LTPs were not correlated (Sun et al. 2008). Mutational analysis of the rice nsLTP1 gene indicated that lipid binding and antimicrobial functions are unrelated (Ge et al. 2003). These data support the view that, with respect to LTP structure, lipid binding and antimicrobial activities are spatially separated.

It was recently suggested that a new subfamily of plant LTPs, *signaling LTPs*, should be formed, consisting of LTPs related to two characterized LTPs with signaling function (Pii et al. 2010). This group would consist of the *Medicago truncatula* LTP MtN5, which is expressed in response to a root pathogenic fungus and in root nodules colonized by the symbiotic bacterium *Sinorhizobium meliloti* (Pii et al. 2009, 2010), together with a closely related protein from *A. thaliana*, defective in induced resistance 1 (DIR1). DIR1 is required for inducing SAR in

distal tissues after initial infection with a necrotizing pathogen (Maldonado et al. 2002). There is also evidence that DIR1 itself might be the mobile signal, as antibodies directed against the tomato DIR1 homolog, Le-DIR1, recognized this protein in the phloem sap of tomato plants (Mitton et al. 2009). Another LTP, azelaic acid induced 1 (AZI1), was recently shown to be required for production of the signal, which induces priming of defense responses for SAR (Jung et al. 2009a). Several genetic studies strongly indicated that a lipid or lipid-derived compound is required for the establishment of SAR in *A. thaliana* (Nandi et al. 2004; Chaturvedi et al. 2008; Shah 2009). However, the identity of the SAR signal, the molecular properties of LTPs, which render them active, and the identity of the bound lipid substrate (if any) in vivo remain to be determined. Interestingly, wheat LTP1 was shown to bind to a plasma membrane-located receptor for elicitins (Buhot et al. 2001). Elicitins are small proteins secreted by oomycete pathogens capable of binding phospholipids and fatty acids in competition with sterols (Ponchet et al. 1999; Osman et al. 2001). Elicitins are able to trigger plant defense responses reminiscent of SAR (Keller et al. 1996). It is tempting to speculate that some LTPs could mediate pathogen recognition and thereby fulfill yet an additional role in plant defense processes.

It was originally suggested that LTPs facilitate intracellular transfer of lipids. However, most LTPs have been extracellularly localized. The following diverse functional data also indicate that LTPs play important roles in the apoplast. This includes direct antimicrobial activity, and for some LTPs, their role in plant defense signaling was shown. DIR1, for instance, appears to be a signaling molecule in itself. Other LTPs may act via binding to plant elicitin receptors. Plant LTPs were assigned additional divergent functions, including a role in beta-oxidation (Tsuboi et al. 1992), cutin synthesis (Pyee et al. 1994), pollen adherence (Park et al. 2000), and somatic embryogenesis (Sterk et al. 1991). In the future, we will have to address the exact mechanism by which LTPs mediate signaling, identify the in vivo substrates of LTPs, and determine the mode of antimicrobial action.

7 Hevein-Like AMPs, Knottins, and Cyclotides

These three types of AMPs are treated together because of their structural similarities. All of them form a triple-stranded β-sheet that is stabilized by at least three disulfide bridges.

7.1 Hevein-Like AMPs

Hevein is the most abundant protein in latex of rubber trees. The mature peptide consists of 43 amino acids with four disulfide bridges and contains a chitin-binding domain (Lee et al. 1991). Homologous chitin-binding domains are found in multidomain proteins, like chitinase (Iseli et al. 1993), and in hevein-like AMPs (Broekaert et al. 1992).

Two classes of hevein-like AMPs exist. The first class of peptides is similar to hevein and contains eight Cys residues. Examples are PnAMP1 and PnAMP2, two peptides that are produced in seeds of *Pharbitis nil* (Koo et al. 1998). PnAMP1 and PnAMP2 are 41 and 40 amino acids long, respectively. Their antimicrobial activity is not dependent on microbial chitin production as oomycete pathogens were also inhibited by these peptides. Fluorescently labeled PnAMP1 rapidly penetrates hyphae, leading to membrane disintegration and disruption of hyphal tips.

The second class of hevein-like AMPs is shorter and contains only six Cys residues. Examples are AcAMP1 and AcAMP2, two peptides from seeds of *Amaranthus caudatus*, consisting of 29 and 30 amino acids, respectively (Broekaert et al. 1992). Both peptides were found to be potent inhibitors of fungal growth when six fungal pathogens and one saprophyte were tested. The antibacterial activity of these peptides is much lower. As in the case of thionins, divalent cations inhibited the antimicrobial activity of these peptides. Intercellular wash fluids from leaves of sugar beet contain another peptide, designated as IWF4 (Nielsen et al. 1997). The chitin-binding activity of this peptide was stronger than that of class I and class IV chitinases (Hamel et al. 1997). IWF4 is 30 amino acids long and inhibits growth of the foliar pathogen *Cercospora beticola* (Nielsen et al. 1997). Its mRNA is constitutively expressed in leaves and flowers but not induced after inoculation of sugar beet leaves with *C. beticola*.

Hevein-like AMPs are produced as preproproteins. They contain a signal peptide that targets them for secretion and a C-terminal extension (De Bolle et al. 1996; Nielsen et al. 1997). Overexpression of *pnAMP-h2* in transgenic tobacco elevated resistance to *Phytophthora parasitica* (Koo et al. 2002). PnAMPs therefore possibly protect seeds of *P. nil* against pathogens.

7.2 Knottins

Knottins are structurally different from hevein-like AMPs in that all three disulfide bridges take part in the reinforcement of the sheet structure and a helix found in hevein-like AMPs is absent (Chagolla-Lopez et al. 1994). The solution structure of a knottin from seeds of *Phytolacca americana* was solved (Gao et al. 2001b).

The 38 amino acids long knottin from *P. americana* has broad-spectrum antifungal activity (Gao et al. 2001). Earlier, two knottins were isolated from seeds of *Mirabilis jalapa* (Cammue et al. 1992). The 37- and 38-amino-acid long *MjAMP1* and *MjAMP2*, respectively, associate to form dimers and effectively inhibit a wide range of fungal pathogens and, to a lesser degree, Gram-positive bacteria. Seeds of *Amaranthus hypochondriacus* contain a knottin that inhibits α-amylase activity (Chagolla-Lopez et al. 1994), demonstrating that these AMPs have multiple biological activities.

An unusual AMP with two knottin motifs was isolated from the cycad *Cycas revoluta* (Yokoyama et al. 2009). The recombinant peptide is capable of binding to chitin and has antifungal and antibacterial activity. Mutant forms of recombinant

CyAMP1 were generated by site-direct mutagenesis of amino acids that are conserved with knottins and hevein-like AMPs. These mutant peptides were no longer able to bind to chitin and lost their antifungal activity. However, the antibacterial activity was maintained, suggesting a different mode of action against prokaryotes.

Knottins are encoded as preproteins and not proteolytically processed like hevein-like AMPs (De Bolle et al. 1995). Expression of *MjAMP2* in transgenic tobacco showed that the peptide is secreted and functional because it inhibits in vitro growth of *Botrytis cinerea* (De Bolle et al. 1996). However, MjAMP2 did not protect transgenic tobacco against infection from *B. cinerea* or *A. longipes*. Further research is therefore needed to determine the role of knottins in protection of plants against pathogens.

7.3 Cyclotides

Kalata B1 was the first identified member of a new family of cyclic AMPs (Saether et al. 1995). These peptides are covalently joined by a peptide bond between the N- and C-terminal amino acids. Cyclotides consist of 27 to 37 amino acids with an embedded cystine knot (Padovan et al. 2010). Unlike other AMPs, cyclotides are not cationic peptides, but they contain a solvent-exposed hydrophobic patch. Two major subfamilies of cyclotides exist. The Möbius subfamily contains a twist in the peptide backbone, owing to the presence of a Pro residue in loop 5 that is preceded by a *cis*-peptide bond located between Cys residues five and six (Craik et al. 2006). The bracelet subfamily does not contain a Pro residue in loop 5, but it contains a short helical segment in loop 3 that lies between the third and fourth Cys residues. Aside from the Cys residues, the Glu residue in loop 1 between the first and second Cys residues is most highly conserved throughout the cyclotide family (Goransson et al. 2009). This Glu residue forms a hydrogen bond network that stabilizes the cyclotide framework for efficient aggregation in membranes (Goransson et al. 2009).

Cyclotides are present in Cucurbitaceae and Apocynaceae, in every analyzed species of the Violaceae, and in a few species of the coffee family Rubiaceae (Gruber et al. 2008). Linear cyclotide-like sequences are present in monocots (Poaceae), suggesting that these peptides evolved prior to the divergence of monocots and dicots. Presence of a single intron in Rubiaceae genes but absence thereof in Violaceae genes suggests that cyclization evolved independently after the divergence of Asterids and Rosids. Within a single species, *Viola hederacea*, 66 different cyclotides were identified (Trabi and Craik 2004). Cyclotide diversity within a single plant family is estimated to be in the order of 10,000 (Craik et al. 2006; Gruber et al. 2008).

Cyclotides are generated from linear precursor proteins that contain one, two, or three cyclotide domains (Dutton et al. 2004; Gillon et al. 2008). Precursor proteins consist of an ER signal sequence, an N-terminal pro-domain, an N-terminal repeat,

the cyclotide domain, and a C-terminal tail. Oak1 is the precursor protein of kalata B1 from the African plant *Oldenlandia affinis*. Foliar extracts from *O. affinis* contain an 11-kDa protein without the ER signal sequence, a 6-kDa processing intermediate without the N-terminal pro-domain, and mature 4-kDa kalata B1. The cyclotide processing sites are highly conserved (Gillon et al. 2008). A protein-disulfide isomerase was shown to be essential for correct oxidative folding of kalata B1 and production of biologically active cyclotides (Gruber et al. 2007). An asparaginyl endopeptidase was shown to catalyze peptide bond formation between the N-terminal Gly and C-terminal Asn residues in kalata B1 (Saska et al. 2007). Violacin A from *Viola odorata* is a naturally occurring linear cyclotide that contains a mutation introducing a stop codon and preventing translation of the key Asn residue required for cyclization (Ireland et al. 2006).

Cyclotides have multiple biological activities. Kalata B1 accelerates contractions during childbirth. Cyclotides, including kalata B1, have antimicrobial activity that is salt-sensitive (Tam et al. 1999). Kalata B1 also has insecticidal activity, disrupting epithelial cells in the midgut of lepidopteran larvae (Jennings et al. 2001; Gruber et al. 2007; Barbeta et al. 2008). Various cyclotides possess cytotoxic, hemolytic, and anti-HIV activities (Chen et al. 2006; Ireland et al. 2006, 2008). This diversity of biological activities together with the marked resistance against chemical, thermal, and enzymatic degradation conferred by the closed cysteine knot structure has sparked interest in using cyclotides as scaffolds for protein engineering and drug design (Craik et al. 2006).

The biological activity of cyclotides depends on membrane interactions. The size of the surface-exposed hydrophobic patch determines cytotoxicity, hemolytic, and anti-HIV activities of cyclotides (Chen et al. 2006; Ireland et al. 2008). Bracelet cyclotides are generally more hydrophobic than Möbius cyclotides with hydrophobic residues on both faces of the molecule (Ireland et al. 2008). Insertion into lipid bilayers differs between members of both subfamilies (Wang et al. 2009). Whereas Möbius cyclotides interact with the membrane via loops 2 and 6, bracelet cyclotides interact via loops 2 and 3. Strategically located charged residues modulate hydrophobic interactions between cyclotides and target membranes and influence the therapeutic index of these peptides (Ireland et al. 2008). Evidently, only a portion of the cyclotide molecule binds to the membrane. These peptides are not buried deeply into the membrane. In the case of bracelet cyclotides, hydrophobicity has been linked to their membrane-disrupting ability (Svangard et al. 2007).

Production of cyclic kalata B1 has been reduced in transgenic tobacco via silencing of asparaginyl endopeptidase (Saska et al. 2007). This would make it possible to test effects of altered cyclotide expression on biotic interactions, but such studies have not yet been performed. It should be noted that another family of circular plant proteins exists that is distantly related to the cyclotides and has trypsin inhibitor activity (Felizmenio-Quimio et al. 2001).

8 Snakins

Yet another class of AMPs was found in solanaceous plants. These AMPs isolated from potato (*Solanum tuberosum*) and closely related *Solanum* species were termed snakins based on their sequence similarity to hemotoxic desintegrin-like snake venoms (Segura et al. 1999). The amino acid sequences of snakins are also related to gibberellin-stimulated transcripts GAST and GASA from a variety of plant species, including *A. thaliana*. The mature snakin-1 (StSN1) and snakin-2 (StSN2) peptides are cationic and contain 63 and 66 amino acids, respectively, with 12 Cys residues (Segura et al. 1999; Berrocal-Lobo et al. 2002). Whereas StSN1 is preceded by a signal sequence, StSN2 is derived from a preproprotein that contains an additional N-terminal acidic peptide and requires proteolytic processing.

Snakins have antifungal and antibacterial activities (Segura et al. 1999; Berrocal-Lobo et al. 2002). StSN1 and StSN2 cause rapid aggregation of Gram-positive and Gram-negative bacteria. Interestingly, StSN1 caused aggregation of *Ralstonia solanacearum* at concentrations that were not toxic to these Gram-negative bacteria (Segura et al. 1999). Although snakins do not lyse artificial lipid membranes, they can promote aggregation of liposomes (Caaveiro et al. 1997). This mode of action is clearly different from other AMPs and responsible for the synergistic activities of StSN1 and potato defensin PTH1 against bacterial and fungal pathogens.

Another AMP that aggregates bacteria prior to killing is hydramacin-1 from the freshwater polyp Hydra (Jung et al. 2009). The structure of hydramacin-1 has been solved and shown to consist of two hydrophobic hemispheres sandwiched by a belt of positive charges. The cationic StSN1 peptide consists of a central hydrophobic stretch flanked by highly polar N-terminal and C-terminal domains (Segura et al. 1999). Further comparisons will have to await the structure of snakins to be solved.

Developmental expression of *StSN1* and *StSN2* mRNAs differs but it overlaps (Segura et al. 1999; Berrocal-Lobo et al. 2002). *StSN1* expression is particularly high in axillary and floral buds, in the stem and in petals, but tubers and carpels also express this gene. *StSN2* is strongly expressed in tubers, petals, carpels, stamen, and leaves, but stems and floral buds also express this gene. Wounding and treatment with the phytohormone abscisic acid induce *StSN2* expression in leaves (Berrocal-Lobo et al. 2002). *StSN2* expression is also induced after infection of tubers with *B. cinerea* but suppressed after inoculation with the bacteria *R. solanacearum* and *Erwinia chrysanthemi*. The expression patterns of *StSN1* and *StSN2* are therefore compatible with roles in constitutive and induced resistance, respectively.

Overexpression of *ScSN1* from *Solanum chacoense* in transgenic potato increased resistance to the fungal pathogen *Rhizoctonia solani* and the Gram-negative bacterium *Erwinia carotovora* (Almasia et al. 2008; Kovalskaya and Hammond 2009). Conversely, silencing of *snakin-2* in *Nicotiana benthamiana* reportedly resulted in an increase in susceptibility to *C. michiganensis* subsp. *michiganensis* (Balaji et al. 2010). These results point toward an important role of snakins in defense of solanaceous plants against pathogens.

9 Conclusions

Plant AMPs are functionally and structurally diverse. Structural features common to plant AMPs are disulfide bridges and secondary structures like α-helices and β-sheets. These structural features generate compact molecules that are resistant to chemical and physical insults and can survive hostile environments like the plant cell wall and the vacuole. Another general feature is that plant AMPs interact with lipids, phospholipids in the case of thionins, sphingolipids in the case of defensins, and various lipids in the case of LTPs. Interactions between other plant AMPs and lipids appear to be less specific. Moreover, other molecular functions, like chitin binding, in the case of hevein-like AMPs and knottins, and interactions with other proteins as observed for defensins and LTPs are important.

Certain plant AMPs, like thionins and cyclotides, are inherently toxic, while others, including defensin and LTPs, are not. The latter category of AMPs has been shown to fulfill important functions in plant signaling. The exact mechanism by which LTPs and defensins modulate plant signaling will be of interest not only to plant scientists.

The vast diversity of Cys-rich AMPs in the plant kingdom suggests that these peptides fulfill important ecological functions. The molecular evolution of the different classes of plant AMPs is incompletely understood. Research on this topic is desperately needed to better understand interactions of plants with symbiotic and pathogenic microbes and with herbivorous and beneficial insects.

In this review, we provide clear evidence that AMPs are an intricate part of the plant immune system, not merely executers of a defense program designed to kill enemies. AMPs therefore fill similar niches in the immune systems of plants and animals, although the molecules involved and the processes are different. As animal defensins are known to link innate and adaptive immune systems (Yang et al. 1999; Biragyn et al. 2002; Funderburg et al. 2007), plant LTPs play essential roles in SAR.

As their animal counterparts, plant AMPs can be exploited for pharmaceutical purposes. In the presence of multiple-drug-resistant bacteria, peptide antibiotics are clearly needed, and plant AMPs may add their share to the antimicrobial cocktail that may be used to fend of infectious diseases.

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Antimicrobial Peptides Produced by Microorganisms

Aline Dias Paiva and Eefjan Breukink

Abstract Antimicrobial peptides comprise a diverse group of ribosomally synthesized molecules that include plant thionins and defensins, insect defensins and cecropins, amphibian magainins and temporins, defensins and cathelicidins from higher vertebrates, as well as fungal defensins, cyanobactins, and bacteriocins. The latter are produced by species of bacteria and certain strains of the Archaea domain, being active in small concentrations, and exhibiting bactericidal or bacteriostatic activity against both human and veterinary pathogens. Nisin is the most well-known bacteriocin and the only one approved for use as food preservative; its mechanism of action is based on the interaction with lipid II, a key molecule in the bacterial cell wall synthesis. Although bacteriocins are traditionally used in the food industry, they show several desired features to biotechnological applications, and they could be used in combined therapy or as substitutes of conventional antibiotics in the control of bacterial infections. Although less documented when compared to antibiotics, the issue of resistance among previously sensitive bacterial strains has to be considered for antimicrobial peptides produced by microorganisms. In this chapter, we discuss some relevant concerns regarding the antimicrobial peptides produced by microorganisms, giving special emphasis to the bacteriocins.

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1 Introduction

Small biological molecules (<10 kDa), including enzymatically synthesized compounds (NRPs) and ribosomally synthesized peptides (AMPs), play an important role in the innate immune response to microbial infection and thus in ensuring first-line defenses in many species, from plant to animal kingdom (Thomson et al. 2004). AMPs are a diverse group of molecules and include plant thionins and defensins, insect defensins and cecropins, amphibian magainins and temporins, defensins and cathelicidins from higher vertebrates, as well as fungal defensins and bacteriocins (Hancock and Sahl 2006).

Antimicrobial peptides produced by microorganisms have been a popular topic of research, and many ribosomally and nonribosomally synthesized peptides have been reported over the last years (Pavlova and Severinov 2006; Sang and Blecha 2008). Many antimicrobial peptides are produced by fungi, including a large variety of nonribosomally synthesized peptides and defensin-like peptides, which are cysteine-rich AMPs consisting of an α-helix and β-sheet structures (Zhu 2008).

The peptides ribosomally synthesized by species of bacteria and certain strains of the Archaea domain are named bacteriocins. Bacteriocins are active even in small concentrations; they are able to exhibit bactericidal or bacteriostatic activity (Drosinos et al. 2006). Bacteriocins differ in size, composition, mechanisms of action and range of antimicrobial specificities, being active against both human and veterinary pathogens, which makes the bacteriocins potentially useful in the food industry and medical treatment (Cleveland et al. 2001).

The use, and sometimes misuse, of antimicrobials in both human and veterinary medicine during the years has resulted in the emergence of multidrug-resistant microbial strains that no longer respond to antimicrobial therapy. So far, resistance has developed to almost all antimicrobial drugs and among clinical isolates of different bacterial species (Rydlo et al. 2006). This process led the World Health Organization (WHO) to announce, some years ago, antimicrobial drug resistance as a main public health concern and a global crisis (WHO 1995), and in order to prevent outbreaks of infectious diseases and to reduce the selection pressure by antibiotics, a renewed focus on antimicrobial agents research is highly desired, including the search for new drugs with alternative cellular targets.

This chapter shows an update on the antimicrobial peptides produced by microorganisms, with focus on ribosomally synthesized peptides, including the differences among them, their production and mode of action, the target strain-dependent resistance mechanisms observed and potential applications.

2 Antimicrobial Peptides Produced by Microorganisms

Microorganisms produce a variety of antimicrobial substances, such as antibiotics and by-products of metabolism (i.e. organic acids, acetoin, hydrogen peroxide, carbon dioxide), exotoxins and ribosomally synthesized peptides, named

defensin-like peptides in fungi and bacteriocins when produced by bacteria (Riley and Wertz 2002; Zhu 2008). Most of the antimicrobial peptides isolated from microorganisms share common biophysical properties that appear to be important to antimicrobial activity, such as cationicity, amphipathicity, and hydrophobicity (Yount et al. 2006; Sang and Blecha 2008).

The net positive charge is essential for the initial electrostatic attraction to the anionic components at cell envelopes and phospholipid membranes of fungi and bacteria. On fungal surfaces, the negative charge is often created by the presence of phosphomannans or related constituents (Salzman et al. 2004) and the negatively charged components of bacterial cell surfaces include lipopolysaccharide (LPS) of -Gram-negative bacteria and teichoic and teichuronic acids of Gram-positive bacteria. Moreover, bacterial membranes are enriched in acid phospholipids, such as phosphatidylglycerol (PG), phosphatidylserine (PS), and cardiolipin (CL), which confer a net negative charge to the membrane, and especially for Gram-positive bacteria, these lipids can form more than 90 % of the membrane. One additional parameter that may contribute to an electrostatic attraction between cationic peptides and their targets is that the membrane potential ($\Delta\psi$) across the bacterial membrane is around 50 % greater in prokaryotes than in most eucariotic cells (Hancock and Rozek 2002).

The amphipathic structure, with clusters of hydrophobic and hydrophilic residues within the tertiary structure of the peptide, correlates with antimicrobial efficacy and peptide toxicity, and most antimicrobial peptides are inherently amphipathic or become amphipathic in anisotropic environments (Yount et al. 2006). The proportion of hydrophobic residues in antimicrobial peptides is around 50 %, and it is important for antimicrobial activity as it governs the extent to which the peptide may partition into lipid bilayers. Although hydrophobicity and amphipathicity are required for effective permeabilization of the bacterial membrane, optimal balance between these two characteristics is essential to peptide activity, since highly amphipathic or hydrophobic molecules tend also to disrupt mammalian cells (Zelezetsky et al. 2005).

2.1 Defensin-Like Peptides

Defensins constitute a major class of cationic antimicrobial peptides in mammals and vertebrates. Fungal genomes encode abundant cysteine-rich AMPs consisting of α-helix-β-sheet structures, collectively called defensin-like peptides. Bioinformatics analysis of fungal genomes revealed six families of fungal defensin-like peptides, among which three families show high degree of structural and sequence similarity to defensin molecules from plants, insects, and invertebrates, suggesting that defensins from fungi and animals could share a common genetic origin. Indeed, cysteine-rich defensin-like peptides have been suggested to be the most diverse group of AMPs existing in all cellular organisms (Zhu 2008).

The first fungal defensin-like peptide, named plectasin, was identified in 2005 by Mygind and coworkers, and it was isolated from the saprophytic ascomycete

Fig. 1 (**a**) Alignment of plectasin with related defensins (tick, *Ornithodoros moubata* defensin A, gi:1362787; mussel, *Mytilus galloprovincialis*, MGD-1, gi:5533299; dragonfly, *Aeschna cyanea*, defensin, gi:259195; spider, defensin, gi:56579998; scorpion, *Leiurus quinquestriatus*, defensin, gi:399696); (**b**) Representative structure of plectasin (adapted from Mygind et al. 2005)

Pseudoplectania nigrella. Plectasin is a 40-amino acid residue fungal defensin and shares structural similarities with defensins isolated from spiders, scorpions, dragonflies, and mussels (Mygind et al. 2005) (Fig. 1).

Defensin-like peptides are synthesized as inactive precursor peptides, with 60–100 amino acid residues, and the active forms, which are generally 30–40 aa in length, arise from regioselective post-translational proteolysis. Defensins have no other modifications to the peptide scaffold, N- or C-terminus (Ganz 2003). The nascent fungal defensin plectasin is a 95 aa peptide that contains a N-terminal signal sequence (residues 1–23), a pro-piece (residues 24–55) and a C-terminal domain (residues 56–95); proteolytic cleavage affords the 40 aa mature toxin, which contains one α-helix and two β-strands stabilized by three disulfide bonds linking Cys4-Cys30, Cys15-Cys-37, and Cys19-Cys39 (Nolan and Walsh 2009).

Defensins are generally active against Gram-negative and Gram-positive bacteria and also against some viruses, fungi, and protozoa. Plectasin has shown potent antimicrobial effect, in vitro and in vivo, against various Gram-positive bacteria (Mygind et al. 2005), being active against antibiotic-resistant strains of *Streptococcus pneumoniae*, with an efficacy comparable to vancomycin and penicillin in mouse model studies of peritonitis and pneumonia (Brinch et al. 2009). The antibacterial spectrum of a plectasin derivative, named NZ2114, also includes the methicillin-resistant *Staphylococcus aureus* (MRSA) (Andes et al. 2009).

Many defensin mechanisms of action result from association with and insertion into cell membranes, which disrupts the membrane integrity and induces cell death by efflux of metabolites and influx of ions (Tanabe et al. 2004). The specific disruption of the bacterial membrane by defensins is believed to occur by

electrostatic attractions between the peptide and the negatively charged membrane, but alternative mechanisms, including targeting of intracellular compounds (Wu et al. 1999; Hancock and Rozek 2002) and interaction with glycosylated proteins (Wang et al. 2004) or carbohydrates (Lehrer et al. 2009), have been proposed.

Recently, the mode of action of plectasin was elucidated, and after a range of genetic and biochemical approaches, cell wall biosynthesis was identified as the pathway targeted by plectasin. This fungal defensin-like peptide acts by binding to the essential bacterial cell wall precursor lipid II, and an equimolar stoichiometric complex is formed (Schneider et al. 2010). Plectasin was the first defensin to be identified as having a target-specific activity, but also the α-defensin human neutrophil peptide-1 (HNP1) binds to lipid II (Leeuw et al. 2010), suggesting that defensins can have specific targets in bacterial membranes and the inhibition of cell wall synthesis could be a common antibacterial mechanism among them.

Defensins in general exhibit a number of advantageous characteristics, such as broad-spectrum activity, rapid action, and anti-inflammatory properties, but there are some obstacles to the large-scale use, including toxicity, pH and salt-dependent activity, poor tissue penetration, and high production costs (Nolan and Walsh 2009). In this sense, some features of plectasin, such as the potent activity in vitro, serum stability and half-life in vivo, low potential toxicity and the fact that plectasin is produced in a fungal expression system (currently employed for industrial-scale productions), give a high potential to the clinical application of this fungal defensin-like peptide (Schneider et al. 2010).

2.2 Cyanobactins

Cyanobacteria produce low molecular weight bioactive peptides, cyclic or linear, with high level of structural variation (Welker and von Döhren 2006; Sivonen and Börner 2008). These peptides are produced by both nonribosomally and ribosomally biosynthetic pathways in cyanobacteria (Burja et al. 2001). The first nonribosomal pathways for cyanobacterial peptides were described in 2000 (Tillett et al. 2000), whereas the first ribosomal pathway was shown in 2005 (Schmidt et al. 2005).

Cyanobactin is one of the largest classes of peptides produced by cyanobacteria, and more than 100 cyanobactins have been identified from symbiotic associations between cyanobacteria and ascidians or from free-living cyanobacteria (Schmidt and Donia 2009). Cyanobacterial strains produce ribosomal cyanobactins which contain heterocyclized amino acids (6–11 aa) and also cyclic peptides which consist solely of unmodified proteinogenic amino acids (7–20 aa), occasionally with prenyl attachments (Leikoski et al. 2010). Oxazoles (A, in Fig. 2) and thiazoles (B, in Fig. 2) are common while oxazolines (C, in Fig. 2) or thiazolines (D, in Fig. 2) occur with a lower frequency. A feature uniting cyanobactins without heterocyclized amino acids in addition to the occasional prenyl attachment is the conserved presence of a proline residue (Sivonen et al. 2010) (Fig. 2).

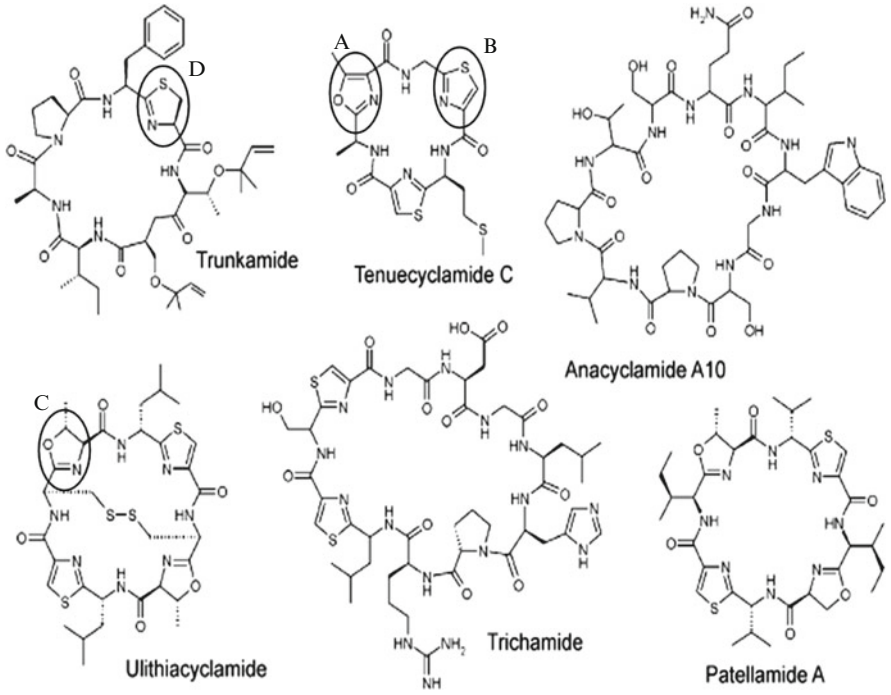

Fig. 2 Chemical structure of some cyanobactins: trunkamide from *L. patella*; tenuecyclamide C, *N. spongiaeforme*; anacyclamide A10, *Anabaena*; trichamide, *T. erythraeum*; ulithiacyclamide and tatellamide A, *Prochloron*. The highlighted structures are A: oxazole; B: thiazole; C: oxazoline; D: thiazoline (adapted from Sivonen et al. 2010)

The cyanobactin biosynthetic genes encoded in gene clusters are approximately 10 kb in size and contain between 7 and 12 genes. The gene order is not strictly conserved, but all of cyanobactin gene clusters contain genes that encode for two proteases, which work in tandem, a short precursor peptide as well as proteins involved in the maturation of the cyanobactins (Donia et al. 2006).

The biosynthetic genes for cyanobactin production have been described in related cyanobacteria *Prochloron*, *Trichodesmium*, *Microcystis*, *Nostoc*, *Lyngbya*, and *Anabaena* (Leikoski et al. 2010). Moreover, one of the protease genes responsible for cyanobactin precursor peptide cleavage was shown to be common among planktonic cyanobacteria, including filamentous heterocystous (*Anabaena*, *Aphanizomenon*, *Nodularia*), filamentous (*Planktothrix*), and colony-forming (*Microcystis* and *Snowella*) cyanobacteria (Leikoski et al. 2009).

Cyanobactins are produced through the proteolytic cleavage and head-to-tail (N–C) cyclization of precursor peptides coupled with modification of specific amino acids (Oman and van der Donk 2010). The cyclic structure is formed via an amide linkage of the α-carbonyl of C-terminal amino acid and α-amino group of the N-terminal amino acid yielding a homodetic cyclic peptide. In cyanobactin biosynthesis, the precursor peptide directly encodes one or more cyanobactins

flanked by the putative recognition sequences at which the precursor peptide is cleaved by two proteases (Schmidt et al. 2005; Lee et al. 2009).

Some cyanobacterial bioactive compound classes are nonribosomally synthesized by peptide synthetases (NRPS) or combined NRPS and polyketide synthases (Sivonen and Börner 2008). The gene clusters responsible for nonribosomal peptide production are bigger than ribosomal peptide synthetase gene clusters. In NRPS, chemical structure of the peptide can be varied by utilization of more than 200 nonproteinogenic amino acids (Sivonen et al. 2010). In NRPS, the enzymes seem to have relaxed substrate specificity, allowing simultaneous production of different variants in the same strain of a cyanobacterium (Welker and von Döhren 2006).

The cyanobactins and cyclic peptides with analogous structures have various reported bioactivities, which is derived from the different structures. Compounds isolated from cyanobacteria have demonstrated antibiotic (Ishida et al. 1997), antiviral (Bokesch et al. 2003) and anticancer (Salvatella et al. 2003) effects; some compounds have activity against multidrug-resistant microorganisms (Ogino et al. 1996) as well as activity against tropical parasites (Linington et al. 2007).

In addition to cyanobactins, another cyanobacterial peptide class named microviridins was recently shown to be synthesized by *M. aeruginosa* and *Planktothrix agardhii*, but their biosynthetic machinery differs from that of cyanobactins. Microviridins are ribosomally synthesized from precursor peptides that are converted into tricyclic depsipeptides through the action of ATP grasp ligases and a transporter peptidase. Similar gene clusters were identified in *Anabaena variabilis*, *Nostoc punctiforme*, and *Nodularia spumigena* as well as in genomes of other bacteria (Philmus et al. 2008).

2.3 Bacteriocins

Bacteriocins are ribosomally synthesized peptides or proteins produced by many Gram-positive and Gram-negative bacteria, and also produced by some Archaea members. According to Klaenhammer, 99 % of all bacteria may make at least one bacteriocin (Klaenhammer 1988). The bacteriocins differ in terms of size, microbial target, mode of action, release, and immunity mechanisms. Several uncharacterized substances produced by bacteria and with bacteriocin-like activity have been also identified and are referred to as bacteriocin-like inhibitory substances (BLIS) (Tagg and Ragland 1991).

Bacteriocins serve to effectively deal with other microorganisms in their environments, conferring ecological advantages in complex bacterial communities (Balakrishnan et al. 2002). Alternatively, they are used to influence the dynamics of bacterial populations, to inhibit the invasion of other strains or to cause the death of neighboring cells, ensuring the survival and perpetuation of the bacteriocin-producing cells (Burkard et al. 2007). Additional roles have been proposed for Gram-positive

bacteriocins, in which they may mediate quorum sensing (Gobbetti et al. 2007) and act as communication signals in bacterial consortia, such as biofilms (Gillor 2007).

The activity spectrum of bacteriocins can be narrow and confined to inhibition of closely related species, or it can be relatively broad, inhibiting the growth of a much wider range of microorganisms, including food-borne pathogens and spoilage microorganisms, such as *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, and *Clostridium tyrobutyricum* (Cotter et al. 2005; Gálvez et al. 2008). In general, the main mechanisms of action for bacteriocins are the pore formation in the target cell membrane, inhibition of cell wall synthesis, and inhibition of enzyme activities (RNAse or DNAse) (Cleveland et al. 2001).

Bacteriocins produced by Gram-positive bacteria differ from those synthesized by Gram-negative bacteria in ecological and evolutionary aspects: for Gram-positive bacteria, the biosynthesis of bacteriocins is self-regulated, and it is not a lethal event, the spectrum of antimicrobial activity is broader, the release of the peptide is controlled by specific regulatory mechanisms, the clusters of genes are generally in the chromosome and include genes encoding structural proteins, and proteins responsible for post-translational modifications, regulation, immunity and transport through the membrane; for Gram-negative bacteria, the production of bacteriocins is a lethal event, the release of the peptide is controlled by common regulatory mechanisms, such as the SOS regulon, and they have genes encoding proteins responsible for cell lysis (Riley and Wertz 2002).

In the following topics, some details about the bacteriocins produced by Gram-negative and Gram-positive bacteria will be discussed, including the nomenclature, classification, biosynthesis, spectrum of activity, and main mechanisms of action.

2.3.1 Bacteriocins Produced by Gram-Negative Bacteria

Surveys of environmental isolates of *Salmonella enterica*, *Hafnia alvei*, *Citrobacter freundii*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* have revealed levels of bacteriocin production ranging from 3 to 26 %. Higher levels of bacteriocin production have been found in other Gram-negative bacteria, such as *E. coli* populations, in which the frequency of bacteriocin production can vary from 10 to 80 % depending on the environment which they were isolated (Gordon et al. 2007), and *Pseudomonas aeruginosa*, in which >90 % of both environmental and clinical isolates produce bacteriocins (Michel-Briand and Baysse 2002).

Proteins or small peptides produced by *Escherichia coli* strains and some related species of *Enterobacteriaceae* are collectively called colicins. These bacteriocins are not post-translationally modified; they have a narrow spectrum of activity and usually require a specific receptor at sensitive cells. Since their discovery, the colicins of *E. coli* have been the most extensively studied Gram-negative bacteriocins, and they serve as a model system for investigating the mechanisms of bacteriocin structure/function, genetic organization, ecology, evolution of bacteriocins and the relationships between the producing and sensitive strains (Hert et al. 2005).

The first bacteriocin discovered was colicin V, produced by *E. coli* and originally described as "principle V" (nowadays, it is classified as microcin V) (Gratia 1925). Classification and nomenclature of colicins are closely related to their principal mode of interaction with sensitive bacterial cells. According to the original scheme, colicins are classified and designated on the basis of their receptor specificity (or of the absence of cross-resistance of the receptor mutants) by capital letters. If more types of colicins are bound to the same receptor, they are further distinguished on the basis of absence of cross-immunity of the producer strains and marked by means of indices added to the type letter (usually by numbers, sometimes by letters). The degree of strain sensitivity to colicin is a function of the average number of receptors per cell, that is, a quantitative character (Smarda and Smajs 1998).

The gene encoding the colicin activity protein forms an operon with the lysis gene, which encodes the protein responsible for releasing the colicin and consequently for killing the producing cells during this process. The immunity gene is downstream from the activity and lysis genes, with opposite transcriptional polarity, and encoding a short sequence involved in immunity protein binding (Cascales et al. 2007). Those genes are located on colicinogenic plasmids (Col plasmids), which can be divided into type A and type B. Type A plasmids are small (6–10 kb) and present in numerous copies per cell; they are mobilizable in the presence of a conjugative plasmid and are amplifiable. Type B are monocopy plasmids of about 40 kb, which carry numerous genes in addition to that encoding colicin activity, and are able to conjugate (Pugsley 1984).

Colicin synthesis reaches its maximum usually during the stationary phase of growth, and the release of this bacteriocin usually involves the lysis of the producer cell [although there are exceptions for which the mechanism of bacteriocin release is still unclear (Cursino et al. 2002)]. The production of most colicins (except for colicins G, H, and Js) is regulated by the SOS system and can be artificially induced by DNA-damaging agents, such as mitomycin C and UV, and also by antibiotics that interfere with DNA replication or cell wall synthesis (Lewin and Amyes 1991), high pressure (Aertsen and Michiels 2005) and single-stranded DNA. The SOS system is a global response, known as an error-prone repair system, which uses the RecA protein as an activator and LexA protein dimers as negative transcriptional regulators (for review, see Michel 2005).

The operon composed of the activity and lysis genes is repressed by LexA, but the colicin gene shows a 20–30 min delay in expression relative to other SOS genes. It has been proposed that the delay in induction may allow cells to repair DNA damage and try to re-establish repression of the operon (Salles and Weinstock 1989a) before lethal induction of the lysis gene, avoiding overt lysis of the colicinogenic strains. Other regulators of colicin expression include cyclic AMP receptor protein-cAMP complex (Salles and Weinstock 1989b), anaerobic conditions mediated by Fnr protein (Eraso and Weinstock 1992), the stringent response due to an increase in ppGpp (Eraso et al. 1996; Kuhar et al. 2001) and temperature (Butala et al. 2008).

In general, colicins have three functionally distinct domains (Fig. 3). These domains are responsible for receptor recognition (R, in Fig. 3a), protein translocation (T, in Fig. 3a), and killing (C, in Fig. 3a) (Cao and Klebba 2002). The central

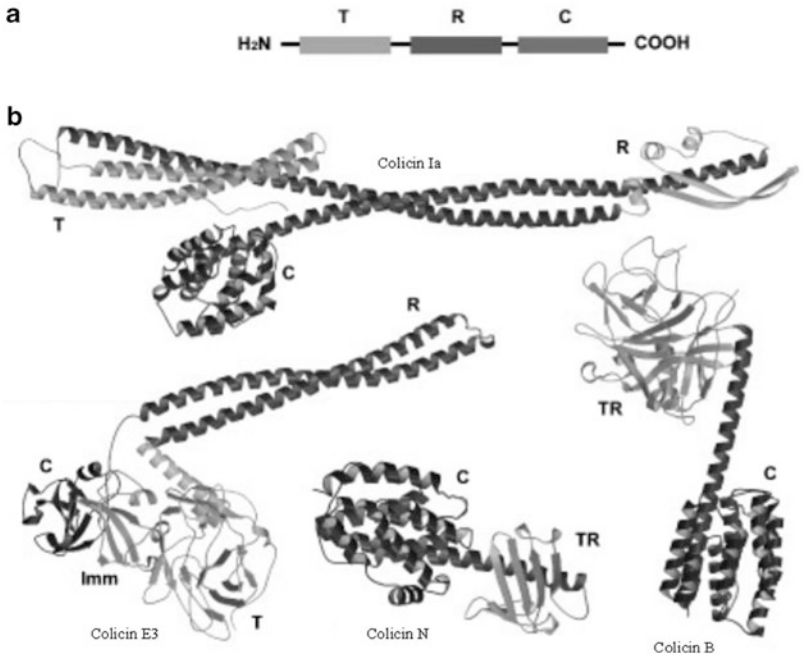

Fig. 3 Colicin multidomains and structures. (**a**) Functional and structural domains in colicins: receptor recognition (R), protein translocation (T) and killing (C). See text for details. (**b**) Colicins' structures with their respective domains: colicin Ia, colicin E3, colicin N, colicin B (adapted from Sharma et al. 2006)

domain comprises about 50 % of the protein, and it is involved in the recognition of specific cell surface receptors on outer membrane of the target cell. The N-terminal domain (<25 % of the protein) is responsible for colicin translocation through the outer membrane and periplasmic space to its target. The remainder of the protein houses the killing or cytotoxic domain, responsible for colicin activity (Zakharova and Cramer 2002).

Colicins first recognize a specific outer membrane receptor at sensitive cells, such as the porins OmpF or receptors normally involved in the uptake of essential nutrients (FepA, BtuB, Cir, and FhuA), using them to translocate through the outer membrane. Then, colicins use translocation systems for passing the periplasm and bacterial inner membrane, in order to reach their targets. Group A colicins (colicins E1–E9) utilize the bacterial Tol-dependent translocation system, a five-protein assembly comprising TolA, TolB, TolQ, TolR, and Pal, while Group B colicins (other colicins) require the Ton-dependent system, consisting of TonB and ExbB–ExbD (Davies and Reeves 1975a, b).

Tol and Ton systems are coupled to the proton-motive force of the inner membrane, and cells carrying mutations in these systems are generally colicin tolerant, since their colicin sensitivity is substantially lowered although not completely lost (Smarda et al. 2002). Part of Tol and Ton systems is anchored to the inner membrane, and the remainder is exposed to the periplasmic space; the

inner membrane partners (TolA or ExbB–ExbD) are used to initiate the pmf-dependent step for colicin translocation through the inner membrane (Housden et al. 2010).

The final target is in the inner membrane for membrane-active colicins and in the cytoplasm for nuclease colicins. The membrane-active colicins can form voltage-activated channels on target cell membranes, resulting in the depolarization of the cytoplasmic membrane (such as colicins A, E1, B, and K), while nuclease colicins act as a nuclease against DNA, rRNA, and tRNA (such as colicins E3, E4, and E6). Other colicins can also inhibit macromolecular synthesis (colicins E1 and K) (Riley and Wertz 2002).

In addition to colicins, some species of Gram-negative enteric bacteria, especially *Escherichia coli* and *Klebsiella*, produce a second type of bacteriocin, known as microcins. They are a heterogeneous group of low molecular weight (<10 kDa) ribosomal peptides, plasmid encoded, with diverse structural features and mechanisms of action. Microcins share some properties with the bacteriocins produced by Gram-positive bacteria, including thermostability, resistance to some proteases, relative hydrophobicity, and resistance to extreme pH (Gillor et al. 2004).

The currently characterized microcins belong to seven classes, A, B, C, D, E, H, and J, recognized on the basis of immune cross-reactions. The available data suggest that microcin genes belonging to one immunity class only slightly differ in nucleotide sequence; that is, the immunity class fully characterizes the nature of a particular microcin. Some microcins are active as unmodified peptides, while other can be post-translationally modified, such as microcins B, C, and J (Pavlova and Severinov 2006).

Microcins are synthesized as pre-pro-peptides, with N-terminal leader sequence, which is cleaved by a signal peptidase. Post-translational modifications to microcin peptides are varied and often required for antimicrobial activity. Amino acids residues that form the mature peptide backbone can be converted to heterocycles (such as to microcin B17 produced by *E. coli*), they can form a ring (microcin J25) and also siderophore or adenosine monophosphate molecules can be attached on C-terminus (microcin E492 and microcin C7, respectively) (Nolan and Walsh 2009) (Fig. 4).

Most *Escherichia coli* microcins are produced at the stationary growth phase, and the only exception is microcin E, which is synthesized in the early logarithmic phase (Pavlova and Severinov 2006). Unlike colicins, microcins are secreted to the extracellular environment, and their production is not inducible by DNA-damaging agents; the microcin antimicrobial activity is kept under environmental conditions which do not support colicin action (de Lorenzo and Aguilar 1984; Tarakanov et al. 2004).

Microcins are primarily active against Gram-negative enterobacteria related to the producer strain, like *Escherichia, Klebsiella, Salmonella, Citrobacter, Enterobacter*, *Erwinia*, *Shigella, Proteus, Serratia*, or *Pseudomonas* (Tarakanov et al. 2004). Sensitivity of some non-agglutinable-type *Vibrio* strains to microcin E492 was also observed at high concentrations of the peptide (de Lorenzo and Aguilar 1984).

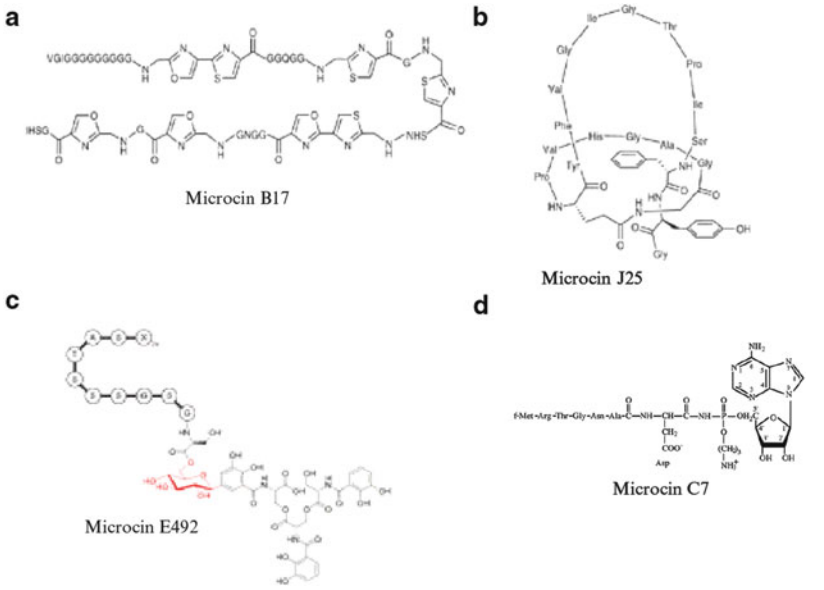

Fig. 4 Structures of post-translational modified microcins. (**a**) Microcin B17, (**b**) Microcin J25, (**c**) Microcin E492, (**d**) Microcin C7. See text for details (adapted from Oman and van der Donk 2010)

In general, microcins can disrupt the membrane integrity of the target cell via pore formation, thereby inducing cell death. Some other microcins can attack intracellular targets, such as DNA gyrase (the blockage of DNA gyrase topoisomerase activity prevents unwinding of supercoiled DNA and halts DNA replication), DNA-dependent RNA polymerase and aspartyl-tRNA synthetase (blocking the protein synthesis) (Nolan and Walsh 2009).

Other bacteriocins have been identified in Gram-negative bacteria, such as pyocins in *Pseudomonas* sp. (Jacob 1954), vibriocin in *Vibrio comma* (Jayawardene and Himsley 1969), alveicins in *Hafnia alvei* (Hamon and Peron 1963), klebicins, or pneumocins in *Klebsiella pneumoniae* (Chhibber and Vadehra 1986), enterocoliticin in *Yersinia enterocolitica* (Strauch et al. 2001), BC1 and BC2 in *Vibrio vulnificus*, IW1 in *V. cholerae* (Shehane and Sizemore 2002), and BLS in *Aeromonas hydrophila* (Messi et al. 2003).

The production of antimicrobial peptides by enterobacteria in general has been suggested to play an important ecological role in the maintenance of the homeostasis in the microbial community of the animal and human intestines (Tarakanov et al. 2004). Bacteriocinogenic *E. coli* strains may have an important function for the interaction between the host organism and its intestinal flora, occupying an ecological niche on the surface of the intestinal mucosa epithelium. *E. coli* utilizes oxygen diffusing through the mucosa into the intestinal lumen, creating suitable conditions for the growth of strict anaerobes, which represent most of the intestinal bacteria. In this situation, the ability of bacteriocin production may be an important factor, in favor of representatives of *E. coli* against other species of the *Enterobacteriaceae* family adapted to the same environmental conditions (Smarda and Smajs 1998).

Several BLIS- or bacteriocin-producer Gram-negative cells are important pathogens, such as *Agrobacterium* (Tate and Sutherland 2002), *Burkholderia solanacearum* (Frey et al. 1996), *Corynebacterium* (Gross and Vidaver 1979), *Erwinia* (Chaung et al. 1999), *Vibrio harveyi* (Prasad et al. 2005), *Xanthomonas perforans* (Hert et al. 2005), and the antibiotic-resistant strains of *Salmonella* spp., *Campylobacter* spp., and *Escherichia coli* (Sit and Vederas 2008).

It is still unclear how pathogenic and nonpathogenic strains compete with each other for colonization, but it may be assumed that the bacteriocin production could be a competitive advantage against other strains, enabling the access to resources within a host and ensuring survival and dominance of the pathogenic bacterium (Prasad et al. 2005). Moreover, for *E. coli* strains, higher frequency of bacteriocin production was found amongst pathogenic isolates when compared with commensal isolates, and often there is a correlation between bacteriocin production and synthesis of virulence factors, such as aerobactin, alpha-haemolysin, and P-fimbria (Cursino et al. 2002).

2.3.2 Bacteriocins Produced by Gram-Positive Bacteria

Gram-positive bacteria produce bacteriocins that are as abundant as and even more diverse than those produced by Gram-negative bacteria. It has been reported that bacteriocins produced by Gram-positive bacteria are restricted to killing other Gram-positive bacteria (with varied spectrum of action) and, in general, they do not have inhibitory activity toward Gram-negative bacteria, fungi, or virus (Klaenhammer 1993; Riley and Wertz 2002).

However, some bacteriocins are also active against certain Gram-negative bacteria, including *Klebsiella pneumonia*, *Pseudomonas* spp., and *Campylobacter jejuni* (Todorov and Dicks 2006). Examples are bifidin I, produced by *Bifidobacterium infantis* BCRC 14602; thermophilin 81, produced by *Streptococcus thermophilus*; plantaricin 35 d, produced by *Lactobacillus plantarum*; lacticin NK24, produced by *Lactococcus lactis* NK24; and bacteriocin AMA-K, produced by *Lactobacillus plantarum* AMA-K (Ivanova et al. 1998; Lee and Paik 2001; Messi et al. 2001; Todorov 2009; Cheikhyoussef et al. 2009).

Bacteriocins with potent activity against antibiotic-resistant bacterial strains, such as vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA), have also been reported (Sit and Vederas 2008). Recently, bacteriocins produced by *L. plantarum* exhibited antifungal activity, showing activity against *Absidia* spp., *Aspergillus niger*, *Epicoccum nigrum*, and *Penicillium* sp. (Todorov 2010). Studies about the bioactivity of bacteriocins against virus are still scarce, but some of them have been recently characterized as potent antiviral peptides, such as bacteriocin ST5Ha, a pediocin-like bacteriocin produced by *Enterococcus faecium*, that is, active against herpes simplex virus type 1 (HSV-1) strain F, an important human pathogen (Todorov et al. 2010).

The genes coding for bacteriocin production are mostly organized in operon clusters, which can be located on the chromosome, on plasmids, or on transposable

elements. The genes are in coordinated expression, and the operons are basically composed of genes for biosynthesis, regulation, self-immunity, a gene encoding an ABC transporter and a gene encoding an accessory protein essential for externalization (Klaenhammer 1993; Rossi et al. 2008).

Like other ribosomally derived antibiotic scaffolds, the bacteriocins produced by Gram-positive bacteria are initially synthesized as biologically inactive precursor peptides; the N-terminal leader sequences are cleaved by proteases to yield the active peptide. The leader peptide is an essential recognition element for the post-translational tailoring enzymes and is also necessary for immunity and export signaling (Nolan and Walsh 2009). Additionally, Gram-positive bacteria have protective mechanisms to limit harm from self-produced bacteriocins: immunity is provided by specific immune proteins and/or by sensing proteins to regulate bacteriocin synthesis or transport (Willey and van der Donk 2007; Draper et al. 2008).

Usually, the production of bacteriocins by Gram-positive bacteria shows secondary metabolite kinetics, and its activity is detected at the end of exponential phase and early stationary phase of growth (Todorov 2010). Normally, the bacteriocin titre decreases at the stationary phase and continues to decrease with prolonged incubation, probably due to proteolytic degradation, protein aggregation, adsorption to the cell surface or feedback regulation (Ondaa et al. 2003; Todorov 2010). For some strains, the bacteriocin production has been determined to be temperature dependant, and according to Dufour et al. (2007), the regulation of bacteriocin expression is not cell cycle dependent, per se, but rather culture density dependent.

Bacteriocins can adsorb to cell surfaces of the producer cells. Some bacteriocins were found to adhere to the cell surface of the producer cells such as pediocin AcH, nisin, sakacin A, leuconocin Lcm and lacticin 3147 (Todorov 2010). In the case of plantaricin C19, produced by *L. plantarum* C19, maximal adsorption to the producer cells was recorded between pH 5.0 and 7.0, with a complete loss of adsorption at pH 1.5 and 2.0 (Atrih et al. 2001).

Some bacteriocins are stable at pH 2.0–8.0, suggesting that activity may not be affected by pH changes during growth. However, constant changes in the pH and medium composition during fermentation may lead to changes in activity levels of bacteriocins. Not all bacteriocins are heat stable, but thermostability at 100 °C has been reported for some of them and may be a result of their low molecular mass or difference in the structures of those peptides (Todorov 2010). For certain bacteriocins, such as leucocin F10, pH influences temperature stability (Parente et al. 1996).

Many factors influence the bacteriocin's efficacy toward the target cell, such as structure and quantity of bacteriocin; physiological status, composition and cell wall structure of the target cell; composition and membrane potential of the target cell; the presence of specific proteases at or nearby the target cells; chemical composition of the environment; amount of target cells and/or cells able to adsorb the bacteriocin; and absence or presence of mutations in some cellular components, as the specific receptors for bacteriocins (Eijsink et al. 2002; Strandberg and Ulrich 2004).

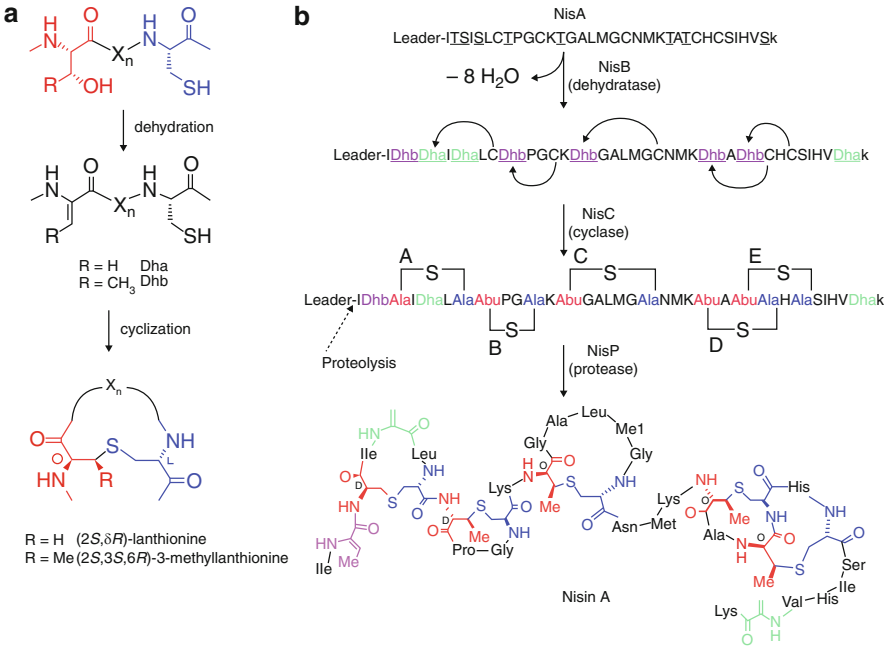

Fig. 5 Post-translational modifications of lantibiotics. (**a**) Dehydration process of serine or threonine residues to give dehydroalanines (Dha) and dehydrobutyrines (Dhb); after cyclization, the lanthionine rings are formed. (**b**) Process of nisin maturation, showing the ribosomally synthesized prepeptide NisA and the benzymatic reactions to form the mature peptide, Nisin A. After cyclization process, the leader peptide is proteolytically removed by the protease (NisP) (adapted from Wiley and van der Donk, 2007)

Based on their primary structure, molecular mass, heat stability, and functional similarity, bacteriocins produced by Gram-positive bacteria can be classified in four major groups (Cotter et al. 2005):

– Class I or lanthionine-containing bacteriocins or lantibiotics: includes small peptides (<5 kDa; 18–39 residues), post-translationally modified to their bioactive forms by multienzyme complexes. First of all, serines (Ser) and threonines (Thr) present in precursor peptides are dehydrated by LanB dehydratases, to give dehydroalanines (Dha) and dehydrobutyrines (Dhb), respectively; LanC cyclases then catalyze the subsequent intramolecular Michael addition of cysteine residues onto these dehydroamino acids, resulting on lanthionine-type thio-ether cross-links (intramolecular covalent bridges), from which lantibiotics derive their name (Pag and Sahl 2002). In some cases, one enzyme (bifunctional LanM-modifying enzymes) carries out both dehydration and cyclization steps (Xie et al. 2004) (Fig. 5). The thio-ether linkages are stable to hydrolysis and are also critical for lantibiotic physiological function (van Kraaij et al. 2000).

– Class II or non-lanthionine-containing bacteriocins or non-lantibiotics: includes small (<10 kDa) membrane-active peptides, with limited or no extensive post-translational modifications. Non-lantibiotics are still divided into three subgroups: class IIa is composed by pediocin-like anti-*Listeria* peptides, with their conserved disulfide bond and N-terminus characterized by a consensus YGNGVXC motif (this consensus region may perhaps cause the bactericidal effect directly and is considered to be involved in the action against *Listeria* strains; however, many other bacteriocins which lack this structural homology with class IIa peptides are still active against *Listeria monocytogenes*); class IIb bacteriocins require a combination of two polypeptides for full antimicrobial activity (e.g. enterocin L50), while class IIc are other bacteriocins (e.g. acidocin B); class IId has been proposed by Gray et al. (2006a) and consists of bacteriocins that are sec dependent, such as thuricin 17 (Gray et al. 2006b) and bacthuricin F4 (Kamoun et al. 2005).
– Class III or bacteriolysins: comprises large, heat-labile proteins (>30 kDa) that catalyze the hydrolysis of bacterial cell walls resulting in autolysis of targeted bacteria (e.g. helveticin J and lactacin B) (Drider et al. 2006; Dobson et al. 2007).
– Class IV: an additional proposed class, which comprises complex bacteriocins that requires lipid or carbohydrate moieties for activity. Little is known about the structure and function of this class (e.g. leuconocin S and lactocin 27) (Vermeiren et al. 2006).

Additionally, two other schemes of lantibiotic classification exist. According to the earliest scheme proposed by Jung (1991), the lantibiotics can be classified in type A and type B: type A lantibiotics includes elongated, amphipathic, flexible and pore-forming lantibiotics (e.g. nisin, bovicin HC5, subtilin, epidermin, gallidermin, Pep5), while type B lantibiotics includes the rigid and globular peptides, that act by inhibition of enzymes (e.g. mersacidin and actagardine, duramycin and its analogues, cinnamycin and ancovenin).

As already mentioned, the gene clusters possess either two (LanB, LanC) or one (LanM) modification enzyme, and this difference on biosynthetic pathway was used to propose a new classification: class I lantibiotics are modified by LanB and LanC (nisin and epidermin); class II lantibiotics possess a GG cleavage site in their leader peptide, and they are modified by LanM enzymes (lacticin and its analogous, several two-peptide lantibiotics); class III lantibiotics are peptides that have no or little antibacterial action and perform other functions, often morphogenetic (SapT, SapB, and their orthologues) (Willey and van der Donk 2007) (Fig. 6).

Recently, Goto et al. (2010) reported the discovery of a new family of lanthionine synthetases, termed LanL, in the mycelial soil bacterium *Streptomyces venezuelae*. Moreover, they have shown that putative lantibiotic biosynthetic gene clusters are widespread in nature and not restricted to Gram-positive bacteria, as long believed, being found in Gram-negative bacteria, such as the proteobacterium *Myxococcus xanthus*, and in cyanobacteria, such as *Nostoc punctiforme* and *Prochlorococcus*.

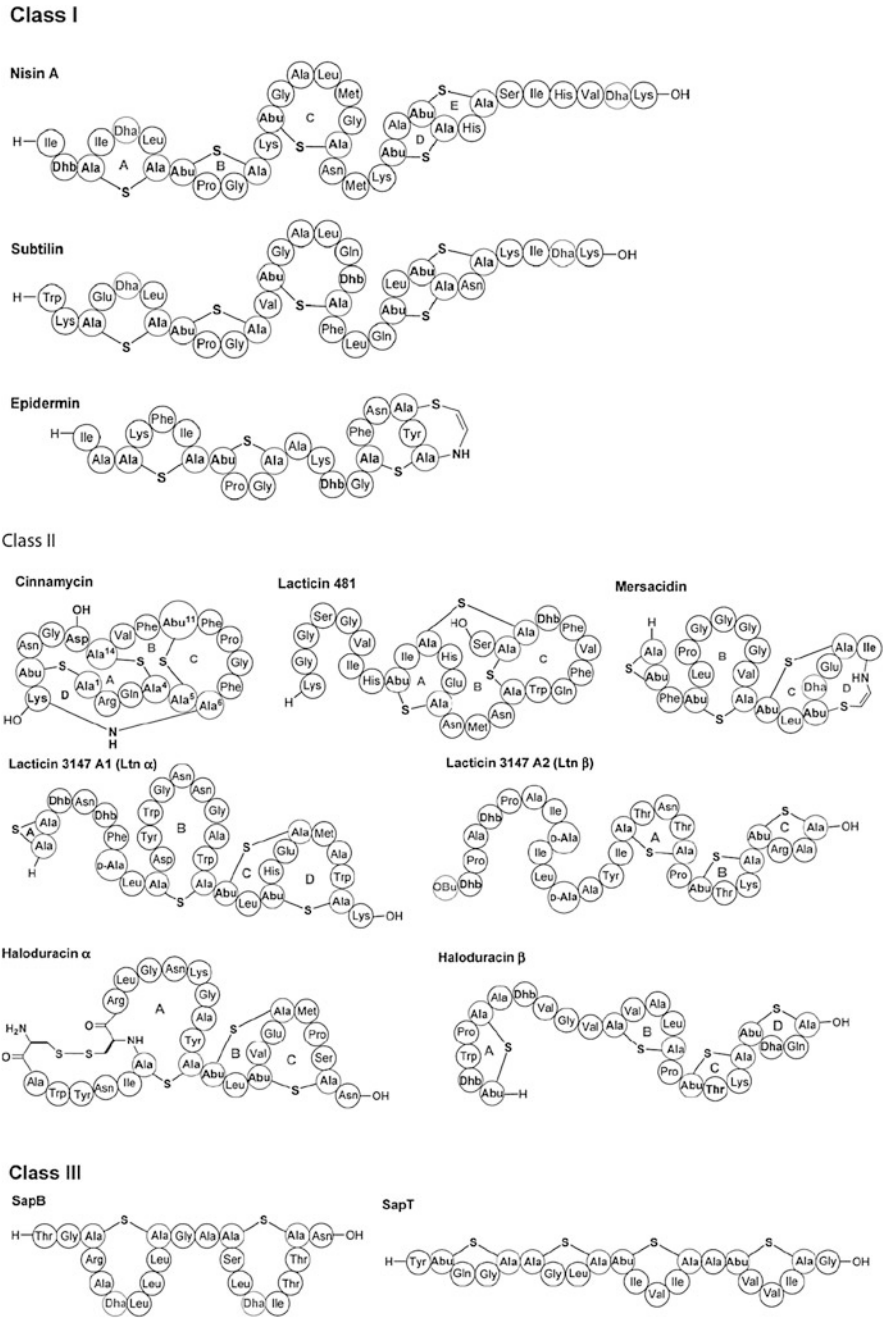

Fig. 6 Examples of lantibiotics, classified according the difference on their biosynthetic pathway (adapted from Wiley and van der Donk, 2007)

Nevertheless, many of those identified lantibiotic-like gene clusters direct the production of peptides that do not have antibiotic activity but that may have other, often unknown, functions as signaling molecules or morphogenetic peptides (Lia et al. 2010); then, Goto and coworkers suggested the name lantipeptides for lanthionine-containing peptides that by structure and biosynthetic routes are related to lantibiotics but that do not have any antimicrobial activity.

Among the bacteriocins produced by Gram-positive bacteria, the most promising are those produced by lactic acid bacteria (LAB), including the genera *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc* and *Bifidobacterium*, isolated from different food matrices such as fermented dairy products, vegetables, fruits, meat, and fish and also from the human and animal gastrointestinal tract (Todorov et al. 2010).

2.3.3 Nisin and Other Bacteriocins Produced by Gram-Positive Bacteria

The most well-known bacteriocin is nisin, a heat-stable antibacterial peptide produced by *Lactococcus lactis* subsp. *lactis* and belonging to the class I bacteriocin. It is a small (3.5 kDa), 34-amino acid, cationic, hydrophobic peptide with five characteristic (beta-methyl) lanthionine rings. It affects primarily vegetative cells and prevents the outgrowth of spores of Gram-positive bacteria. Until now, five natural nisin variants (A, Z, Q, U, and F) have been identified (de Kwaadsteniet et al. 2008), and nisin provides a paradigm for studies of lantibiotic structure, biosynthesis, and mode of action of antimicrobial peptides (Nolan and Walsh 2009).

Microorganisms susceptible to nisin include other lactic acid bacteria, *Bacillus*, *Clostridium*, *Staphylococcus*, *Listeria*, and *Streptococcus* genera (Cleveland et al. 2001), *Actinomyces*, *Corynebacterium*, *Gardnerella*, *Mycobacterium*, *Campylobacter*, *Haemophilus*, *Helicobacter*, and *Neisseria*. Nisin is often used in combination with other synergistic preservation methods, including pH reduction and addition of salt in high concentration (Rayman et al. 1983), preheating of the product (Boziaris et al. 1998), addition of chelating agents (e.g. EDTA) (Branen and Davidson 2004) and detergents (e.g. Tween 80) (Joerger 2003), to increase its antimicrobial activity against Gram-negative bacteria, yeasts, or molds.

In 1969, nisin was accorded international acceptance as food additive by the Codex Alimentarius Commission (JECFA 1969). In 1988, it received GRAS (generally recognized as safe) status, being approved by the US Food and Drug Administration for certain food applications (FDA, 1988). The Science Committee on Food (SCF) evaluated the safety of nisin in 1990 and allocated it an ADI of 0.13 mg/kg body weight/day (SCF 1992). In 1995, nisin (code E234) was authorized for food preservation in the European Union by Directive 95/2/EC on food additives other than colors and sweeteners. In 2001, FDA affirmed nisin as GRAS for use as an antimicrobial agent on cooked meat and poultry products when used at a level that delivers a maximum of 250 ppm of nisin in the finished products (FDA 2001).

So far, nisin is the only bacteriocin which has been approved for use in over 50 countries as food preservative, including the USA, European Union, Australia, and

New Zealand (Delves-Broughton 2005). Nisin has been used in dairy products, canned foods (vegetables, soups), hot baked products (crumpets), and pasteurized liquid eggs (Delves-Broughton et al. 1996). The approval of nisin for food use is a logical consequence of the fact that nisin is produced by *L. lactis*, a food grade lactic acid bacterium that has a long history of use as a component of starter cultures in dairy industry.

Nisin is readily inactivated by trypsin, pancreatin and alpha-chymotrypsin (Jarvis and Mahoney 1969), it is not detected in human saliva 10 min after the consumption of 0.005 mg nisin/kg (Claypool et al. 1966) and the intestinal microbiota in human flora-associated rats is not affected after consumption of nisin (Bernbom et al. 2006), suggesting that ingested nisin is completely digested to amino acids prior to absorption. According to Reddy et al. (2004), nisin does not possess any subchronic or chronic toxicity, reproductive/developmental toxicity, genotoxicity, and carcinogenicity and can be safely used.

Nisin is not the only bacteriocin produced by *L. lactis*. Other lantibiotics produced by the same genera include the single peptide lacticin 481 and the two-component system lacticin 3147 (de Vuyst and Leroy 2007). Non-lantibiotic bacteriocins from *L. lactis* include pediocin-like bacteriocins (class IIa) such as lactococcin MMFII, two-peptide component bacteriocins (class IIb) such as lactococcin G and M, thiol-activated bacteriocins (class IIc) such as lactococcin B, and heat-labile, Lactococcus-specific bacteriocins (class IId) such as lactococcin A (diplococcin) and lactococcin 972 (Oppegard et al. 2007).

In general, type A lantibiotics kill bacteria via pore formation, using specific receptors, also referred to as docking molecules. These receptors enhance bacteriocin activity and provide target cell specificity, increasing the antimicrobial activity and resulting in selective toxicity of type A lantibiotics. The bacteriocin nisin has at least five different antimicrobial activities based on both high-affinity targets and low-affinity membrane interactions (Pag and Sahl 2002). Nisin uses the membrane pore formation and the inhibition of cell wall synthesis to kill sensitive cells. Nisin binds with high affinity to the lipid II molecule, a hydrophobic carrier for peptidoglycan monomers, using this compound as a specific receptor to integrate into the bacterial membrane and subsequent pore formation, and this interaction with lipid II compromises the incorporation of murein precursor units into the cell wall (Brötz et al. 1998; Breukink et al. 1999; Wiedemann et al. 2001).

Lipid II plays an essential role in bacterial cell wall synthesis, carrying the sugar-peptide subunits from the cytosolic side of the membrane—the site of lipid II synthesis—to the peptidoglycan synthesis machinery outside the cell (Fig. 7). Lipid II is also the target for antibiotics, such as ramoplanin and vancomycin, and other bacteriocins, such as mutacin, pediocin, subtilin, gallidermin, epidermin, mersacidin, bovicin HC5 and haloduracin. Recently, Carroll et al. (2010) demonstrated the activity of nisin and lacticin 3147 against *Mycobacterium* sp., and, according to the authors, the binding of lantibiotics to the mycobacterial lipid II is not compromised by the extensively modified lipid II structure in mycobacteria.

The interaction between nisin and lipid II starts specifically with the high-affinity binding of nisin with its N-terminal to the hydrophilic head group (pyrophosphate)

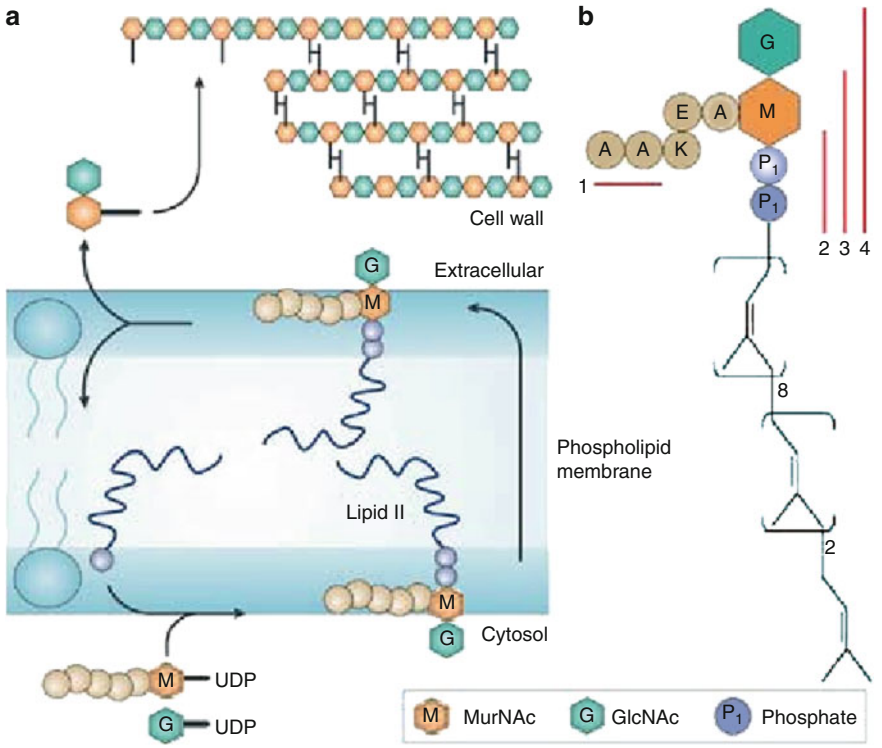

Fig. 7 Cell wall assembly. (**a**) The cell wall biosynthesis starts on the cytosolic side of the bacterial plasma membrane. UDP-activated precursor sugars are assembled on a polyisoprenoid carrier and the coupling of which molecule produces lipid II; lipid II is transported across the membrane and peptidoglycan subunits are transferred to the growing peptidoglycan chain; the polyisoprenoid carrier is recycled back to the cytoplasmic side, and the cycle is completed. (**b**) Lipid II structure, showing the polyisoprenoid anchor (eight *cis*-conformation isoprene units, two units in the *trans*-conformation and the terminal isoprene unit) and the pentapeptide. *Red bars* indicate the minimal binding sites in lipid II of glycopeptide antibiotics (1), nisin (2), ramoplanin (3), and mersacidin (4). GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid (Breukink and de Kruijff 2006)

of the cell wall precursor. The N-terminus of nisin interacts with lipid II, and its C-terminus inserts into the membrane (Hsu et al. 2002). The interaction between initially formed nisin-lipid II complexes in the membrane results in the formation of complexes that consist of several nisin and lipid II molecules, which assemble further into larger complexes; the conversion of the large complexes into a pore requires the cooperative insertion of the nisin molecules into the lipid bilayer. The resulting nisin-lipid II pore was supposed to contain eight nisin and four lipid II molecules (Hasper et al. 2004) (Fig. 8).

Furthermore, independently of lipid II binding, nisin can impair microbial membranes at micromolar concentrations (Breukink et al. 1999) or displace cationic autolytic enzymes from their anionic binding sites in the Gram-positive cell wall, resulting in premature lysis of nascent cell wall septa (Bierbaum and Sahl 1985),

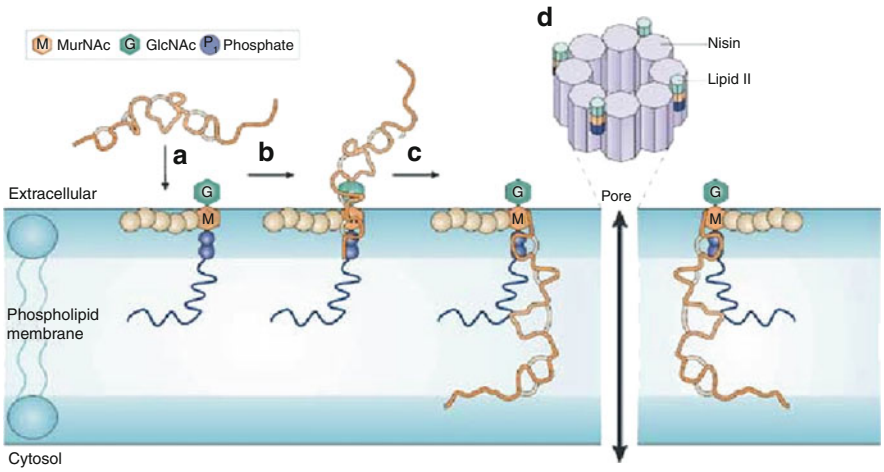

Fig. 8 Nisin-lipid II interaction. (**a**) Nisin reaches the bacterial membrane. (**b**) Nisin's N-terminal binds to the hydrophilic head group of lipid II, with high affinity. (**c**) The pore formation starts, and nisin adopts a transmembrane orientation. (**d**) During or after assembly of four 1:1 (nisin:lipid II) complexes, four additional nisin molecules are recruited, and the final pore complex is formed (Breukink and de Kruijff 2006)

and it can also promote the release of some enzymes, such as *N*-acetylmuramoyl-*L*-alanine amidase and *N*-acetylglucosaminidase, which hydrolyze the cell wall by binding to teichoic, teichuronic, and lipoteichoic acids (Héchard and Sahl 2002). Nisin also inhibits the outgrowth of bacterial spores, and although it does not inhibit the initiation of germination, nisin's action results in the uncoupling of two important steps necessary for the spore outgrowth: the establishment of oxidative metabolism or membrane potential and the shedding of external spore structures (Gut et al. 2008). Recently, Gut and co-workers (2011) showed that nisin utilizes lipid II as the target during inhibition of spore outgrowth, and the membrane disruption induced by nisin is important to inhibit the development of spores into vegetative cells.

Despite the same target, several type A lantibiotics can have different mechanisms of action. Mutacin is able to bind to lipid II, inhibiting the cell wall synthesis, but in the absence of pore formation (Smith et al. 2008), while the pore-forming activity of bovicin HC5 is clearly dependent on membrane thickness, being detected only in thinner membranes (Paiva et al. 2011). It is important to mention that bovicin HC5 maintains its antibacterial activity independent on the membrane thickness, by binding to lipid II and recruiting some lipid II molecules as a pre-pore-like structure, and as a consequence, inhibiting the bacterial cell wall biosynthesis (Paiva et al. 2011). These varied mechanisms of action demonstrated by lantibiotics that share the same target can be combined toward sensitive cells and might explain the differences observed in sensitivity to the lantibiotics depending on the bacterial strain tested.

Type B lantibiotics can act by increasing of membrane permeability on target cells, protein inhibiting the peptidoglycan synthesis at the transglycosylation level, forming a complex with the membrane-bound lipid II [mersacidin and actagardin (Brötz et al. 1998)], and also by inhibiting of bacterial phospholipase A2 (duramycin-C (Héchard and Sahl 2002)). Lacticin 3147, a two-component type B lantibiotic, requires the presence of both components to exert its maximal antimicrobial activity: one of the peptides, that resembles type B lantibiotic mersacidin, acts by inhibiting the cell wall synthesis, while the other peptide, more similar to the type A lantibiotic, is responsible for pore formation via interaction with lipid II molecule (Wiedemann et al. 2001, 2006).

Yoneyama et al. (2009a, b) described a mode of action for lacticin Q, a pore-forming type B lantibiotic that apparently does not require lipid II or another docking molecule to exert its antimicrobial activity in nanomolar range. According to the authors, lacticin Q quickly binds to the outer membrane leaflets [a process not restricted, but accelerated by the presence of negatively charged phospholipids (Yoneyama et al. 2010)], forming an amphiphilic α-helical structure; lacticin Q forms pores (average diameter of more than 4.6 nm) accompanied by lipid flip-flop and consequently protein leakage; some bacteriocin molecules migrate from the outer to the inner membrane leaflets when the pore closes; this antimicrobial mechanism was called "huge toroidal pore" (HTP), and the features of the lacticin Q mode of action are more similar to the mode of action of self-defense antimicrobial peptides from multicellular eukaryotes, as magainin 2, than to those of typical lantibiotics. Despite this mode of action, lacticin Q still exhibits selective toxicity, but the mechanisms used by this bacteriocin to recognize sensitive cells remain unclear.

Among the class II bacteriocins, the bacteriocins produced by *Pediococcus acidilactici*, such as pediocin PA-1/AcH, are the most studied. The subclass IIa bacteriocins dissipate the proton-motive force, preventing the occurrence of energy synthesis reactions (Mcauliffe et al. 2001), or they act through the formation of small pores in the membrane, with subsequent intracellular material efflux (Drider et al. 2006; Oppegard et al. 2007). Some bacteriocins belonging to subclass IIa are also active against viruses, and although the mode of action is not completely known, possible explanations could be the aggregation of virus particles, blockage of receptor sites on the host cell, or inhibition of key reactions in the multiplication cycle (Wachsman et al. 2003).

Subclass IIb bacteriocins require the combined activity of both partners to dissipate membrane potential, by forming hydrophilic pores and consequently increasing the permeabilization of target cells or by specific cationic/anionic pore formation, with consequent collapse of transmembrane potential (Héchard and Sahl 2002). Some two-peptide bacteriocins can also interfere with cellular ATP production (Willey and van der Donk 2007). Subclass IIc bacteriocins, with no structural similarity among the group members, have different modes of action, including membrane permeabilization, specific inhibition of septum formation and pheromone activity (Héchard et al. 2001).

It is still unclear if class II bacteriocins require a target molecule on the sensitive cells. In this respect, it has been recognized that antibacterial peptides, which do not

have a specific receptor, are equally active in their D and L forms, and some class II bacteriocins synthesized as D-enantiomers have been shown to be inactive, indicating a stereospecific interaction with a target molecule (Nes and Holo 2000). In addition, with nisin as an example, it was proven that some lantibiotics require a specific target to be active in the nanomolar range, and activity in nanomolar range has been demonstrated by class II bacteriocins. These findings suggest that this bacteriocin class needs a receptor-like molecule on sensitive cells in order to exert maximum antimicrobial activity, and the components of the sugar phosphotransferases systems, such as the MptD, a membrane subunit of the mannose permease EII_t^{Man}, have been considered (Héchard et al. 2001; Dalet et al. 2001).

Class III bacteriocins catalyze the hydrolysis of bacterial cell walls, promoting the autolysis of sensitive bacteria (Dobson et al. 2007), and the mode of action of class IV bacteriocins remains unclear (Vermeiren et al. 2006).

As mentioned to Gram-negative bacteria, some bacteriocin-producer Gram-positive cells are pathogenic strains, such as *Listeria monocytogenes*, *Enterococcus* sp., and *Clostridium perfringens* (Rood and Cole 1991). Several bacteriocin loci are found in association with IS sequences or near cell wall-associated serine protease genes. According to Dupuy et al. (2006), it is possible that some common molecular mechanism is involved in the regulation of synthesis of toxins and bacteriocins, such as sigma factors in *Clostridium* (sigma factors are sequence-specific, DNA-binding subunits of RNA polymerase, that ensure the recognition of appropriate promotor sites (Helmann and Moran 2002)).

In addition to bacteriocin production, some Gram-positive bacteria also produce peptides with antibacterial and antifungal activity, the cyclic dipeptides. Ström et al. (2002) described the antifungal cyclic dipeptides, cyclo (L-Phe-L-Pro) and cyclo (L-Phe-trans-4-OH-L-Pro) produced by *Lactobacillus plantarum* MiLAB 393, and active against *Aspergillus nidulans*; beyond *L. plantarum*, the cyclic dipeptides are also produced by other LAB, such as *P. pentosaceus* and *L. sakai* (Magnusson et al. 2003).

3 AMPs Versus NRPs

Although antibiotic therapy is still the first choice to combat microbial infections in humans and animals, the prevalence of bacterial resistance to conventional antibiotics is a growing public health concern. The emergence of multidrug-resistant bacteria can lead to severe consequences like treatment failure and increased mortality. AMPs are being investigated as potential substitutes to conventional antibiotics in the hope of developing novel antimicrobial agents to control antibiotic-resistant bacterial strains (Sang and Blecha 2008).

The AMPs differ from NRPs according to their synthesis, mode of action, toxicity and resistance mechanisms. The synthesis of NRP secondary metabolites can depend, in some degree, on genome size. A correlation of 23 bacterial genomes indicated that microorganisms with larger genomes (>5 Mb) are likely to produce

NRP metabolites, and those with smaller genomes (<3 Mb) produce no or few NRPs; nevertheless, additional efforts are still necessary to determine this correlation (Donadio et al. 2007).

Compared with conventional NRPs, which are generally active against bacteria or fungi, AMPs often exert activity against a broad spectrum of microorganisms including bacteria, fungi, parasites, enveloped viruses and even some cancer cells (Matanic and Castilla 2004). Unlike conventional antibiotics, which generally are directed against specific proteins (easily mutated), AMPs kill microbes at micromolar concentrations mainly by pore-forming mechanisms (Hancock and Sahl 2006; Wang et al. 2010), and, consequently, the target cell membrane functions are impaired by AMP action: the membrane potential is reduced, the osmotic balance is disturbed and the insertion of the peptide into membranes increases pressure, impairing numerous membrane-associated biological processes (Sang and Blecha 2008).

Along with the different modes of action of AMPs and therapeutic antibiotics, the selection pressures for developing resistance have also been different. Whereas most AMP-resistance mechanisms do not provide complete protection and result in only reduced susceptibility to the peptide, bacterial strains bearing antibiotic-resistance determinants often exhibit no detectable susceptibility to the corresponding antibiotic. Many bacterial antibiotic-resistance mechanisms involve specific regulatory proteins that bind cognate antimicrobial compounds and activate the resistance genes (Zhang et al. 2001; Peschel and Sahl 2006).

4 Resistance Mechanisms

As with any other antimicrobial compound, the issue of resistance also has to be considered for peptides produced by microorganisms. Although the resistance in this case is less well documented, microorganisms have developed several countermeasures aimed at subverting the actions of antimicrobial peptides prior to their accessing or gaining entry to interior targets (Peschel and Sahl 2006; Yount et al. 2006). Resistance mechanisms to antimicrobial peptides include modifications of cell wall components, inactivation by peptide degradation or modulation of efflux pumps (Bals 2000).

Anionic bacterial cell wall molecules, such as peptidoglycan, teichoic acids, lipid A or phospholipids, are formed by complex biosynthesis processes, and it is unlikely that microorganisms could replace these essential macromolecules with novel structures that would be less favorable for AMP interactions (Ernst et al. 2001). So, several resistance strategies used by microorganisms are based on reducing the net anionic charge of the bacterial cell wall, reducing the affinity of AMPs. Dorrer and Teuber (1977) were the first to describe that some pathogens, like *Pseudomonas fluorescens*, are able to decrease the anionic phospholipid levels (PE, PG, CL) and increase cationic-ornithine-amide constituents on cytoplasmic membranes.

Staphylococcus aureus, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Listeria monocytogenes* and other bacteria are able to modify teichoic acid with esterification of D-alanine residues (this is achieved by the products of the *dltABCD* operon) (Poyart et al. 2003; Kristian et al. 2005). *S. aureus* uses a similar concept to change the net negative charge of the major membrane lipid phosphatidylglycerol into a net positive charge by lysine modification (Peschel et al. 2001). The production of lysyl-PG requires the enzyme MprF, and the *mprF* gene is present in many bacterial genomes, that is, assumed to represent an important evasion strategy (Weidenmaier et al. 2003).

According to Papo and Shai (2005), in Gram-negative bacteria, lipopolysaccharide (LPS) is the first protective determinant that limits peptide binding and insertion into bacterial membranes. *Salmonella enterica* and *Pseudomonas aeruginosa*, for example, incorporate positively charged aminoarabinose into lipid A, the conserved integral membrane component of LPS, enhancing the membrane cationic charge and thereby reducing affinity of AMPs for the bacteria (Miller et al. 2005). The gene *pagP* encodes an acyltransferase that catalyzes lipid A palmitoylation in *Salmonella*, a modification that reduces antimicrobial peptide accessibility to the cytoplasmic membrane. Analogous mechanisms are also used by other Gram-negative bacteria, such as lipid A acylation and 4-amino-4deoxy-L-arabinose and palmitate derivation of lipid A in *E. coli* (Zhou et al. 1999), aminoarabinose and myristoylation modifications of LPS in *Pseudomonas* (Ernst et al. 1999), loss of LPS O-specific side chains in *Bordetella bronchiseptica* (Banemann et al. 1998).

The presence of capsular polysaccharide or glycocalyx surrounding microorganisms is one of the most effective resistance mechanism and one of the major virulence determinants of many bacterial pathogens, since it provides an initial barrier to the attachment of antimicrobial peptides. Capsular polysaccharide is formed from the oligomerization of anionic monomer subunits, being negatively charged and widely heterogeneous, which represents an efficient way to bind antimicrobial peptides with great avidity, removing these peptides from solution and limiting the access to their targets (Yount et al. 2006). Friedrich et al. (1999) have described that alginic acid, a highly anionic exopolysaccharide, produced by virulent strains of *P. aeruginosa*, interferes with AMP's efficacy in vitro; similar mechanisms are also employed by *Klebsiella*, *Haemophilus*, *Streptococcus*, *Legionella*, and *Staphylococcus* species.

The production and secretion of proteolytic agents against antimicrobial peptides is a generalized resistance mechanism documented for some microorganisms, especially for pathogenic strains (Malloy et al. 2005). For example, PhoQ, a downstream component of the PhoP/PhoQ regulon, was identified as an outer membrane endopeptidase in *Salmonella* (Guina et al. 2000) and shares similarities with other outer membrane protease families, such as Omp T or protease VII of *E. coli* (Sugimura and Nishihara 1988) and Pla of *Yersinia* (Sodeinde et al. 1992); all of them cleave peptides between basic residue pairs and the carboxy-terminal aspect of basic amino acid residues preceding nonpolar residues (Yount et al. 2006). Many other bacterial species, including *Streptococcus pyogenes*, *Pseudomonas aeruginosa*,

Enterococcus faecalis, *Proteus mirabilis*, *Porphyromonas gingivalis*, *Prevotella* spp., and *Staphylococcus aureus* also produce proteases that cleave AMPs (Peschel and Sahl 2006).

Efflux pumps or drug-export systems are fundamentally involved in resistance to membrane-active antimicrobial peptides. In *Neisseria gonorrhoeae*, the energy-dependent efflux system named *mtr* mediates resistance to antibacterial peptides of diverse structures, and MtrCDE complex ejects also antibiotics, organic dyes and detergents (Shafer et al. 1998). In *Yersinia*, the efflux of AMPs appears to involve a potassium antiporter formed by RosA and RosB proteins, and *rosA*/*rosB* regulon is induced upon exposure to antimicrobial peptides (Bengoechea and Skurnik 2000). Efflux systems have also been described as resistance mechanisms in Gram-positive bacteria and fungal pathogens: the plasmid-encoded gene *qacA* mediates staphylococcal resistance to many antimicrobial agents via an energy-dependent efflux pump (Kupferwasser et al. 1999), and ABC-type transporters have been implicated in fungal resistance to antimicrobial peptides (Andrade et al. 2000).

Other resistance mechanisms include the capacity to form biofilms, which prevents the access of antimicrobial peptides to the cells (Otto 2006; Peschel and Sahl 2006), and also the use of complex mechanisms that specifically counteract antimicrobial peptide targeting of intracellular targets, such as mutation in the *gyrB* gene of *E. coli*, that confers a significant reduction in susceptibility to the antimicrobial peptides targeting DNA gyrase, by reducing binding and activity of this enzyme (del Castillo et al. 2001).

Some pathogens can employ multiple mechanisms, at the same time, to resist to AMPs. *Neisseria*, for example, uses multiple distinct mechanisms simultaneously to resist to cationic peptides, including membrane-directed efflux systems (*mtrCDE*), phosphoethanolamine transferase (*lptA*) modification of lipid A head groups, and the expression of *pilMNOPQ* operon involved in pilin synthesis (Tzeng et al. 2005).

Development of resistance to AMPs is not frequently associated to currently used antibiotics resistance. However, Crandall and Montville (1998) noted that nisin-resistant *L. monocytogenes* cultures were more sensitive to penicillin and ampicillin, and according to Carlson et al. (2001), the exposure of *Salmonella* to the bacteriocin microcin-24 resulted in microcin-resistant cells that exhibited resistance to multiple common antibiotics. Mantovani and Russell (2001) also reported that nisin-resistant mutants of *Streptococcus bovis* exhibited a 1,000-fold higher resistance to ampicillin than the original nisin-sensitive isolates, although they were still sensitive to vancomycin and bacitracin.

The primary mode of action of vancomycin and bacitracin is the blockage of bacterial cell wall synthesis, via binding to lipid II (binding site D-alanine-D-alanine) or inhibition of dephosphorylation of the peptidoglycan carrier C_{55}-isoprenyl pyrophosphate, respectively. Thus, the resistance to nisin reported by Mantovani and Russell (2001) does not seem to be related only to lipid II levels, as this would be expected to result in cross-resistance to vancomycin. According to Kramer et al. (2006), acquired nisin resistance is a very complex feature and sensitive cells can employ diverse simultaneous mechanisms to resist to nisin; although alteration of lipid II structure does not occur, the nisin resistance mechanisms include prevention

of nisin from reaching the membrane, that is, the lipid II molecule, change of extracellular pH, change of elongation and saturation of membrane phospholipids, extrusion of nisin out of the cytoplasmic membrane. Some Gram-positive bacteria, such as *Clostridium botulinum*, *Streptococcus thermophilus*, and *Bacillus cereus*, have been shown to be resistant to nisin due their ability to synthesize nisinase, a specific nisin-degrading enzyme (Alifax and Chevalier 1962; Rayman et al. 1983).

The antimicrobial resistance mechanisms described above illustrate the complex host-microorganism relationship as it defines infection or immunity. These concepts also emphasize that microbial resistance to AMPs may be difficult to reproduce and to study in vitro, as some microbial responses in vivo are dependent on strategies and conditions relevant to immunoavoidance in specific physiological niches (Yount et al. 2006).

5 Applications of Antimicrobial Peptides

The widespread use of antibiotics for both animal and human applications over the last 50 years has led to the emergence of microbial resistance and to the dissemination of resistance genes among pathogenic microorganisms, and several strains are already immune against many or all currently available antibiotics. In order to prevent outbreaks of infectious diseases and to reduce the selection pressure by antibiotics, antimicrobial peptides have attracted researchers' attention because of their broad-spectrum activity, mechanisms of action and low resistance development, which indicate a promising future for extensive application of these peptides (Gordon et al. 2005).

The field of antimicrobial peptides produced by microorganisms is expanding and substantial advances are expected in the years to come. Although bacteriocins are traditionally associated with food applications, they also offer potential biotechnological applications, because they are stable at low pH values and to heating, easy to produce and show little or no activity against eukaryotic cells, features which have led to their evaluation as the most promising class of peptides to be used as antibiotic substitutes.

Due to the mentioned concerns and expectations, in the next topics, we discuss some applications of antimicrobial peptides produced by microorganisms in fields of animal husbandry, food industry, and clinical applications.

5.1 Animal Husbandry

Antibiotics have been extensively used in animal research since they were discovered, as therapeutic agents or growth promoters, and their efficacy and cost-effectiveness contribute to their popularity. Nevertheless, treatment of animals with antibiotics leads to antibiotic residue problems in the environment and veterinary products (Molina et al. 2003), and the prophylactic use of antibiotics in areas

such as feed supplements and in the control of bovine mastitis will be probably and hopefully limited in the European Union in the years to come (Crispie et al. 2005).

In this sense, a number of strategies to reduce or eliminate pathogens or to improve growth and feed conversion that do not involve antibiotics have been explored (Bedford 2000), and agents such as bacteriocins, probiotic microorganisms, and bacteriophages have been studied or proposed as potential alternatives (Joerger 2003), because of the relative safety of their uses and because usually they do not leave residues in the environment nor the animal meat (Simon 2005).

The aim of using growth promoters in cattle feed is alter ruminal fermentation, by decreasing Gram-positive species that produce large amounts of hydrogen (a precursor of methane), and ammonia (a wasteful end product of protein degradation) (Russell and Strobel 1989). In vitro experiments indicated that nisin has similar effects on ruminal fermentation when compared with monensin, the most common ionophore antibiotic used as feed additive (Callaway et al. 1997). Jalc and Laukove (2002) introduced nisin into an artificial rumen system and detected some changes in fermentation parameters such as an increase in hemicellulose degradation and acetate and propionate production, which contribute to the improvement of microbial balance in this environment.

Some topically applied agents have been tested, in substitution of conventional used antibiotics, in the treatment of animal diseases, especially bovine mastitis. The therapeutic effect of formulations containing nisin was assessed for the treatment of mastitis, and considerable reductions of *S. aureus* and *E. coli* viable cells were observed (3.9 and 4.2 log cycle, respectively) (Sears et al. 1992). Wu and coworkers (2007) demonstrated significant increases in cure rates of infections caused by *S. agalactiae*, *S. aureus*, and other pathogens (90.1 %, 50 %, and 65.2 %) when the cows were treated with nisin Z, via intramammary administration; moreover, after 48 h of treatment, no bacteriocin residue was detected in milk.

Previous reports also indicate that the addition of lacticin 3147 to teat seal offers protection against mastitis infection in vivo (Ryan et al. 1998). Twomey et al. (2000) evaluated a formulation containing lacticin 3147 in order to determine the concentration of bacteriocin required to reduce the mastitis incidence, and a dosage of 32800 AU/4 g of teat seal reduced the mastitis incidence in about 45 %, when compared to the control (not treated). The therapeutic potential of intramammary infusions containing *L. lactis* viable cells (lacticin 3147 producer) was evaluated in cows with subclinical and clinical mastitis, and the cure observed was 47 % and 35 %, respectively, while the cure obtained with conventional treatment using antibiotics was 61 % (Klostermann et al. 2008).

5.2 Food Industry

Consumers' demands by preservative-free, safe, and an extended shelf-life to the food products and also the food legislation have made the task of providing high-quality products even more challenging to the food industry (Papagianni 2003;

Rydlo et al. 2006). Currently, artificial chemical preservatives are employed to limit the number of microorganisms capable of growing within foods, but the consumer awareness of potential health risks associated with these substances have stimulated research of new effective alternatives to be used as biopreservatives (Hagiwara et al. 2010).

Because of the long history of using LAB in the processing of fermented foods, such as dairy products and meats, the antimicrobial and safety information for LAB in food preservation is widely accepted (Sit and Vederas 2008). Bacteriocin-producer bacteria have been used as starter cultures or cocultures, offering a double advantage: they provide peculiar flavor to fermented products by the peptidolytic and transamination activities (key factors in the formation of aroma and taste compounds) (Fernández de Palencia et al. 2004), and also serve to extend the product shelf-life, by the conversion of lactose to lactic acid and by the production of antimicrobial compounds, as bacteriocins and bacteriocin-like inhibitory substances (Khan et al. 2010). Bacteriocin-producing *L. lactis* has been experimentally tested in the manufacture of several cheese varieties (Rilla et al. 2003; Garde et al. 2006) and other fermented products (Diop et al. 2009).

Not only the bacteriocin-producer bacteria but also the bacteriocins produced by LAB species are considered suitable alternatives for food preservation in a variety of food products of animal and vegetable origin, because bacteriocins are harmless to eukaryotic cells, are usually pH and heat tolerant and are digested by proteolytic enzymes present in the organism (Gálvez et al. 2007; Khan et al. 2010).

The incorporation of bacteriocin-producer bacteria in the manufacture of fermented food provides an attractive and economic alternative to the addition of purified bacteriocins. However, in situ bacteriocin production is not always successful. Sometimes the growth and bacteriocin-producing ability of the producer cell is severely affected due to the food environment, and use of purified or semi-purified bacteriocin preparations can be more beneficial. Furthermore, when the product under consideration is an unfermented one, purified, semi-purified, or crude forms of bacteriocins (produced ex situ) shall be the method of choice (Khan et al. 2010).

On the other hand, the addition of purified bacteriocins to the complex food environment is not simple, since many factors can cause complete or partial loss of the antimicrobial activity. The activity may be affected by processing and storage conditions, as well as by the intrinsic environment of the food product which may result in resistance of target pathogens to bacteriocins or can cause changes in the solubility and charge of bacteriocins, binding to constituents of food or destruction of activity by proteases (Rydlo et al. 2006).

Moreover, the use of purified or semi-purified preparations of bacteriocins as food preservatives have legal implications: any new bacteriocin intended to be used as food preservative is considered as an additive and needs prior approval by the regulatory authorities, requiring detailed safety information supported by toxicological data, proof of efficacy in foods, description of manufacturing process, as well as the maximum safe levels (Cleveland et al. 2001).

Currently, only the bacteriocin nisin has regulatory approval for use in foods, as previously mentioned. The most used commercial form of nisin is named

NisaplinTM, which contains 2.5 % of the active ingredient (Nisin A), 77.5 % NaCl, and 12 % nonfat dry milk (Chen and Hoover 2003). Some other bacteriocins, such as pediocin PA-1/AcH (available as ALTA 2341) and lacticin 3147, are also commercially available, but they are marketed as fermentates of LAB having the GRAS status, and are not approved as food additives (Gálvez et al. 2008).

Despite its success as a food preservative, nisin has reduced activity against *L. monocytogenes* and it undergoes oxidation and degradation in neutral and basic conditions (Stergiou et al. 2006). These stability issues make nisin unsuitable for use as a preservative in meat, poultry, and fish products (Sit and Vederas 2008), characteristics that stimulate the examination of other bacteriocins, especially the ones with broad spectrum of activity, temperature, and heat stability (Rydlo et al. 2006; Khan et al. 2010). In the future, protein engineering, genetic engineering, and/or chemical synthesis may lead to the development of new antimicrobial peptides with properties based on some features of the original peptides but with optimized potency and stability under the typical incubation conditions in food (Rydlo et al. 2006).

The development of packaging films having antimicrobial properties is one innovation which has been increasingly studied (Mauriello et al. 2004). The antimicrobial substances are either simply coated on the surface of films or incorporated in the matrix of the films. There is a long list of antimicrobials which have been tested in the preparation of antimicrobial films, but bacteriocins are the most promising compounds (Joerger 2007). Nisin is the most frequently tested antimicrobial in the preparation of antimicrobial films (Joerger 2007), but other bacteriocins, such as pediocin and enterocins, have also been investigated for the preparation of such films (Khan et al. 2010).

5.3 Clinical Application

The emergency of multi-resistant organisms is a major concern and the main reason for efforts to find alternative regimens, including the identification of novel antimicrobial agents and/or some antibiotic combinations, known as combination therapy. This combination of common therapeutic antibiotics with ribosomal antimicrobial peptides is a promising aspect in improving antimicrobial therapy and is generally used to increase the activity of both antimicrobials, to reduce the increasing selection pressure by antibiotic and to broaden the antimicrobial spectrum (Cirioni et al. 2006).

Previous studies have reported the synergistic interaction among sublethal levels of peptides and pointed out the capacity of some peptides to synergize with antibiotics. The mechanism of this positive interaction is not completely known, even though it might be due the effect of antimicrobial peptides against the same or closely related targets (Todorov 2010; Cirioni et al. 2006). Synergistic effects have been observed in vitro when some antimicrobial peptides are combined with

clinically used antibiotics and tested against several clinically isolated bacterial strains (Giacometti et al. 2005).

Sublethal levels of ciprofloxacin were combined with bacteriocin ST5Ha and a strong enhancement of the bioactivity was observed, resulting in a stronger inhibition of *Listeria ivanovii* ATCC19119 than when ciprofloxacin or bacteriocin ST5Ha was used alone; according to the authors, the mechanism by which the cationic peptide increases the effectiveness of the antibiotic is through dissipation of the proton gradient responsible for the extrusion of these antibacterial compounds, resulting in a synergistic effect (Todorov et al. 2010).

The presence of those synergistic effects make the antimicrobial peptides, specially bacteriocins or other peptides produced by bacteria, potentially valuable as an adjuvant for antimicrobial chemotherapy against antibiotic-resistant bacterial strains, reducing the level of MIC of the antibiotics required for the treatment of infectious diseases in human and veterinary medicine and overcoming the development of resistant strains (Giacometti et al. 2005; Todorov 2010).

However, in spite of the good results obtained with the use of antimicrobial peptides in vitro, in general, the in vivo application of antimicrobial peptides is limited due to the loss of their function in serum, partially because their short half-life owing to enzymatic degradation and binding to serum components (Imura et al. 2007; Meng and Kumar 2007; Rotem and Mor 2009); moreover, there are few data available on the in vivo toxicities of the antimicrobial peptides, the stability of the peptide/peptide-formulation in vivo has not been studied in details and also the costs of production on a large scale has been an obstacle until now (Brogden et al. 2003).

Based on this, many structure-function studies of antimicrobial peptides have focused on developing synthetic antimicrobial peptides or their congeners. The fundamental issues of stability, toxicity and cost are being targeted by a number of distinct strategies, including creating mimics of AMPs using starting materials that are cost effective and that create active, relatively small antimicrobials that are not susceptible to protease degradation and have the same antimicrobial properties (Rydlo et al. 2006); creating extremely small replicas of AMPs with low overall charge and that are protected from degradation by protecting groups and incorporation of unusual amino acids (like D-amino acids); and C-terminal amidation and/or cyclization (which are believed to improve stability and activity against targeted microorganisms, as shown in natural bacteriocins, plant cyclotides, and primate θ-defensins) (Bansal et al. 2008).

The smaller, safer, and more stable bioactive antimicrobial peptides usually have lower MICs and broader spectrum activity than that of natural peptides, neutralize LPS, promote wound healing, have synergistic activity with conventional antibiotics, no or less hemolytic activity (used as a measure of toxicity against mammalian cells), and very few side effects have been reported (Cirioni et al. 2006). They have been approached to be used in a wide range of clinical application including infective, inflammatory and immune system-based diseases (Zhang and Falla 2006).

Nevertheless, a rational comprehension of the role of the structural, biochemical, and physical factors which influence the activity of antimicrobial peptides are indispensable to the goal of using and/or designing AMPs with therapeutic value

(Hoskin and Ramamoorthy 2008). Different biophysical parameters (net charge, chain length, helicity, and hydrophobicity) should be considered during the design of new drugs (Wang et al. 2010), and other challenges involving AMP-based drug development include environmental concerns (Keymanesh et al. 2009), the higher cost of peptide synthesis (Hancock and Sahl 2006), detailed investigation of mechanisms of action, stability, cytotoxicity, and immunogenicity, as well as the microbial resistance, which although considered less frequent than to conventional antibiotics, have been identified and should be considered in developing and using AMP-based drugs (Gunn 2008).

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LL-37: An Immunomodulatory Antimicrobial Host Defence Peptide

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Abstract Cationic host defence peptides (CHDP) are conserved peptide components of the innate immune system. These peptides, also known as antimicrobial peptides, were originally discovered and described on the basis of their direct microbicidal properties. However, it has become increasingly clear that CHDP, such as the human cathelicidin hCAP18/LL-37, have an extensive range of immunomodulatory properties that can be complementary to microbicidal activity or may even represent their major antimicrobial function. As a result of its capacity to interact with cells involved in host defence LL-37 can modulate both innate inflammatory processes and interact with the generation of adaptive immunity. CHDP have been implicated in a variety of disease processes at diverse organ sites and are attracting increasing attention as templates for the development of novel immunomodulatory antimicrobial therapeutics. This chapter will focus on the antimicrobial and immunomodulatory properties of hCAP18/LL-37 and the underlying mechanisms involved in the bioactivity of this peptide.

Keywords Cationic host defence peptides • Antimicrobial peptides • Innate immunity • Host defence • Cathelicidins • LL-37 • mCRAMP

1 Mammalian Cationic Host Defence Peptides

Cationic host defence peptides (CHDP) are evolutionarily conserved, small, positively charged peptide components of innate host defences. In mammals, CHDP are represented by two main classes of peptide: the defensins and cathelicidins. The multiple different defensins are believed to share a common ancestral gene and can

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be subdivided into α-, β- and θ-defensins, based on the organisation of three characteristic cysteine disulphide bonds in the mature peptide fragment of the prepropeptide (reviewed in Taylor et al. 2008). In contrast, cathelicidins are not grouped as a family on the basis of the mature peptide structure, which displays considerable diversity, but rather by the presence of an evolutionarily conserved N-terminal cathelin domain in the propeptide (reviewed in Zanetti 2004). Mammals express a plethora of defensins [with humans expressing six α-defensin genes and having over forty predicted β-defensin genes (Taylor et al. 2008)], and multiple cathelicidins are seen in some species. However other species, including humans and mice, express only a single cathelicidin. Although steadily more reports detailing bioactivities of defensins are emerging, the immunomodulatory properties of cathelicidin peptides will be the focus of this chapter.

The defining features of cathelicidins are an N-terminal signal sequence, a conserved cathelin domain and a variable C-terminal domain which, upon cleavage, becomes the mature functional peptide. The cathelin domain was named on the basis of its capacity as a cathepsin L inhibitor, and the cleaved cathelin protein has been described as a cysteine protease inhibitor with some microbicidal properties in its own right (Zaiou et al. 2003). The mature cathelicidin peptides range from 12 to 88 amino acids in length and take various forms including linear peptides with the capacity to form amphipathic α-helical structures, disulphide bond-stabilised β-hairpin structures and proline-rich structures (Zanetti 2004). The sole human cathelicidin human cationic antimicrobial peptide of 18KDa (hCAP18) generates a 37-amino-acid peptide called LL-37 as its primary mature product (Gudmundsson et al. 1996), which adopts an α-helical structure in lipid membranes and in physiological ionic environments (Johansson et al. 1998).

2 Human Cathelicidin hCAP18/LL-37

hCAP18 is encoded by the *CAMP* gene on chromosome 3p21.3. After removal of the signal peptide, the propeptide may be initially stored or immediately cleaved by proteinase 3 to form LL-37, the 4.5 kDa mature peptide fragment (Sorensen et al. 1997a, 2001). Although LL-37 is the major mature form, smaller fragments such as KS-30, KS-22, LL-29, KR-20, RK-31, LL-23 and KS-27 may also be formed by serine proteases (e.g. kallikreins) in keratinocytes and sweat (Murakami et al. 2004; Yamasaki et al. 2006), and cleavage by gastricin in the semen can lead to the formation of the ALL-38 form (Sorensen et al. 2003). These alternatively processed forms have variations in their balance of microbicidal and immunomodulatory properties (Braff et al. 2005), demonstrating a mechanism of in vivo functional control and illustrating the therapeutic potential to modulate function through peptide sequence manipulation.

hCAP18/LL-37 is produced at highest concentration by neutrophils [~630 μg/10^9 cells (Sorensen et al. 1997b)] where it is stored in propeptide form in the secondary granules. However, expression can also be induced in epithelial cells, keratinocytes,

monocytes, macrophages, mast cells, NK cells, γδT cells and B cells (reviewed in Bowdish et al. 2006). hCAP18/LL37 can be detected in a broad range of tissues and bodily fluids including plasma, bone marrow, airway surface fluid, skin, sweat, reproductive tract, semen, urine, breast milk and vernix (reviewed in Bowdish et al. 2005a).

The expression of hCAP18 is subject to complex transcriptional and post-transcriptional control, with upregulation in response to inflammatory and infectious stimuli [such as lipopolysaccharide (LPS), IL-6 and IL-1α (Frohm et al. 1997; Erdag and Morgan 2002; Nell et al. 2004)] and to wounding (Dorschner et al. 2001). The precise mechanisms remain to be fully determined; however, recent studies have clearly shown the importance of the active vitamin D metabolite 1,25-dihydroxyvitamin D3 (1,25(OH)D3) as an inducer of hCAP18 expression, acting via a vitamin D response element in the *CAMP* gene promoter (Wang et al. 2004; Gombart et al. 2005; Martineau et al. 2007; Hansdottir et al. 2008). The observation that expression of the hydroxylase CYP27B1, which converts 25-hydroxyvitamin D3 to the active 1,25(OH)D3, can be upregulated by TLR stimulation (Liu et al. 2006) indicates a mechanism for vitamin D-dependent upregulation of *CAMP* expression in response to inflammatory and infectious stimuli. Other mechanisms of control include butyrate-enhanced histone acetylation at the *CAMP* promoter, resulting in AP-1-mediated transcription (Kida et al. 2006), recruitment of the PU.1 transcription factor to the *CAMP* promoter in response to vitamin D, butyrate or lithocolic acid (Termen et al. 2008), and the identification of nuclear factor for interleukin-6 expression sites (Gudmundsson et al. 1996).

The importance of hCAP18/LL-37-mediated protection against infectious diseases in vivo can be seen in patients with the rare condition morbus Kostmann, in whom neutrophils are deficient in hCAP-1/LL-37 and who are more susceptible to infection (Putsep et al. 2002), and in the association between hCAP18/LL-37 levels and susceptibility to infection in dermatological pathologies (reviewed in Schauber and Gallo 2008). Whereas expression is not increased in response to the inflammation in atopic dermatitis and increased susceptibility to infection is observed, high levels of hCAP18/LL-37 (Ong et al. 2002) are associated with a relative protection from skin infections in psoriasis. However, pathologically high LL-37 levels may be harmful to the host and have been proposed to contribute to the pathogenesis of psoriasis by conferring antigenicity to self-RNA and self-DNA (Lande et al. 2007; Ganguly et al. 2009). Increased levels of hCAP18/LL-37 have also been reported in pulmonary infection, cystic fibrosis (CF) lung disease and bronchiolitis obliterans syndrome (Schaller-Bals et al. 2002; Chen et al. 2004; Anderson et al. 2008). Although these could represent a protective microbicidal response, dysregulated LL-37-mediated immunomodulatory effects might contribute to pathology, with the severity of CF lung disease found to correlate with increased LL-37 levels in the lung persisting between exacerbations (Chen et al. 2004). Indeed altered posttranslational processing of hCAP18, associated with an increase in stratum corneum tryptic enzyme, contributes to disease pathogenesis in acne rosacea (Yamasaki et al. 2007), demonstrating a pathological role for the angiogenic properties of this CHDP (Koczulla et al. 2003). In addition, recent studies demonstrate that LL-37

bound to DNA, generated by neutrophil extracellular trap formation, may play a key role in the pathogenesis of systemic lupus erythematosus (Lande et al. 2011; Garcia-Romo et al. 2011). Thus, in common with other key controllers and modulators of inflammation, LL-37 has potential to protect the host from infection but to be detrimental when expression is excessive or dysregulated.

Additional evidence for the critical, non-redundant protective effects of cathelicidins in vivo comes from studies of mCRAMP (mouse cathelin-related antimicrobial peptide, encoded by *Camp*), the murine orthologue of hCAP18. Genetically modified mice deficient in mCRAMP expression ($Camp^{-/-}$) demonstrate increased susceptibility to infections of the skin, intestinal tract, cornea, urinary tract and lung (Nizet et al. 2001; Iimura et al. 2005; Chromek et al. 2006; Huang et al. 2007; Yu et al. 2010) and are more susceptible to dextran sulphate sodium-induced colitis (Koon et al. 2011; Tai et al. 2012). Interestingly, regulation of murine cathelicidin expression diverges from that observed in humans, as mCRAMP is not regulated by vitamin D (Gombart et al. 2005), but has been shown to be HIF1α (hypoxia-inducible factor 1 alpha)-responsive (Peyssonnaux et al. 2005). Nevertheless, these studies show considerably more severe effects upon host defence than knockout models deficient in single β-defensins (Morrison et al. 2002; Moser et al. 2002), where there may be considerable redundancy, and demonstrate multi-organ effects of cathelicidin deficiency in vivo. Additional evidence of in vivo antimicrobial function was demonstrated using gene augmentation with hCAP18/LL-37 to enhance the clearance of *Pseudomonas aeruginosa* from the murine lung (Bals et al. 1999), a study that also demonstrated the therapeutic potential of these peptides. Although this research clearly indicates the importance of cathelicidins to host defence, the precise mechanisms underpinning these observations remain uncertain.

As with many antimicrobial peptides, the minimum inhibitory concentrations (MIC) for LL-37 against microbes in vitro [in the range of 10–250 μg/ml (Travis et al. 2000; Johansson et al. 1998; Saiman et al. 2001; Li et al. 2006; Pompilio et al. 2011)] are much higher than the physiological concentrations that have been described in vivo at uninflamed mucosal sites (<2 μg/ml hCAP18/LL-37, of which it is unclear what proportion is mature peptide). In addition, LL-37 can be inhibited by the presence of cations (Bowdish et al. 2005b), serum apolipoprotein (Wang et al. 1998), DNA and F-actin (Weiner et al. 2003; Bucki et al. 2007). Indeed, in the presence of concentrations of divalent cations (Ca^{2+}, Mg^{2+}) found in the human body, even 100 μg/ml LL-37 (exceeding levels observed at inflammed mucosa) was not microbicidal for *Staphylococcus aureus*, or *Salmonella* typhimurium (Bowdish et al. 2005b) nor for *P. aeruginosa* (Barlow et al. 2010) against which cathelicidin-mediated in vivo protection has been observed (Bals et al. 1999; Yu et al. 2010). The question therefore arises as to how cathelicidins function as antimicrobial agents in vivo. While antimicrobial effects might be mediated through direct microbicidal properties at sites of localised high peptide concentrations or through synergy with other antimicrobial agents, perhaps the most important functions are indirect inflammomodulatory and immunomodulatory effects (Fig. 1).

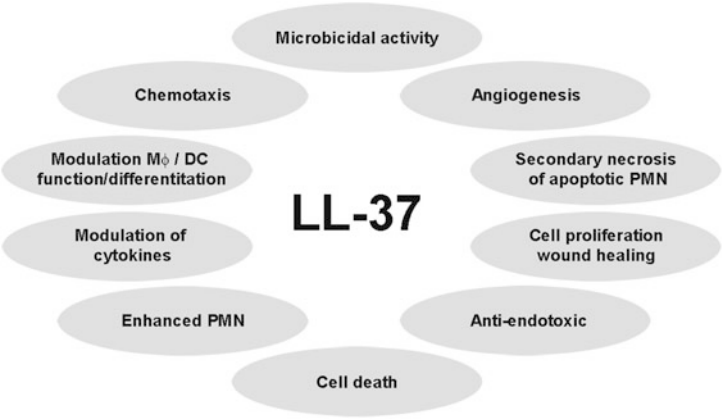

Fig. 1 LL-37 is a multifunctional component of host defence

3 Microbicidal Activity

LL-37 was initially described and characterised as an antimicrobial peptide, with the focus placed squarely on its microbicidal functions. LL-37 has been reported to be microbicidal against a broad range of gram-positive and gram-negative bacteria, including *P. aeruginosa*, *S. aureus* and *E. coli* (Travis et al. 2000; Johansson et al. 1998; Saiman et al. 2001; Li et al. 2006) and the yeast *Candida albicans* (den Hertog et al. 2005), and to inhibit biofilm formation by *P. aeruginosa* (Overhage et al. 2008). However, the capacity for direct bactericidal activity in physiologically relevant environments and lower concentrations has been questioned (Bowdish et al. 2005b; Barlow et al. 2010; Pompilio et al. 2011). Additional studies have also demonstrated antiviral activity of LL-37 in vitro and/or in vivo against HIV (Bergman et al. 2007), vaccinia virus (Howell et al. 2004), influenza virus (Barlow et al. 2011) and respiratory syncytial virus (Logermann et al. 2012), indicating broad-spectrum antimicrobial potential.

The microbicidal properties of CHDP have been variously attributed to three main mechanisms (Henzler Wildman et al. 2003), with the focus primarily on bacterial membranes: (a) a "barrel-stave" pore formation where hydrophobic surfaces interact with membrane lipid acyl chains while hydrophilic regions align to form a pore which may enlarge as more monomers are added, (b) a "carpet model" with transient toroidal pore formation induced through CHDP-mediated membrane curvature strain at sites of high local peptide concentration and (c) an alternative "carpet model" characterised by detergent-like bilayer disruption eventually leading to the formation of micelles at high peptide concentrations. LL-37 appears to function by the toroidal pore formation, binding to the negatively charged bacterial surfaces and adopting a stable α-helical conformation at the polar/nonpolar interface, aligned parallel to the membrane surface (Henzler Wildman et al. 2003). Studies evaluating the properties of LL-37 analogues and truncated peptides have

demonstrated that hydrophobicity and the propensity to form α-helices is critical to microbicidal function, but that the helical sense (using enantiomeric peptides) is not (reviewed in Burton and Steel 2009). In addition, the core microbicidal region has been defined as amino acids 17–32 (Li et al. 2006), with this truncated peptide having enhanced microbicidal activity in comparison to full length LL-37 (MIC ~100 μg/ml against *E. coli* K12, compared to 200 μg/ml for LL-37). The membrane defects induced by CHDP are proposed to allow leakage of intracellular contents, although whether this alone induces death or whether subsequent intracellular translocation of the peptide to interact with internal targets (Hancock and Rozek 2002) is also critical remains to be determined. Although bacteria appear less able to develop resistance to CHDP than to conventional antibiotics, various resistance strategies have been reported. These include the production of proteases capable of cleaving LL-37 (e.g. SpeB of *Streptococcus pyogenes*, metalloproteases of *Pseudomonas aeruginosa*, gelatinose by *Enterococcus faecalis* and ZapA from *Proteus mirabilis* (Nyberg et al. 2004; Schmidtchen et al. 2002)], membrane modifications (e.g. *Neisseria meningitidis* lipid A modifications (Jones et al. 2009), PmrA-PmrB-based modification of *P. aeruginosa* LPS structure (McPhee et al. 2003)) and the capacity of *Shigella* spp. to downregulate hCAP18/LL-37 production by host cells (Islam et al. 2001) [a process that could be counteracted by the therapeutic use of phenylbutyrate (Sarker et al. 2011)].

In specific protected environments, such as leukocyte phagolysosomes, high concentrations of peptide and controlled ionic environment may be well suited for direct effects on bacterial pathogens (Rosenberger et al. 2004; Martineau et al. 2007). In addition, alterations to in vitro bacterial culture conditions, designed to more closely mimic those present in mammalian tissues by increasing carbonate concentration, can alter the sensitivity of *S. aureus* and *E. coli* to LL-37 (Dorschner et al. 2006). This suggests that adaptations occurring in invading organisms may increase their susceptibility to innate microbicidal CHDP defences in vivo. Furthermore, LL-37 can act synergistically with other CHDP (Chen et al. 2005) and has been shown to synergise with conventional antibiotics (Leszczynska et al. 2010). However, the mechanisms of antiviral activity are less clear, and LL-37 had protective effects in a mouse model similar to that of a current first line anti-influenza therapeutic, despite very modest in vitro antiviral activity (Barlow et al. 2011). Furthermore, at mucosal surfaces in vivo, the capacity of LL-37 to play a fundamentally microbicidal role seems unlikely given the expression levels of LL-37, the presence of serum proteins, DNA and F-actin and the concentrations of cations. It is in these contexts that the additional bioactivities of LL-37 may prove to be of greatest significance to host defence.

4 Modulation of Cytokine Expression

Mammalian cells respond to a range of different microbial components or pathogen-associated molecular patterns (PAMPs) via innate pattern recognition receptors (PRR) including Toll-like receptors (TLR), RIG-I-like receptors (RLR)

and nucleotide-binding domain leucine-rich repeat containing receptors (NLR) [reviewed in (Kawai and Akira 2010)]. LPS and lipoteichoic acid (LTA) from gram-negative and gram-positive bacteria are powerful, well-studied proinflammatory PAMPs that can be released by dying bacteria. These PAMPs can activate leukocytes and epithelial cells to promote an initially protective inflammation, but can induce harmful inflammation and sepsis. The properties of LL-37 appear to extend beyond pathogen killing, to include mopping up and detoxifying liberated endotoxin upon microbial death to limit damage to host tissues. LL-37 has been shown to bind and neutralise both LPS and LTA and to modulate downstream TLR signalling, downregulating expression of PAMP-induced genes (Nagaoka et al. 2001; Scott et al. 2002; Rosenfeld et al. 2006; Mookherjee et al. 2006), even when the peptide was not applied for up to 90 min after PAMP stimulation (Scott et al. 2002). Interestingly these effects are observed at peptide concentrations lower than those required for microbicidal activity (typically 1–5 μg/ml). However, these modulatory effects of LL-37 appear to be PRR specific. LL-37 inhibited TLR4 and TLR2/1 agonists but not TLR2/6, TLR5, TLR7 and TLR8 agonists in peripheral blood mononuclear cells (Molhoek et al. 2009). Furthermore, LL-37 complexed with self-DNA and self-RNA can induce TLR7-, TLR8- and TLR9-dependent inflammatory responses in dendritic cells (Lande et al. 2007; Ganguly et al. 2009), LL-37 upregulated TLR9 expression and induced type I IFN responses in keratinocytes independent of DNA-LL-37 complex formation (Morizane et al. 2011), and LL-37 has been proposed to enhance (Lai et al. 2011) or inhibit (Hasan et al. 2011) TLR3-dependent responses to viral RNA or synthetic mimics. The precise points in the TLR signalling pathways at which LL-37 functions have not been clearly defined to explain all these functions, but the anti-inflammatory activities presumably underpin the protective effects of LL-37 in animal models of sepsis (Cirioni et al. 2006, 2008). The use of analogues and truncated peptides has demonstrated that the LPS-neutralising activity of LL-37 resides primarily in the C-terminal portion of the peptide and resulted in the generation of a 24-amino-acid peptide derivative with similar efficacy to LL-37 in terms of LPS and LTA neutralisation, but lower proinflammatory activity (Nell et al. 2006). These studies highlight the potential for development of cathelicidin-based peptides as novel anti-endotoxic therapeutics.

Inflammatory responses induced by PAMPS are driven by classic proinflammatory cytokines (e.g. TNF-α) and by chemokine-dependent recruitment of leukocytes. Interesting, while LL-37 can inhibit PAMP-induced TNF-α responses, it can also promote the production of chemokines [e.g. IL-8, MCP-1; (Scott et al. 2002; Tjabringa et al. 2003; Braff et al. 2005; Mookherjee et al. 2006; Filewod et al. 2009)] and has potent direct chemotactic properties for neutrophils, monocytes, memory T cells and mast cells in vitro and in vivo (Yang et al. 2000; Niyonsaba et al. 2002; Tjabringa et al. 2006; Kurosaka et al. 2005; Soehnlein et al. 2008). In addition LL-37 can induce degranulation in mast cells, resulting in the release of histamine, prostaglandin D2 and leukotriene B4, increasing vascular permeability and further promoting infiltration of leukocytes to the site of inflammation

(Niyonsaba et al. 2001). While optimal induction of chemokine production by monocytes, epithelial cells and keratinocytes occurs at ~25–50 μg/ml and involves activation of MAPK pathways (Tjabringa et al. 2003; Bowdish et al. 2004), the optimal direct chemotactic activity is observed in response to 2–25 μg/ml and functions through FPRL-1, CXCR2, MrgX2 and perhaps other unidentified G-protein-coupled receptors (Yang et al. 2000; Niyonsaba et al. 2002; Kurosaka et al. 2005; Zhang et al. 2009; Subramanian et al. 2011). Importantly, in contrast to the microbicidal properties, the chemotactic properties of LL-37 are not inhibited by serum (Yang et al. 2000).

LL-37 has also been shown to enhance responses to IL-1β and GM-CSF in peripheral blood mononuclear cells, but antagonise the responses to IFN-γ, IL-4 or IL-12 (Yu et al. 2007), to promote caspase-1-dependent posttranslational processing and release of IL-1β by LPS-primed monocytes (Elssner et al. 2004) and induce a caspase-1-independent processing of IL-18 from keratinocytes acting synergistically with β-defensins (Niyonsaba et al. 2005). These functions all suggest that, rather than being conventionally anti-inflammatory or proinflammatory, LL-37 can "rebalance" inflammatory responses in a concentration- and stimulus-dependent manner. Such complexity highlights the need to examine the effects of potential cathelicidin-based therapeutics in a pathogen-specific manner.

5 Leukocyte Differentiation and Function

The nature and extent of any inflammatory response is dictated by the functional properties of the participating innate and adaptive immune effector cells, including neutrophils, macrophages, monocytes, dendritic cells and lymphocytes. The appropriate responses of these cells, and the resolution of their responses, are critical to the successful outcome of an inflammatory response while avoiding host damage and chronicity. In addition to roles in the chemotaxis and cytokine responses of these effector cells, LL-37 also has the capacity to alter their differentiation and function in a number of other important ways.

Neutrophils are the key, innate immune effector cells that are the major cellular constituent of the early-phase response to inflammatory stimuli. In keeping with observations in other cells types, LL-37 can both promote neutrophil IL-8 responses in a MAPK p38 and extracellular signal-regulated kinase (ERK)-dependent manner (Zheng et al. 2007) and inhibit cytokine responses to Toll-like receptor (TLR) agonists and whole bacteria (Alalwani et al. 2010). However, in addition, recent studies have shown that exposure to 5–20 μg/ml of LL-37 can induce dose-dependent increases in neutrophil intracellular calcium mobilisation (Zheng et al. 2007; Zhang et al. 2009), induce the generation of reactive oxygen species [ROS; (Zheng et al. 2007)] and/or amplify ROS production in response to PMA or whole bacteria (Alalwani et al. 2010). Significantly decreased ROS production in *Camp*$^{-/-}$ murine neutrophils underscores the role of the endogenous peptide in this process (Alalwani et al. 2010). Given the importance of ROS as effector molecules in the

direct microbicidal function of neutrophils and the additional capacity of LL-37 to enhance neutrophil phagocytosis (Alalwani et al. 2010), these results suggest that LL-37 can prime and enhance neutrophil antimicrobial functions. Furthermore, LL-37 was shown to induce expression and release of human α-defensins (human neutrophil peptides 1–3) from live and apoptotic neutrophils (Zheng et al. 2007; Li et al. 2009). These α-defensins have recently been shown to also have effective anti-inflammatory properties in vitro and in vivo (Miles et al. 2009) and are likely to act in concert with the inflammomodulatory effects of LL-37 to modify the responses of macrophages and other cells.

LL-37 has been clearly shown to modulate the inflammatory responses of macrophages and monocytes, as described earlier; however, LL-37 is also capable of modulating macrophage differentiation (van der Does et al. 2010). While LL-37 exposure during the in vitro generation of human monocyte-derived macrophages (MDMs) promoted a more proinflammatory M1 phenotype, LL-37 could also redirect fully M2 phenotype-differentiated MDMs to produce more IL-12p40 and less IL-10. This bioactivity of LL-37 was localised to the C-terminus of the peptide, and LL-37 internalisation by the cells was necessary to modulate phenotype. In addition, the vitamin D-regulated antimycobacterial activity of human monocytic cells, attributed in part to the activity of CHDP (Martineau et al. 2007; Sonawane et al. 2011), has recently been demonstrated to involve LL-37-mediated autophagy of the infected cells (Yuk et al. 2009). Expression of LL-37 was shown to be critical both for the infection-induced transcription of autophagy-related genes Beclin-1 and Atg5, and for the colocalisation of mycobacterial phagosomes with autophagosomes. These studies demonstrate that both LL-37 expression by monocytic cells and the exposure of these cells to external sources of this peptide can modulate the antimicrobial and immunomodulatory properties of these cells.

In addition to their multiple roles in innate immunity, it is becoming clear that CHDP can modulate the adaptive immune response (reviewed in Bowdish et al. 2005a). Immunisation of mice with a plasmid fusing LL-37 to a tumour antigen generated enhanced antigen-specific humoral and cytotoxic responses and prolonged survival in a tumour model in vivo (An et al. 2005). LL-37 fusion plasmids were found to be significantly more effective than the tumour antigen plasmid alone, or co-administration of separately encoded plasmids for LL-37 and the tumour antigen, but the mechanisms remain unclear. Direct modulation of lymphocyte activity and/or proliferation, although demonstrated for defensins (Tani et al. 2000), is not a reported property of LL-37. Indirect mechanisms, such as alteration of the local cytokine environment should be considered, but a likely explanation may be found in the effects of LL-37 on dendritic cell (DC) differentiation and function. LL-37 has been shown to modulate DC differentiation from monocytic precursors in vitro, with LL-37-primed DC displaying significantly upregulated endocytic capacity, modified phagocytic receptor expression and function, upregulated co-stimulatory molecule expression (including CD86 expression in the absence of DC maturation) and enhanced Th-1 responses in vitro (Davidson et al. 2004), as well as modifying the nature of adaptive immune responses in vivo (Davidson, Schwarze, Wang, unpublished data). LL-37 therefore has the capacity to induce the differentiation

of immature DC "primed" to skew the nature of the adaptive response. Thus, LL-37/tumour antigen fusion proteins may function by delivering both the target for the adaptive immune response and a CHDP to generate a "primed" DC to same cell in a temporally appropriate manner for an enhanced adaptive response. These effects of LL-37 involved signalling via an unidentified G-protein-coupled receptor (Davidson et al. 2004), while related DC phenotype-modulating properties have been shown to require internalisation of LL-37 by the DC (Bandholtz et al. 2006). In addition to the effects of LL-37 on DC differentiation, LL-37 has been shown to inhibit LPS-induced maturation of differentiated DC (Kandler et al. 2006) in a manner consistent with its anti-endotoxic activities, but to promote DC activation in response to DNA and RNA (Lande et al. 2007; Ganguly et al. 2009). In the latter studies, LL-37 was demonstrated to bind non-inflammatory self-DNA and self-RNA and promote its uptake into DC in a manner that resulted in retention in early endocytic vesicles and activation of both plasmacytoid and myeloid DC, via TLR7, TLR8 and TLR9. These findings suggest a possible mechanism by which dysregulation of or exposure to high levels of LL-37 might be involved in breaking self-tolerance and driving autoimmunity in psoriasis and SLE. However, the initiation of LL-37 overexpression in psoriasis remains unclear as do the mechanisms by which tolerance is maintained in the context of inflammatory levels of LL-37 and dead cells in the healthy individual. These studies demonstrate the capacity of LL-37 to modulate DC differentiation and function in an inflammatory environment and reiterate the contrasting effects of this cathelicidin on cellular responses to diverse stimuli.

It is therefore clear that by modifying the influx, functional responses and differentiation of inflammatory effector cells, LL-37 can orchestrate and modulate responses to infectious and inflammatory signals. However, in addition to these properties, recent studies have demonstrated that this peptide can also influence inflammation through effects on cell death.

6 Modulation of Cell Death

Although CHDP can rapidly permeabilise prokaryotic membranes, most natural peptides are relatively less toxic to eukaryotic cells, an observation proposed to relate to the essentially neutral outer surface of eukaryotic membranes and their cholesterol content (reviewed in Lai and Gallo 2009). This affords host cells a degree of protection from the lytic effects of such peptides. However, negatively charged erythrocytes are more susceptible, presenting a challenge in the design of novel therapeutic derivatives (reviewed in Burton and Steel 2009), and CHDP can be cytotoxic to mammalian cells in a manner specific to cell type and its concomitant stimuli.

LL-37 has long been known to have cytotoxic effects on peripheral blood leukocytes at concentrations above 125 μg/ml, even in the presence of 10 % foetal bovine serum [FBS; (Johansson et al. 1998)], but it was unclear whether this death was due simply to primary necrosis resulting from peptide-induced membrane

damage or an induction of programmed cell death. LL-37 can enter eukaryotic cells by an active process requiring endocytic machinery (Lau et al. 2005) and can facilitate the cellular entry of nucleic acids (Sandgren et al. 2004; Zhang et al. 2010) and DNA dyes (Elssner et al. 2004; Tomasinsig et al. 2008) without inducing cell lysis, suggesting temporary membrane disruption or pore opening mediated by this cathelicidin in live cells. Exposure to higher concentrations of LL-37 can induce apoptosis of airway epithelial cells in a dose-dependent manner (with substantial cell death at $\geq$50 μg/ml) in vitro and in murine airway epithelial cells in vivo (Lau et al. 2006; Barlow et al. 2006). This induction of cell death by high concentrations of LL-37 involves Bax translocation to the mitochondria and is partially dependent on caspases (Barlow et al. 2006, 2010). The presence of high-density lipoproteins from human serum could block entry of LL-37 into the epithelial cells, inhibiting this LL-37-induced cell death and the IL-8 production by these cells (Lau et al. 2006). LL-37 has also been shown to induce death in Jurkat T leukaemia cells, although requiring exposure to higher concentrations of peptide (50–200 μg/ml). This was demonstrated to be mediated via a caspase-independent and calpain- and AIF-dependent apoptosis that involved Bax activation and translocation to the mitochondria (Mader et al. 2009), but also associated with significant levels of necrosis (with propidium iodide entry into the cells) at the higher peptide concentrations in another study (Aarbiou et al. 2006). However, no cell death was induced in primary human lymphocytes or monocytes, at more physiologically relevant levels of LL-37 (up to 50 μg/ml) in the presence of 10 % FBS (Davidson et al. 2004; Bowdish et al. 2004). Furthermore, LL-37 has been found to protect primary keratinocytes from induction of apoptosis by camptothecin, an effect mediated by a cyclooxygenase-2-dependent mechanism involving production of inhibitor of apoptosis 2 protein (Chamorro et al. 2009), and to inhibit tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in intestinal epithelial cells in vitro (Otte et al. 2009), demonstrating the cell-type specificity of cathelicidin-mediated effects on cell death.

The extent to which direct induction of eukaryotic cell death at high peptide concentrations might modulate innate or adaptive immune responses in vivo remains unclear. However, a recent study has demonstrated that more physiological, inflammatory concentrations of LL-37 (10–30 μg/ml) can preferentially induce death in airway epithelial cells that have been infected with *P. aeruginosa* (Barlow et al. 2010). This enhanced susceptibility of infected cells to peptide-induced death was associated with mitochondrial depolarisation and a later-stage activation of caspase-3 and caspase-9 that were only observed in the presence of both peptide and infection, and required invasion of the epithelial cell by live bacteria. The internalisation of *P. aeruginosa* by epithelial cells and the induction of apoptosis in infected pulmonary cells in vivo have been proposed to be important in innate host defence against this organism (Pier et al. 1997; Grassme et al. 2000). In addition, apoptosis in the context of infection has been proposed to modulate the nature of subsequent adaptive immune responses, promoting a TH17 response (Torchinsky et al. 2009). However, intriguingly LL-37 has also been shown to mediate increased epithelial cell stiffness, diminishing intracellular localisation of *P. aeruginosa*

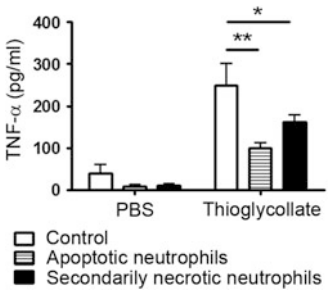

Fig. 2 *LL-37-induced secondarily necrotic neutrophils are anti-inflammatory in thioglycollate-induced sterile peritonitis*. 6–8-week-old Balb/c mice were injected intraperitoneally with 0.5 ml 10 % thioglycollate or 0.5 ml PBS concomitantly with human neutrophils, either (1) control-no neutrophils, (2) apoptotic neutrophils or (3) neutrophils previously induced to undergo post-apoptotic secondary necrosis by exposure to 25 μg/ml LL-37. Peritoneal lavage collected at 3 h post-injection was evaluated for TNF-α by ELISA. Significance was assessed with two-way ANOVA with Bonferroni's multiple comparison post-tests used to compare mice with and without PMN injection under the same conditions (PBS/thioglycollate); *, $p \leq 0.05$; ***, $p \leq 0.001$, $n = 4$ per group

(Byfield et al. 2011a) Thus, LL-37 may contribute to innate defence against epithelial cell-invading microbes by diminishing epithelial cell invasion and by inducing the death of infected, compromised epithelial cells to both deny microbes a safe niche for replication and invasion of the host tissue and modulate the nature of subsequent adaptive immune responses.

The control of cell death is critical in maintaining homeostasis and in host responses to infection and inflammation but also for the resolution of inflammatory responses. Despite the key roles played by neutrophils in innate immunity, uncontrolled or persistent neutrophilia is detrimental to the host. Neutrophils undergo spontaneous apoptosis and have a short half-life that can be modulated by a broad range of substances, including bacterial products (e.g. LPS) and cytokines (e.g. GM-CSF) (Bianchi et al. 2006). Control of neutrophil death and the anti-inflammatory effect that apoptotic neutrophils have on phagocytosing macrophages are critical in the resolution of inflammatory responses (Savill et al. 2002). LL-37 can antagonise the effects of LPS on neutrophil survival (Li et al. 2009) and has been shown to modulate neutrophil death directly. Although initially proposed to be an inhibitor of neutrophil apoptosis (Nagaoka et al. 2006; Barlow et al. 2006), the principal effect of LL-37 is the rapid induction of secondary necrosis of apoptotic neutrophils, occurring at concentrations of peptides as low as 1 μg/ml (Li et al. 2009; Bjorstad et al. 2009; Zhang et al. 2008). This property was retained by C-terminal partial peptides and was also evident for mCRAMP. In contrast to expectation, LL-37-induced secondarily necrotic neutrophils had anti-inflammatory effects in vitro on activated macrophages (Li et al. 2009) and in vivo in a murine thioglycollate-induced sterile peritonitis (Fig. 2). The maximal anti-inflammatory effects were observed in association with LL-37-mediated release of granule contents from the apoptotic cells, induced by exposure to higher concentrations of LL-37 (25 μg/ml). These effects were independent of the anti-endotoxic activity of the

peptide used to induce secondary necrosis (Li et al. 2009) and may result from the release of both LL-37 and α-defensins from the apoptotic neutrophils (Miles et al. 2009). Although other granule contents could have deleterious effects, LL-37-mediated release of CHDP from apoptotic neutrophils may enhance the apoptosis-driven resolution of inflammation.

Thus, the capacity of LL-37 to modulate the induction of cell death and modalities of death should be considered as one of the inflammomodulatory properties of this cathelicidin. Interestingly these properties are complemented by peptide-mediated enhancement of cell proliferation, indicating that LL-37 has the potential to generate both protective cell death and repair in an inflammatory environment.

7 Cellular Proliferation and Angiogenesis

The expression of LL-37 is upregulated at sites of wounding and has been shown to play roles in cell proliferation, wound healing and angiogenesis. hCAP18/LL-37 was found to be strongly expressed in healing skin, but absent from chronic skin ulcers, and to promote re-epithelialisation of wounds in organ-cultured human skin (Heilborn et al. 2003). LL-37 and mCRAMP also enhanced re-endothelialisation, limiting neointima formation, after stent implantation (Soehnlein et al. 2011). Intriguingly this latter observation was still observed in mice lacking active forms of neutrophil-derived serine proteases (proteinase-3, cathepsin G and neutrophil elastase), raising interesting questions about the active product and proteolyic processing of mCRAMP in this system in vivo in these studies. However, mCRAMP has also been found to promote atherosclerosis, with deposition of this cathelicidin on inflamed endothelium mediating enhanced inflammation at these sites (Doring et al. 2012). LL-37 has been shown to induce keratinocyte migration in vitro at concentrations as low as 100 ng/ml [in the absence of serum (Carretero et al. 2008; Tokumaru et al. 2005)], associated with MAPK and matrix metalloproteinase-dependent epidermal growth factor receptor (EGFR) activation, and to enhance re-epithelialisation at skin wound sites in vivo (Carretero et al. 2008). This cathelicidin can also promote fibroblast proliferation (Tomasinsig et al. 2008), but inhibits collagen production by dermal fibroblasts and may have anti-fibrotic properties in wound healing, with the degree of fibrosis in dermal keloids found to be inversely correlated with the expression of hCAP18/LL-37 (Park et al. 2009). Furthermore, in studies using airway epithelial cells, LL-37 promoted wound healing in a dose-dependent manner by stimulating epithelial cell migration and proliferation at concentrations as low as 1 μg/ml, but interestingly only in the presence of serum (Shaykhiev et al. 2005). In addition to these wound healing properties, LL-37 has been shown to induce the proliferation of endothelial cells and neovascularisation in vitro and in vivo, with decreased vascularisation observed during wound repair in $Camp^{-/-}$ mice (Koczulla et al. 2003).

The capacity of LL-37 to modulate cell proliferation has stimulated a number of studies to evaluate the effects of this peptide on tumour growth and metastasis

(reviewed in Wu et al. 2010b). LL-37 derivatives have been proposed to have tumouricidal activity, via induction of apoptosis (Okumura et al. 2004). However, increased expression of hCAP18/LL-37 has been found in breast, ovarian and lung carcinomas (Heilborn et al. 2005; Coffelt et al. 2008; von Haussen et al. 2008), correlating with vascular density (Coffelt et al. 2008), and proposed to be mitogenic, with LL-37-dependent activation of the IGF-1R implicated as a possible mediator of increased migratory and invasive potential of malignant cells (Girnita et al. 2011). Transfection of epithelial cell lines (HEK293 and HaCaT cells) with hCAP18 enhanced cellular proliferation in vitro (Heilborn et al. 2005). Similarly, recombinant LL-37 stimulated proliferation of ovarian cell lines (Coffelt et al. 2008), although this occurred exclusively in the presence of serum and the enhanced proliferation observed at 1 μg/ml LL-37 was lost for two of the three cell lines at higher concentrations of peptide. The growth of anchorage-independent lung carcinoma cell lines in vitro was shown to be enhanced after the addition of ng/ml concentrations of LL-37, but significantly diminished by 20 μg/ml of peptide (von Haussen et al. 2008). In addition, LL-37 has been proposed to promote ovarian tumour progression by enhancing invasion, matrix metalloproteinase expression and the recruitment of multipotent mesenchymal stromal cells (Coffelt et al. 2008, 2009a), and tumours derived from transformed cells injected into nude mice showed significantly faster growth when engineered to overexpress hCAP18 (von Haussen et al. 2008). However, in contrast, exogenous LL-37 demonstrated anti-proliferative properties for gastric carcinoma cells, inducing cell cycle arrest, and had direct anticancer activity in vivo in a gastric cancer xenograft model (Wu et al. 2010a). Thus, although this cathelicidin can clearly impact upon tumour growth in model systems, the cell-type specificity and net effect of its properties in vivo remains to be determined.

8 Mechanisms of Immunomodulatory Activity

The pleiotropic effects of LL-37 in modulation of host defence responses raise questions about the mechanisms that could underpin such a broad array of bioactivities. At the simplest level, the anti-endotoxic properties of LL-37 are at least partly a consequence of direct, charge-based binding of LPS as discussed above, inhibiting interaction between LPS and its binding protein and/or receptor. However, even for this property, additional mechanisms are required to explain the selective LL-37-mediated inhibition of specific LPS-induced proinflammatory genes, without inhibition of LPS-induced genes that antagonise inflammation (Mookherjee et al. 2006), and a variety of receptor-specific and alternative mechanisms for LL-37-mediated immunomodulation have been proposed (Fig. 3).

A classical receptor-ligand mechanism has been proposed for LL-37, functioning through formyl receptor-like 1 (FPRL1), a G-protein-coupled receptor (GPCR). This receptor interaction was initially identified as the mechanism for LL-37-mediated chemotaxis of leukocytes (Yang et al. 2000). FPRL-1 has also been implicated in LL-37-mediated wound healing (Carretero et al. 2008), angiogenesis

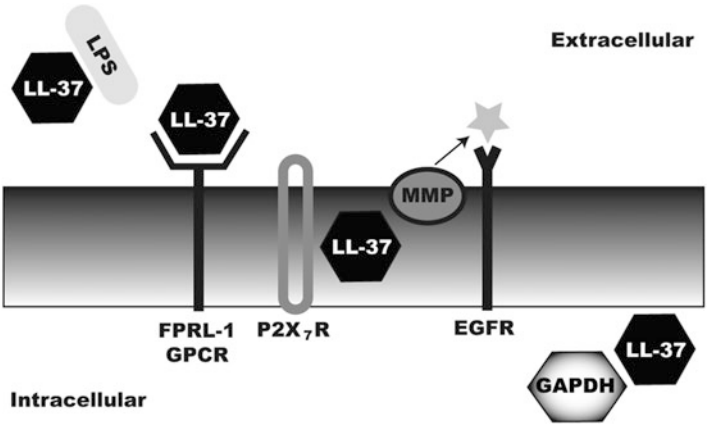

Fig. 3 *LL-37: mechanisms of immunomodulatory activity*. LL-37 may function via (a) direct sequestration of ligand (e.g. LPS), (b) classical receptor-ligand mechanisms, (c) interaction with diverse membrane-bound receptors and (d) intracellular mechanisms

(Koczulla et al. 2003), inhibition of neutrophil apoptosis [in one study (Nagaoka et al. 2006)] and in activating MAPK and enhancing invasiveness of ovarian carcinoma cells (Coffelt et al. 2009b). However, additional mechanisms occurring concomitantly have been implicated for many of these properties, while a recent study has described CXCR2 as an alternative receptor for LL-37-mediated neutrophil and monocyte chemotaxis (Zhang et al. 2009). The GPCR MrgX2 has been identified as a key LL-37 receptor on mast cells, and unidentified other GPCRs have also been proposed as receptors for LL-37 (Lau et al. 2005) and implicated in LL-37-mediated modulation of DC differentiation (Davidson et al. 2004) based on inhibition of LL-37-mediated effects by pertussis toxin. Furthermore, utilisation of GPCR by cathelicidins has been excluded in other studies, implicating alternative mechanisms and receptors, including $P2X_7R$, EGFR, IGF-1R and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

The purinergic receptor $P2X_7R$ has important roles in the regulation of inflammatory processes (Lister et al. 2007). Activation by ATP (described as its principal ligand) reversibly opens large non-selective pores involving $P2X_7R$ and pannexin 1, which can enable ion flux across the cell membrane. The $P2X_7R$ has been identified as responsible for LL-37-mediated posttranslational modification and release of IL-1β from LPS-primed monocytes (Elssner et al. 2004), experimentally implicating LL-37 as an alternative direct ligand for this receptor. $P2X_7R$ activation has also been implicated in LL-37-mediated modulation of neutrophil apoptosis (Nagaoka et al. 2006; Barlow et al. 2006), endothelial cell stiffening (Byfield et al. 2011b) and the mitogenic properties of LL-37 on fibroblast proliferation (Tomasinsig et al. 2008). However, the latter study demonstrated that LL-37 could restore pore-forming activity to a truncated $P2X_7R$, which could not itself generate the classical non-selective pore (Tomasinsig et al. 2008). This activity, its independence from pannexin 1 and the equivalent mitogenic activity of similarly

structured orthologues and the D-enantiomer of LL-37, lead to a proposal of functional interaction between $P2X_7R$ and amphipathic peptides with appropriate helix-forming propensity, mediated by binding of transmembrane segments, and opening pores through mechanisms distinct from that of ATP-stimulated $P2X_7R$. LL-37 has also been proposed to function through activation of metalloproteinases in the cell membrane, by as yet undefined mechanisms, with consequent cleavage of soluble membrane-bound EGFR ligands and transactivation of EGFR. This mechanism has been implicated in LL-37-mediated induction of IL-8 expression (Tjabringa et al. 2003; Braff et al. 2005), wound healing, keratinocyte migration and enhanced cellular proliferation (Carretero et al. 2008; Tokumaru et al. 2005; Shaykhiev et al. 2005). Common to these and other studies is the activation of MAPK pathways by LL-37 (Bowdish et al. 2004); a downstream signalling event that can also be observed following FPRL-1 ligation by LL-37 (Coffelt et al. 2009b) and has can been implicated in LL-37-mediated modulation of TLR responses (Molhoek et al. 2009). The potential for LL-37 to modulate multiple signalling processes via interactions with transmembrane domains of diverse membrane-bound receptors may help to explain its pleiotropy and the apparently key nature of the amphipathicity of this peptide, irrespective of helical sense (Braff et al. 2005; Tomasinsig et al. 2008). However, a role for promiscuous receptors cannot be excluded and other properties of LL-37 require peptide entry into the eukaryotic cell. These include the induction of chemokine expression (Lau et al. 2005), altered MDM/DC differentiation (van der Does et al. 2010; Bandholtz et al. 2006) and peptide-mediated cell death (Lau et al. 2006). The identification of GAPDH as a novel intracellular receptor for LL-37 (Mookherjee et al. 2009) may be significant in this regard, but the full extent of intracellular effects mediated by this peptide and the mechanisms involved remain to be determined. Membrane integration of cathelicidin in the absence of peptide internalisation by the cell might also be fundamental to the cathelicidin-mediated induction of secondary necrosis in apoptotic membranes (Li et al. 2009; Bjorstad et al. 2009). Clearly the mechanisms of immunomodulation employed by LL-37 are complex and may be atypical, and elucidation will be important to furthering our understanding of these intriguing peptides.

9 Conclusions

The sole human cathelicidin hCAP18/LL-37 is a multifunctional CHDP with direct microbicidal potential and the capacity to modulate inflammation and immune responses through a broad range of mechanisms. It has been implicated in host defence and disease pathogenesis in multiple systems and conditions and represents both a fascinating target for clinical intervention and promising template for the development of novel antimicrobial, immunomodulatory therapeutics. Early clinical trials using synthetic analogues of CHDP were designed to maximise microbicidal activity, but achieved only moderate efficacy (Lipsky et al. 2008),

perhaps due to failure to recognise the importance of their immunomodulatory functions. A recent approach, using non-microbicidal analogues that retained other bioactive functions, has demonstrated effective host defence augmentation in mice (Scott et al. 2007). These studies suggest that realising the full therapeutic potential requires further research to more clearly understand the precise mechanisms of action underpinning the inflammomodulatory and immunomodulatory properties and the in vivo effects of these peptides' pleiotropic functions in specific clinical conditions.

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Wound Repair and Antimicrobial Peptides

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Abstract Wounding of protective barriers is a major insult to the organism and immediately sets in motion a complex cascade of cellular responses in order to re-establish tissue integrity. Antimicrobial proteins, AMPs, are important components in the innate immune system and play a vital role in this process. The defensin family of proteins and the human cathelicidin, hCAP18, are the most documented AMPs in human and the focus of this review. Still, many proteins display antimicrobial activity, suggesting that the capacity to defend against microbes has been a driving force during evolution. In addition to direct killing of microbes, AMPs are involved in the inflammatory reaction through chemotaxis and control of cytokine response. Furthermore, recent data also show that AMPs have growth-factor like effects and signal via receptors promoting angiogenesis and re-epithelialization. Thus, the role of AMPs in wound healing is only beginning to be understood and may be far-reaching.

Abbreviations

AMP	Antimicrobial protein
EGFR	Epidermal growth factor receptor
FPRL1	Formyl peptide receptor-like 1
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	Mitogen activated kinases
P2X7	Purinergic receptor
PI3K	Phosphoinositide 3-kinase
PDC	Plasmocytoid dendritic cell

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SLPI Secretory leukocyte protease inhibitor
TLR Toll-like receptor
TGF-β Transforming growth factor beta

1 Introduction

Epithelia are barriers that protect the organism from the hostile environment, shielding self from non-self. Wounding of such barriers represents a major threat and immediately evokes a plethora of responses to restore the integrity of the tissue. Wound healing in higher organisms is a fundamental and complex biological process that has been fine-tuned during evolution. It may seem like a paradox that this process in some aspects represents a compromise, such as higher organisms are unable to repair the tissue to completion. In this sense we have traded perfection for speed and the cost is scarring. That being said, wound healing is a beautifully and precisely orchestrated process that integrates fundamental pathways and recruits the most powerful players of the immune system including antimicrobial proteins.

Physiologic wound repair is characterized by sequential and partly overlapping phases that can be schematically divided into initial homeostasis tightly linked to the inflammatory phase which is followed by the proliferative phase during which new granulation tissue is formed and the epithelium is regenerated. The whole process is completed during an extended remodelling phase when excess tissue is removed and the mature scar is formed. Precisely how these phases are integrated and regulated at the molecular level is not yet completely understood (Li et al. 2007). Delayed or impaired wound healing is a significant cause of morbidity and a more detailed knowledge about the mechanisms involved would facilitate the development of effective treatments which today are largely lacking (Menke et al. 2007).

In the early 1990s Gallo et al. reported that the pig cathelicidin PR-39 was capable of inducing proteoglycans in wounds and speculated on a putative impact on wound healing (Gallo et al. 1994). Since then accumulating data from several groups do support the notion that antimicrobial proteins, AMPs, are important in this process. Their potential roles are many, and may vary during the different phases of wound repair and also according to the tissue affected. We will go through the temporal sequences of the repair process and discuss which AMPs might be involved and how. In the present paper we will concentrate on skin wound healing and the AMPs that are present in skin, but also briefly discuss wound-related APMs in other epithelia such as the lung and cornea.

2 AMPs in Skin

An increasing number of proteins with antimicrobial activity have been identified in human skin. The source of AMPs varies, they are produced by structural resident cells as well as by infiltrating migratory cells. In neutrophils, AMPs are stored in preformed granules and released upon demand and in other cells such as keratinocytes and other leukocytes there is de novo synthesis. In addition to the "classical" AMPs, such as the human cathelicidin, hCAP18 and the defensins, an impressing list of molecules with other established biological functions such as neuropeptides, chemokines and protease inhibitors have now also been coined as antimicrobial proteins. One might speculate that antimicrobial activity has been a critical driving force during evolution and that this property has conferred a survival advantage for proteins involved in microbial defense, which thus has lead to their enrichment in the human genome. Subsequently, such proteins may have evolved to function in more complex biological pathways and whether they are classified as AMPs or otherwise would then depend on which function was discovered first. Current findings that cathelicidins and defensins in addition to their direct antimicrobial activities, also have effects on host cells and participate in receptor mediated signalling support this hypothesis.

2.1 Cathelicidins

The cathelicidins belong to a family of mammalian proteins that share a conserved cathelin-like domain at the N-terminus and a highly variable cationic C terminal peptide, typically 20–40 amino acids, that is proteolytically released through the activity of serine proteinases (Sorensen et al. 2001; Yamasaki et al. 2006). For a detailed background, the reader is referred to comprehensive reviews (Zanetti et al. 2000; Radek and Gallo 2007). In humans there is a single cathelicidin gene, wheras in other species such as pigs and cattle, there are multiple. The human cathelicidin, hCAP18 releases a 37 a-a long mature peptide, LL-37, which has broad antimicrobial activity as well as other biological effects (Agerberth et al. 1995; Cowland et al. 1995; Zanetti et al. 1995). Cathelicidin derived peptides display features that are common among antimicrobial proteins such as net positive charge and an amphipathic structure to allow interaction and disruption of the microbial membrane. At high concentrations, the peptide exhibits in vitro toxicity also towards eukaryotic cells. However, multiple factors in the tissue such as serum components protect the host cells from direct exposure to the peptide and net toxicity in vivo is thus unclear (Sorensen et al. 1999). Mammalian cathelicidins were initially identified from neutrophils and are now known to be constitutively expressed in most leucocytes. In epithelia, there is induction upon demand, such as inflammation, injury and infection (Frohm et al. 1997).

2.2 Defensins

Defensins are cystein-rich AMPs that fall into three distinct categories: α-defensins, β-defensins and θ-defensins. The mature peptides share features such as disulfide bridges. α-defensins are constitutively produced by neutrophils (HPN1-4), Paneth cells of the gut (HD5 and HD6) and epithelial cells of the female reproductive tract (HD5). In humans, β-defensin, hBD1-4, have been primarily identified in epithelial cells including skin, whereas the circular θ-defensins are not produced in humans. For a detailed background please be referred to selected reviews (Ganz 2003; Selsted 2004). Most defensins show broad antimicrobial activity against bacteria, fungi and some viruses and act by permeabilization of microbial membranes. In addition, like cathelicidins, the defensins also have effects on host cells and are involved in diverse inflammatory responses linking the innate and the adaptive immune systems.

3 The Wound Repair Process

3.1 The Acute Inflammatory Phase

Upon injury, the physical barrier is disrupted exposing the organism to potential hazardous invasion of microorganisms. This sets off the innate immune system where the AMPs are key players. In intact uninjured skin epithelium there is only low constitutive expression of AMPs, but following injury, these are rapidly upregulated (Frohm et al. 1997; Dorschner et al. 2001; Heilborn et al. 2003). The efficient and timely production of AMPs in skin and other epithelia is likely of major importance in the protection against pathogens in vivo, even though it has proven challenging to provide conclusive in vivo evidence. Support for the notion that functional antimicrobial effectors are critical in acute wounding was suggested by experimental data from mice with a disrupted cathelicidin gene, that were unable to efficiently eliminate a bacterial pathogen and developed large and necrotic ulcerations upon microbial challenge (Nizet et al. 2001). In human, it was recently shown that patients with atopic dermatitis fail to upregulate hCAP18 upon wounding which may underlie the susceptibility to infections in their skin so often traumatized by scratching (Mallbris et al. 2010). Interestingly, several studies have shown that the different AMPs have slightly different antimicrobial profiles and may act in synergy (Nagaoka et al. 2000). Their mode of action differs, but involves non-specific direct interaction with the microbial cells membranes.

The acute inflammatory response following injury typically lasts up to 48 h, but may persist longer. Following the initial hemostasis, there is influx of leukocytes into the tissue, first the neutrophils which are a rich source of antimicrobial molecules, proteases and reactive oxygen species. Experimental data show that neutrophils migrating into an acute wound induce a transcriptional program that

orchestrates the repair process (Singer and Clark 1999). However, neutrophils may function as a double-edged sword, when exaggerated, the inflammatory response will be detrimental to healing. Animal studies actually suggest that depletion of neutrophils does not delay repair of sterile incisional wounds, but rather accelerates re-epithelialization. Following the neutrophils, monocytes, which differentiate into macrophages, become after a few days the dominating cell type in the wound tissue. Several factors in the wound environment, including fibrin degradation products, proteases, cytokines and notably also AMPs, contribute to the recruitment of these inflammatory cells. LL-37 as well as the defensins exhibit significant chemotactic activity (Agerberth et al. 2000; De Yang et al. 2000). To further facilitate the recruitment of migratory cells, AMPs also have the capacity to upregulate chemokines and chemokine receptors (Scott et al. 2002; Lai and Gallo 2009). AMPs also function as important modulators linking the innate immediate response and the adaptive immune system (Davidson et al. 2004). In this way AMPs may modulate and amplify the inflammatory response following injury.

Ideally, the acute inflammatory reaction is self-limiting and should prepare the wound for healing. When the inflammatory phase is prolonged and becomes chronic, it will have a detrimental effect on the repair process There will be excessive tissue degradation through the sustained activation of matrix metalloenzymes (Wysocki et al. 1993) and a reduced concentration of factors to promote cell proliferation and formation of new tissue (Falanga 1992). The causes for failing to limit the inflammatory response in the chronic ulcer are manyfold and not entirely known but likely involve the presence of bacteria. Here, AMPS may play an important role not only through direct killing of microbes, but also in their capacity to neutralize microbial derived products such as lipopolysaccharide, LPS, and lipoteichoic acid, LTA, thus inhibiting subsequent cellular responses which may perpetuate the inflammatory reaction (Scott et al. 1999; Larrick et al. 1994). In line with these findings are recent data demonstrating that LL-37 actually suppresses the production of proinflammatory cytokines from dendritic cells exposed to microbial stimuli, such LPS, LTA and flagellin (Kandler et al. 2006; Mookherjee et al. 2006). Still, the net biologic effects exerted by AMPs are complex and vary between target cells. In lung epithelial cells, it was recently shown that the cathelicidin LL-37 rather augments the inflammatory response upon microbial exposure through promoting the uptake of LPS with subseqeuent production of proinflammatory cytokines (Shaykhiev et al. 2010). Clearly, in vitro findings are difficult to translate into physiologic effects in vivo and the overall biologic consequences will be defined by the microenvironment and the temporal and spatial context.

3.2 Regulation of AMPs and TLRs

Toll-like receptors, TLRs, are key molecules in the innate immune system mediating critical signals during injury. TLRs are typically activated in epithelial barriers upon microbial exposure and in turn induce the expression of AMPs.

Specifically, activation of epithelial cells with agonists for several TLRs: TLR2, TLR4 (Wang et al. 2003; Vora et al. 2004), TLR 5 and TLR9 (Miller et al. 2005) can induce the expression of human beta-defensins which is dependent on NF-kB signalling and activation of TLR9 stimulates the production of hBD-2 in airway epithelium (Platz et al. 2004).

The main transcriptional regulator of hCAP18/LL-37 in human skin (Weber et al. 2005) is 1,25-dihydroxyvitamin D3 that binds directly in the promoter region of the hCAP18 gene. It was recently shown that in addition to upregulating the expression of AMPs, injury potentiates the function of TLR2 through a vitamin D dependent mechanism. (Schauber et al. 2007). The importance of vitamin D was also stressed by recent data showing that vitamin D treatment even further enhances the expression of hCAP18 in acute wounds (Heilborn et al. 2010). Thus, vitamin D and AMPs produced in the same epithelial cells constitute a powerful biological unit serving to protect and repair the skin barrier if needed.

In contrast to skin, the expression of hCAP18 is differentially regulated in the gastrointestinal epithelium where short fatty acids but also microbial DNA control the production of hCAP18/LL-37. This emphasizes the vital role of the microenvironment in controlling AMP production and how AMPs have evolved to make optimal use of the specific conditions in different biological niches (Schauber et al. 2003, 2004; Islam et al. 2001).

Recently, it was shown that plasmocytoid dendritic cells (PDCs) rapidly infiltrate the skin upon injury (Gregorio et al. 2010). PDCs are a rare population of circulating cells not found in normal intact/non-inflammatory skin and which are involved in the antiviral defense through production of large amounts of type 1 interferons. Now it was shown that PDCs may be important in mediating early inflammatory responses following injury and also to promote re-epithelialization of skin wounds. LL-37 peptide is key in activating and recruiting PDCs into the skin and the production of hCAP18/LL-37 at the wound site may thus further support wound healing through this mechanism (Lande et al. 2007).

3.3 Angiogenesis

Formation of new tissue requires new blood vessel formation and angiogenesis is an integral component of wound healing. Relevant to this process were findings that hCAP18/LL-37 directly stimulated the proliferation of endothelial cells and supported functional neoangiogenesis in an animal model (Koczulla et al. 2003). The mechanism that was proposed for mediating this effect involved binding of LL-37 to the G protein-coupled receptor formyl peptide receptor-like 1, FPRL1, expressed on endothelial cells, since blocking the receptor through neutralizing anti-FPRL antiserum and by pertussis toxin abolished the effect. Downstream signalling seemed to involve the mitogen activated kinase pathway, MAPK and to some extent also the phosphoinositide 3-kinase, PI3K, pathway (Koczulla et al. 2003).

Angiogenetic capacity was recently demonstrated also for HBD-2,that was shown to stimulate migration and proliferation as well as tube formation of endothelial cells (Baroni et al. 2009). The in vivo relevance and relative importance for AMPs in supporting the outgrowth of new blood vessels during wound healing however, remains to be established. Considerable biologic redundancy is likely at play, and as multifunctional proteins, AMPs may be team-players participating in this process.

3.4 Re-epithelialization

Re-epithelialization begins within hours after wounding and is a critical feature of the repair process since formation of a new protective interface re-instates the integrity of the organism. The epithelial cells undergo a profound phenotypic change, loose their attachment to the surrounding tissue and neighbouring cells and move laterally into the wound. Initial migration is followed by proliferation. The precise molecular signals regulating these processes are not completely known but involve local release of growth factors from infiltrating cells, fibroblast and keratinocytes. In order to allow efficient migration through the wound, the production of tissue degrading enzymes such as matrix metalloproteinases is important. A balance between degradation and tissue formation is needed to avoid excessive degradation, which is thought to be at play in non-healing ulcers.

During recent years several studies have reported on the involvement of AMPs in re-epithelialization and wound closure, stimulating the migration as well as the proliferation of the epithelial cells. Wounding of mucous membranes show accelerated healing rate as compared to skin and histatins in human saliva, which are known antifungal agents, have been shown to stimulate wound closure through a receptor mediated mechanism and downstream activation of the ERK pathway (Oudhoff et al. 2008).

In human skin, it was shown that hCAP18 is produced in the migrating epithelial cells in sterile ex-vivo wounds that heal in cell culture which demonstrated that inflammation is not a prerequisite for the induction of hCAP18 in skin (Heilborn et al. 2003) Furthermore it was shown that re-epithelialization was blocked in a concentration dependant manner when adding anti-hCAP18 antiserum to the tissue (Fig. 1). The lack of hCAP18 immunoreactivity in the epithelium of non healing leg ulcers further suggested that hCAP18 may be important in the wound healing process (Heilborn et al. 2003).

In a diabetic mouse model simulating impaired wound healing, it was shown that adding LL-37 peptide to an experimental ulcer accelerated its closure, lending further support to the potential in vivo significance of hCAP18/LL-37 in the repair process (Carretero et al. 2008). Subsequently it has been shown that cathelicidins are capable of stimulating wound healing also in other systems such as lung epithelium (Shaykhiev et al. 2005), intestinal epithelium (Otte et al. 2009), cornea (Huang et al. 2006; Yin and Yu 2010) and also in rat gastric ulcers

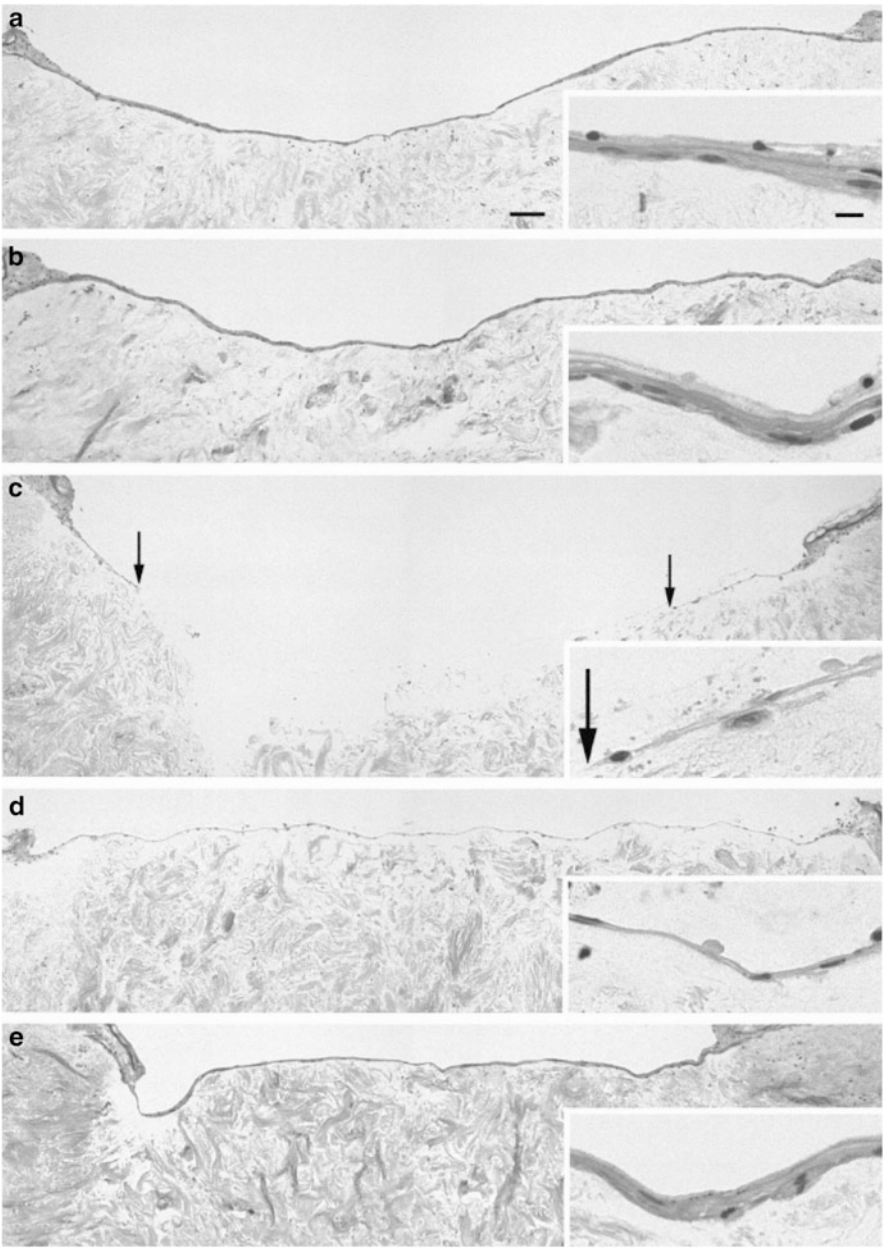

Fig. 1 *LL-37 antibody inhibited re-epithelialization in a concentration-dependent manner in the organ cultured full-thickness ex vivo wound model*. (**a**) Normal control wound completely re-epithelialized at 7 days. *Inset* shows higher magnification of the epithelium of the right wound margin comprising two to three cell layers. (**b**) *Preimmune* serum, at a final IgG concentration equal to the 1:10 dilution of the LL-37 anti-serum, did not affect re-epithelialization or the

(Yang et al. 2006). The effects seem to be mediated via activation of signalling pathways involving transactivation of the epidermal growth factor receptor, EGFR leading to activation of MAP kinases (Tjabringa et al. 2003; Shaykhiev et al. 2005) and via the G protein-coupled receptor FPRL1. The involvement of other receptors cannot be excluded and novel data show that LL-37 is a partial agonist for the type I insulin-like growth factor receptor, IGF1R, inducing downstream signaling confined to the ERK pathway and not affecting phosphatidylinositol 3 kinase/Akt signaling (Girnita et al. 2012). Furthermore, functional experiments demonstrated that LL-37 activating IGF1R signalling increased the migratory capacity of cells consistent with a potential role in epithelial wound closure (Girnita et al. 2012). In this context it was recently reported that growth factors such as insuling-like growth factor and transforming growth factor-alpha in turn stimulate the expression of cathelicidins and defensins in keratinocytes, demonstrating cooperation of multiple forces to optimize healing (Sorensen et al. 2003).

A mitogenic effect for neutrophil defensins on epithelial cells was shown already in the early 1990s (Murphy et al. 1993). This has later been substantiated reporting a direct stimulatory effect for HNP1-3 on lung epithelial cells and in vitro wound closure (Aarbiou et al. 2002; Aarbiou et al. 2004). Like cathelicidins, beta-defensins are also upregulated during re-epithelialization of the cornea, further substantiating the notion that induction of AMPs is a physiologic and general response to epithelial injury (McDermott et al. 2001). In an in vivo pig model for infected diabetic wounds, application of human beta-defensin -3 peptide was recently shown to significantly enhance wound closure in addition to reducing the bacterial load, lending further support for a role for defensins in wound healing (Hirsch et al. 2009).

In addition to effects on the epithelial cells in promoting wound closure, exciting new data suggest that AMPs may also be involved in osteogenesis (Zhang 2010). Thus, it was shown that blood monocytes, when treated with physiologic concentrations of LL-37 peptide, underwent differentiation towards a novel cell type called monoosteophils, capable of forming new bone. A potential implication for repair and healing of fractures was proposed. These results point to a growth-factor like role for cathelicidins in cell biology and are in line with data from several reports exploring a potential role for LL-37 in cancer development (Weber et al. 2009; Coffelt et al. 2009).

Fig. 1 (continued) appearance of the epithelium in control wounds at 7 days or the appearance of the epithelium. (**c**–**e**) Adding polyclonal anti-LL-37 IgG affected re-epithelialization in a concentration-dependent manner. (**c**) The highest concentration of LL-37 anti-serum (1:10) severely impaired re-epithelialization and inhibited wound closure. Higher magnification demonstrates a thin, profoundly affected epithelium. *Arrows* indicate the epithelial edges. (**d**) At 1:100 dilution of LL-37 anti-serum, the wound bed was covered with a single layer of keratinocytes with a fragile appearance at day 7. (**e**) At 1:1,000 antibody dilution re-epithelialization occurred at a level equal to that of the controls. *Scale bars*: (**a**–**e**) 50 μm; *insets* 10 μm. Used with permission from the publisher

3.5 *Tissue Remodeling*

Following the acute phases of wound healing, the tissue undergoes extensive remodelling during a prolonged period to produce a mature scar. In this process the activity of fibroblasts is crucial in fine-tuning the balance between matrix production and degradation to optimize tissue structure and function. The potential involvement of AMPs in this process has not been studied in detail, but an interesting observation was recently reported demonstrating that LL-37 has the capacity to suppress collagen synthesis in human fibroblast induced by transforming growth factor beta, TGF-β (Park et al. 2009). This finding opens up for the notion that AMPs may participate during all phases of wound healing and also for a putative function for cathelicidin peptides in the regulation of fibrosis and control of scar formation.

4 Wound Healing and Cancer

Wound healing and cancer share fundamental features and tumors have been called wounds that do not heal (Dvorak 1986). The molecular machinery that directs wound healing is also activated during tumor development and it is plausible that mechanisms driving the controlled repair of tissue are at work also in abnormal and uncontrolled tissue growth and spread. However, unlike in the wound microenvironment, the cancer milieu is abnormal and characterized by cellular mutations and epigenetic alterations resulting in a different outcome compared with physiologic tissue repair.

Cellular mechanisms implicated in mediating non-antimicrobial effects of cathelicidins include signalling through G protein coupled receptors such as FPRL1 and the purigernic ion channel P2X7 as well as transactivation of the EGFR (Tjabringa et al. 2003). These and other pathways are likely involved in mediating the wound healing promoting effects of AMPs (Yin and Yu 2010) and are also involved in the biology of cancer. Several recent reports suggest a role for the human cathelicidin LL-37 in tumor development demonstrating growth-factor like effects and implicating signalling through oncogenic pathways (Heilborn et al. 2005; von Haussen et al. 2008; Coffelt et al. 2009). The defensins have not been as extensively studied in this respect, but there are reports showing overexpression in transformed lung epithelium (Aarbiou et al. 2004) and in tumor-associated blood vessels (Kawsar et al. 2010).

Current thinking stresses a strong link between inflammation and cancer and here AMPs may be positioned in the crossfire between the two being involved in the physiologic inflammatory response and tissue repair as well as possibly being utilized by transformed cells to promote their growth and spread.

Another relevant aspect is apoptosis, where LL-37 was recently shown to inhibit apoptosis in keratinocytes in vitro (Chamorro et al. 2009). Suppression of apoptosis

is a fundamental aspect of cancer and important also for wound healing (Xue et al. 2004). Still, the picture is complex since LL-37 has been shown to mediate contrasting effects on apoptosis in different cellular systems (Barlow et al. 2006; Nagaoka et al. 2006). Thus, the net in vivo effects are difficult to predict and dependent on multiple factors such as cell type, tissue environment as well as the spatial and temporal exposure to the peptide. Current findings (see above) linking LL-37 to IGF1R signaling further strengthens its link to tumor growth (Girnita et al. 2012).

5 Other AMPs

A number of proteins detected in skin and other barrier organs display antimicrobial activity when tested in vitro. In relation to wound healing, there is limited data so far, but worth mentioning is secretory leukocyte protease inhibitor, SLPI, a cationic serine protease inhibitor. SLPI is constitutively expressed in glandular epithelia and induced in keratinocytes in hyperproliferative conditions such as psoriasis and wound healing. In mice lacking SLPI, experimental mucosal wounds healed significantly slower compared with wild type, suggesting that SLPI may play a role in tissue repair (Angelov et al. 2004). Interestingly, deletion of SLPI was also associated with an enhanced cellular inflammatory infiltrate in the wound bed, which is in line with the notion that sustained inflammation is detrimental for repair.

Neutrophil gelatinase–associated lipocalin, NGAL, is an antibacterial protein packaged in neutrophils, but also induced in hyperproliferating keratinocyte in psoriasis and in squamous cell carcinoma (Mallbris et al. 2002). Like hCAP18, its expression is upregulated by growth factors, IGF-1 and TGF-α, suggesting that it may play a role in tissue regeneration even though experimental evidence is lacking. Psoriasin is another AMP that is overexpressed in keratinocytes and is responsive to keratinocyte derived growth factors (Gläser et al. 2004). Interestingly it is implicated in cancer development, and is upregulated in skin wounds (Kesting et al. 2010) but has not been studied specifically in wound healing.

Overall it is to be expected that many molecules that function in the innate immune system in barrier organs will show activity towards microorganisms and possibly also help repair the epithelium if needed. Thus, the most likely scenario is one of redundancy and cooperation and it will be a real challenge to develop experimental systems to evaluate the relative contribution and importance of individual molecules.

6 Future Perspectives

Following their discovery in the 1980s, our perception of mammalian AMPs has undergone profound changes. From being regarded as purely antimicrobial effector molecules, i.e. endogenous peptide antibiotics, they are now emerging as versatile

players providing a vital link between the innate and the adaptive immune systems. The full functional repertoire of the most studied mammalian AMPs, the defensins and the cathelicidins, is still unclear and their potential involvement in diverse biological processes including wound healing opens up new perspectives and possibilities.

In view of the rapidly developing resistance to existing antibiotics, much hope and resources are invested in trying to develop AMPs into pharmaceutical drugs. It is believed that microbial resistance to AMPs is less likely to become a clinical problem compared with conventional antibiotics, which would be a huge benefit. However, one could only speculate and the consequences of widespread clinical use of AMPs as drugs are difficult to predict. Especially, extensive use over prolonged periods may create a different scenario compared with the endogenous controlled usage of AMPs which has been fine-tuned during evolution.

Regarding non-antimicrobial activities induced by AMPs, new pieces of this fascinating puzzle are steadily emerging. Still, it seems that we are only in the beginning to grasp the full functions of AMPs in normal physiology let alone disease. So far most studies have reported on expression patterns in different tissues and the majority of functional studies are performed in vitro. The few animal models, e.g. the cathelicidin deficient mouse (Nizet 2001), have generated valuable information and we expect to see an increase in such more complex system to test the in vivo relevance and potential of primary observations and hypotheses.

In wound healing it seems obvious that induction of AMPs represents a consistent and physiologic response to injury. We are beginning to understand the mechanisms for their induction at the transcriptional level, but much less of posttranscriptional regulation. LL-37 is perhaps the most documented AMP in skin injury. The complete lack of immunoreactive hCAP18/LL-37 in chronic ulcer epithelium is puzzling and in sharp contrast to the pronounced induction in acute skin wounds. What are the mechanisms to suppress translation of hCAP18 protein in chronic ulcers? What is the role of micro-organisms in this process? Is there a rational for using LL-37 in the treatment of chronic ulcers? Even with support from animal models, the hypothesis could only be ultimately tested in human. In the complex process of wound repair, it is difficult to predict the respective contribution and importance of individual molecules. Most likely there is redundancy and cooperation between the AMPs.

AMPs are fascinating molecules with a long history in biology, but with a short history in biomedical science. The initial discovery of AMPs represented a paradigm shift in biology and the start of a new era in understanding innate immunity. The research field is growing and the number of researchers engaged in studying AMPs from different perspectives is increasing rapidly. We can only hope that their combined efforts will help us understand and make use of the full potential offered by AMPs.

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WAPing Out Pathogens and Disease in the Mucosa: Roles for SLPI and Trappin-2

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Abstract The interface between the external environment and the body's internal structures is defined by the mucosal tissue and the viscous lining fluid that is responsible for maintaining its integrity and protecting internal structures from damage or infection. Human mucosal fluids include seminal fluid, cervical mucus, bronchial and nasal secretions and tears whose composition is particularly complicated. Here we will focus on just two related molecules that are present in the mucosal lining fluid, namely, secretory leucocyte protease inhibitor (SLPI) and trappin-2/elafin, that are responsible for many of the homeostatic and host defence functions of these uniquely situated viscous sols. This review will focus on our increasing understanding of these two molecules from a simple role as local antibiotics that respond to pathogen invasion to major orchestrators of cellular interplays, host defence mechanisms and immune homeostasis.

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1 SLPI

1.1 The Gene and Molecule

Early understanding of SLPI structure and function was complicated by at least four interrelated factors (Seemuller et al. 1986). Firstly, multiple forms seemed to exist in vivo. Secondly, the mucosal environment where SLPI is present is often full of mucous, leucocytes and degradative enzymes. Thirdly, isolation of molecules from tissues often involved the use of trypsin or non-specific protease digestion steps. Finally, inhibitors of similar activity were isolated from anatomically distinct body compartments. Thus, bronchial mucus inhibitor (BMI), human seminal inhibitor I (HUSI-I), cervical mucus inhibitors (CUSI), antileucoprotease (ALP), secretory leucocyte protease inhibitor (SLPI) and mucus proteinase inhibitor (MPI) proved to be identical or derived from a mature inhibitory protein encoded by a single gene of the human genome (Fritz 1988). Human and mouse SLPI are relatively conserved at both the genomic and protein level. The human gene is composed of ~2.6 kb and is organised into four exons, which transcribes a 399-base-pair message to a 132-amino acid protein (Stetler et al. 1986). Similarly, the mouse gene is composed of four exons, which transcribes a 396-base-pair coding sequence to a 131-amino acid protein (Kikuchi et al. 1998). Human SLPI is located on chromosome 20, and the mouse orthologue is located to the syntenic chromosome 2 H (Kikuchi et al. 1998). In both species the functional domains of the SLPI molecule are distributed across the exons; exon 1 codes for the secretion signal, exon 2 the trypsin inhibition domain, exon 3 the elastase inhibitory domain and exon 4 the 3′ untranslated region. Grutter and co-workers eloquently wrote that 'SLPI has a boomerang-like shape with both wings comprising two well separated domains of similar architecture' in their paper outlining the 2.5 Å crystal structure of SLPI binding to bovine α-chymotrypsin (Grutter et al. 1988). Each domain is, relatively conserved, cysteine rich and has high homology to the whey acidic protein (WAP) genes found in rodent milk (Campbell et al. 1984). However, despite the presence of two separate WAP domains, it is the C-terminal that is responsible for the antielastase, antichymotrypsin and antitrypsin activities and that leucine 72 is a key residue involved in the interaction (Kramps et al. 1990; Eisenberg et al. 1990). In keeping with this, both full length and a truncated C-terminal (1/2 SLPI) SLPI could also inhibit cathepsin G activity (Renesto et al. 1993). However, SLPI and its active site variants do not bind or inactivate proteinase 3(PR-3), but instead get cleaved in the N-terminal domain at alanine-16 (Rao et al. 1993). This is further complicated by the species specific potency of SLPI against these proteases (Wright et al. 1999a).

1.2 Expression and Binding Interactions

Numerous studies have evaluated the tissue distribution of SLPI in humans using specimens from surgically treated patients or autopsy where normal tissue is selected

from gross appearance and further examination by light microscopy. These studies utilise specific antisera to localise signal in tissue by immunocytochemistry or to detect in biological fluids using ELISA. SLPI is expressed in numerous areas of the respiratory tract including the submucosal glands of the nose and bronchus, non-ciliated cells of the bronchus, terminal and respiratory bronchioles and alveolar duct (Franken et al. 1989; Fryksmark et al. 1982). Willems et al. used two separate antibodies to localised SLPI along the elastic fibres of the alveolar septa and walls of the bronchi, bronchioles, blood vessels and extracellular matrix (Willems et al. 1986; Kramps et al. 1989). Using a gold labelling technique to demonstrate increased resolution in serous cells of the bronchial submucosal glands, SLPI was located in granules, including structures such as the endoplasmic reticulum and nuclear envelope. This study could only detect SLPI in the Clara cells of bronchial epithelium (De Water et al. 1986). SLPI has also been detected in lung secretions including bronchoalveolar lavage (Kouchi et al. 1993; Ohlsson et al. 1992), broncholavage (Kouchi et al. 1993) and sputum sol phases (Piccioni et al. 1992).

SLPI is also expressed in reproductive mucosa where it has been localised to the epithelium of the upper cervix (Schill et al. 1978) and in seminal fluid (Moriyama et al. 1998). More specifically others have demonstrated SLPI expression in the cervical crypts, together with high concentrations in cervical mucus which varied throughout the menstrual cycle with increased concentrations during the ovulatory compared to follicular phases (Casslen et al. 1981; Moriyama et al. 1999). Interestingly during pregnancy SLPI is increased in cervical tissue and is particularly high in the cervical plug which also has a high molar ratio of SLPI to elastase. Denison and co-workers demonstrated dramatic increases in the levels of SLPI (~200-fold) in amniotic fluid over the course of pregnancy and suggested that the major source is the decidua parietalis cells (Denison et al. 1999). These studies together with demonstration of SLPI in foetal membranes suggest a protective role (involving structural integrity and inhibition of proinflammatory responses) for SLPI during the menstrual cycle and pregnancy (Helmig et al. 1995).

Expression of SLPI has been demonstrated in many other mucosal tissues and lining fluids including salivary glands (Ohlsson et al. 1984; Shugars et al. 2001; Cox et al. 2006), middle ear (Carlsson & Ohlsson 1983; Lee et al. 2006), maxillary sinus (Fryksmark et al. 1985), intestine, (Bergenfeldt et al. 1996), colon, (Nystrom et al. 2001), human skin (Wiedow et al. 1993), nasal secretions (Westin et al. 1999a), peritoneal fluid (Shimoya et al. 2000), stomach (Wex et al. 2004), gingival crevicular fluid (Cox et al. 2006; Nakamura-Minami et al. 2003) and cornea (Nielsen et al. 2005).

The binding interactions of SLPI are not limited to forming 1:1 molar complexes with proteases such as elastase, chymotrypsin and trypsin. Indeed, binding activities for SLPI are not just limited to the extracellular milieu but have also been reported at the plasma membrane and within the intracellular space. Extracellular binding interactions include those to the pathogen-associated molecular patterns bacterial lipopolysaccharide (LPS) (Ding et al. 1999), mannan-capped lipoarabinomannans and phosphatidylinositol mannoside (Gomez et al. 2009) together with numerous glycosaminoglycans (Fath et al. 1998; Ying et al. 1997) and classes of immunoglobulin (Hirano et al. 1999). Intracellular binding interactions include binding to DNA

(Miller et al. 1989; Taggart et al. 2002) and to IRAK, IκBα and IκBβ (Lentsch et al. 1999a). Interactions at the plasma membrane include annexin-II (Ma et al. 2004), scramblase-1 (Tseng & Tseng 2000; Py et al. 2009) and scramblase-4 (Py et al. 2009).

1.3 Antimicrobial Activity

SLPI has moderate antimicrobial actions against a variety of human bacterial pathogens including *Escherichia coli, Staphylococcus aureus, S. epidermidis, Pseudomonas aeruginosa* and *Mycobacterium bovis* (Wiedow et al. 1998; Hiemstra et al. 1996; Nishimura et al. 2008). SLPI is less potent (on a molar basis) than lysozyme or defensin with 50 % inhibitory concentrations against *E. coli* of 4.7 μM, 1.8 μM and 1.4 μM, respectively, with the antimicrobial domain residing in the N-terminal (Hiemstra et al. 1996). SLPI has also been shown to have antimicrobial activity against metabolically active fungi, in particular *Aspergillus fumigatus* and *Candida albicans*. Interestingly metabolically quiescent *A. fumigatus* conidia were resistant to SLPI in this study. The antifungal activity is reported to be equal to lysozyme and defensins and also appears to reside in the N-terminal portion of the molecule (Wiedow et al. 1998; Tomee et al. 1997).

In contrast to the relatively consistent parallel studies investigating bacterial and fungal activity, the antiviral activity of SLPI has proved much more complicated. McNeely and colleagues identified a protein in saliva that could protect monocytes against HIV infection which following analysis was confirmed to be SLPI (McNeely et al. 1995; Shugars et al. 1997). Since then reports have both supported (McNeely et al. 1997) and refuted (Turpin et al. 1996) this work. Following this, studies focused on two main aspects of this compelling argument: (1) clinical studies attempting to relate SLPI levels to transmission of HIV and viral load and (2) mechanistic studies attempting to explain the precise conditions necessary for activity. Thus, SLPI was increased in saliva and plasma of HIV-infected individuals compared to uninfected controls (Baqui et al. 1999). In a study of pregnant HIV-positive South African women, those who had higher levels of SLPI in vaginal fluid had lower perinatal transmission rates to their babies (Pillay et al. 2001). In a similar but larger study of 602 saliva samples from 188 infants over the first 3 months following birth, increased SLPI was associated with a reduced risk of HIV transmission from breast milk (Farquhar et al. 2002).

1.4 Unique Role in Inflammation: Priming Innate Immunity and Tissue Remodelling

Cell culture studies have identified a plethora of cytokines, drugs and hormones that modulate the levels of SLPI when introduced to the bathing medium. In human

airway cells, Abbinante-Nissen et al. found that neutrophil elastase (NE) was a potent inducer of SLPI transcript. Furthermore, other neutrophil products, such as cathepsin G, myeloperoxidase and lysozyme, had little or no effect on SLPI transcript levels. In contrast, two non-neutrophil proteases, trypsin and pancreatic elastase, also increased SLPI transcript levels at higher doses than that required of NE. These authors also showed that tumour necrosis factor-alpha (TNF-α) and interleukin (IL)-8 induced little or no SLPI transcript levels (Abbinante-Nissen et al. 1993). Using Clara cells and alveolar type II cells and measuring SLPI protein as an end point, we showed both a constitutive and IL-1β- or TNF-α-induced production of SLPI (Sallenave et al. 1994). Interestingly, glucocorticoids can induce SLPI transcript in human airway epithelial cells with a descending potency of fluticasone > triamcinolone > or = dexamethasone > methylprednisolone > hydrocortisone (Abbinante-Nissen et al. 1995). This study also demonstrated that elastase and fluticasone together induce synergistic increases in SLPI. Indeed, the ability of glucocorticoids to induce SLPI may be partly responsible for their anti-inflammatory action. Furthermore, progesterone has been shown to upregulate SLPI mRNA and protein through a mechanism involving its transactivation of the SLPI gene through the progesterone receptor (PR), via induction of basic transcription element-binding protein-1 (BTEB1) gene and co-recruitment of BTEB1 and the PR coactivator cAMP-response element-binding protein (CBP) to the SLPI promoter (Velarde et al. 2006; King et al. 2003).

The late 1990s saw a dramatic change in the way we viewed SLPI. Before then SLPI was considered an antimicrobial molecule with potent antiprotease activity; however, the seminal work of Jin and colleagues in macrophages demonstrating the ability of LPS to induce SLPI and furthermore that SLPI could suppress LPS-induced activation of NF-κB and synthesis of TNF-α/nitric oxide suggested that SLPI had immunomodulatory activity as well (Jin et al. 1997). In a later paper, the same group also demonstrated that LPS-induced SLPI was an early (~30 min) and prolonged response (remaining at 72 h). The LPS inducible proteins IL-10 and IL-6 could also upregulate SLPI but IL-1β and TNF-α could not. Finally, the Gram-positive cell wall constituent LTA could also stimulate SLPI production (Jin et al. 1998). There are multiple mechanisms responsible for these effects including the ability of SLPI to inhibit NF-κB activation by stabilisation of IRAK, IκBα and IκBβ proteins, despite increasing the amount of phosphorylated and polyubiquitinated IκBα (Taggart et al. 2002; Lentsch et al. 1999a). This is supported by the anti-inflammatory activity of a non-secretable form of SLPI when transfected into macrophages (Zhu et al. 1999). Others have suggested that SLPI can prevent the p65 subunit of NF-κB binding to its consensus sequence in the promoters regions of target genes. It is unclear which domain of the SLPI molecule mediates the anti-inflammatory action as one study suggests that oxidation of SLPI inhibits this action (Taggart et al. 2002), whereas site-directed mutants of the oxidisable methionine residue (Met 73) could still inhibit LPS-induced TNF and nitric oxide responses (Yang et al. 2005). In vivo Mulligan and co-workers have suggested that the leucine 72 residue which is essential in determining antiprotease

function is vital, and their studies implicate the antitrypsin activity in SLPI (through a Lysine 72 mutant) to have a greater suppressive effect on the inflammatory response than wild-type SLPI (Mulligan et al. 2000).

Generation of mice deficient in SLPI at the beginning of the millennium has enhanced our knowledge of the in vivo effects of this molecule. The first of these studies suggested a role for SLPI in linking host defence with wound repair (Ashcroft et al. 2000). SLPI-deficient mice have deficient cutaneous wound healing with increased inflammation and elastase activity with enhanced local production of TGF-beta. In a similar model, Zhu has suggested an alternative pathway dependent on proepithelin and its cleaved product epithelin which have opposite effects during inflammation (Zhu et al. 2002). Proepithelin blocks TNF-α-induced neutrophil activation and oxidant and protease release, whereas epithelin inhibits the growth of epithelial cells and induces IL-8 production by neutrophils. In this way proepithelin complexed with SLPI cannot be cleaved with elastase to epithelin, and SLPI null mice can be rescued with proepithelin. Angelov and co-workers identified differences in the mechanisms of wound healing in a combined dermal scarring and oral non-scarring model (Angelov et al. 2004). Here an absence of SLPI results in markedly impaired oral wound healing associated with increased inflammation, raised elastase activity and decreased matrix deposition through increased MMP activity suggesting deregulated proteolysis. Intriguingly, TGF-beta expression is increased in cutaneous model (Zhu et al. 2002), but decreased in the oral model (Angelov et al. 2004) pointing to the ability of SLPI to improve wound healing by very different local mechanisms. The link is particularly pertinent in a cardiac transplant model of ischemia/reperfusion injury where SLPI-deficient hearts had profound abnormalities in early contraction and high protease expression and TGF-beta expression (Schneeberger et al. 2008). Interestingly, systemic SLPI could not rescue this phenotype, whereas including SLPI in the preservation solution prior to transplantation reversed the phenotype suggesting a dual inhibitory effect on protease and TGF-beta expression might be the underlying mechanism (Schneeberger et al. 2008).

The identification of these homeostatic functions for SLPI encouraged others to investigate its role during inflammation and infection. In a model of endotoxin shock, SLPI-deficient mice had significantly higher mortality possibly due to increased levels of macrophage IL-6, HMG-1 and NF-κB compared to wild-type cells (Nakamura et al. 2003). Similarly B cells isolated from null mice showed increased proliferation and IgM production suggesting that SLPI acts to attenuate excessive inflammatory responses. However, in a model of infection, SLPI null mice were highly susceptible to *Mycobacterium bovis*, when given by the respiratory route, suggesting a role in driving the local inflammatory response to clear pathogens (Nishimura et al. 2008).

In addition to gene deletion, functional studies have also supplemented recombinant SLPI either through overexpression (e.g. adenoviral) or administration of the purified protein. Thus, adenoviral gene delivery of SLPI can protect against ischemic

brain injury (Wang et al. 2003) and has also been shown to attenuate NF-κB-dependent inflammatory responses to atherogenic stimuli (Henriksen et al. 2004). By transfecting multiple clones of the highly metastatic subline (H-59) to overexpress SLPI, Wang and colleagues showed that these cells' ability to elicit a host proinflammatory response in the liver was markedly decreased, as evidenced by reduced TNF-α production and vascular E-selectin expression, relative to controls (Wang et al. 2006). Consistent with these findings, recombinant SLPI administered systemically could suppress inflammation associated with joint damage (Song et al. 1999) and attenuate hepatic ischemia/reperfusion injury (Lentsch et al. 1999b) in rats and mice, respectively. Furthermore, local delivery of SLPI to ovine lung by aerosol has been shown to prevent allergen-induced pulmonary responses in a model of asthma (Wright et al. 1999b), and topical administration to the eye in guinea pigs suppressed the recruitment of eosinophils and decreased the severity of conjunctivitis (Murata et al. 2003).

It has been unclear for sometime whether SLPI has proinflammatory/immune stimulatory effects that are distinct from its direct antimicrobial activity. In models of resolving inflammation where neutrophil apoptosis has been shown to stimulate macrophage clearance (Savill et al. 1989), SLPI seems to play a proinflammatory role. Firstly, murine macrophages produce SLPI during clearance of apoptotic cells (Odaka et al. 2003), and SLPI (together with lactoferrin) is secreted by activated neutrophils (Jacobsen et al. 2008). Recently a functional study by Subramaniyam and colleagues has suggested that SLPI inhibits apoptosis therefore prolonging the life of neutrophils during inflammation (Subramaniyam et al. 2011). In support of an immune stimulatory role for SLPI, Gomez and co-workers have shown that SLPI may act as a pattern recognition receptor for mycobacteria which acts to stimulate phagocytosis (Gomez et al. 2009). Thus, it seems that the proinflammatory actions of SLPI are dependent on the type of pathogen and on the progress of the inflammatory response.

1.5 Key Roles in Mucosal Tissue: Ovarian and Gastric Cancer

An emerging literature identifying a role for SLPI in cancer has developed over the last few years. Initial evidence for this came from mRNA differential display systems identifying changes in a SLPI gene variant in the highly metastatic murine carcinoma cell line IMC-HA1 (Morita et al. 1999). The gene was isolated as SLPI-α and SLPI-β and was found to be differentially expressed with SLPI-α expressed ubiquitously in tumours but SLPI-β having lower expression in normal tissues and distinct expression in certain tumours (e.g. P388 leukemias). In a separate study, the repression of SLPI was shown to be under the control of the cell growth regulator interferon regulatory factor (IRF)-1 suggesting that it might be a downstream target modulating cell growth properties (Nguyen et al. 1999). Changes in SLPI levels have been associated with cancer. For instance, SLPI is expressed in a number of tumour environments including ovarian endometriomas (Suzumori et al. 2001;

Shigemasa et al. 2001), head and neck squamous cell carcinomas (Westin et al. 1999b), cervical adenocarcinoma (Tian et al. 2004) and gastric cancer (Cheng et al. 2008) but decreased in prostate cancer (Xuan et al. 2008).

A role for SLPI in cancer has been suggested in a variety of studies. Devoogdt et al. have suggested a pro-malignant role as transfection of low malignant lung carcinomas with SLPI produced a highly malignant phenotype both in vitro and in vivo (Devoogdt et al. 2003), and moreover, the protease inhibitory function was essential for that activity. In a later study, the same author has found that overexpression of a protease-dead SLPI resulted in more aggressive ovarian cancers (Devoogdt et al. 2009). This tumour-promoting effect of SLPI is thought to mediate the pro-tumourigenic effects of TNF-α as SLPI expression and tumour size was decreased in TNF-α-deficient mice (Devoogdt et al. 2006). In being a TNF responsive gene, SLPI may even impact on the 'cancer immunoediting hypothesis' which suggests that the local tumour microenvironment might induce cancer cell variants with increased resistance to the immune system (Dunn et al. 2002).

2 Trappin-2/Elafin

2.1 The Gene and Molecule

Trappin-2 protein was purified and characterised from human lung secretions and skin tissues in the 1980s and 1990s under a variety of names, such as elafin, BSI-E, elastase-specific inhibitor (ESI), precursor of elafin–ESI (PELESI) and skin-derived antileucoprotease (SKALP) (Hochstrasser et al. 1981; Kramps & Klasen 1985; Wiedow et al. 1990; Sallenave & Ryle 1991; Sallenave et al. 1992). The trappin-2 gene encodes a secreted 9.9-kDa non-glycosylated 95-residue cationic protein (Saheki et al. 1992; Sallenave & Silva 1993), comprising an N-terminal domain (38 residues) or cementoin domain (Nara et al. 1994) and a C-terminal inhibitory whey acidic protein (WAP)-type domain (57 residues) (Bairoch & Apweiler 1997). The N-terminal domain contains several repeated motifs with the consensus sequence Gly–Gln–Asp–Pro–Val–Lys that can anchor the whole molecule to extracellular matrix proteins by transglutaminase-catalysed cross-links. By doing so, it is believed trappin-2 shields the elastic tissues from locally secreted enzymes (e.g., NE), which overwhelm the tissues at times of inflammation/infection (Nara et al. 1994). The C-terminal domain is structurally similar to the SLPI domains (about 40 % sequence identity with each SLPI domain). Trappin-2 is encoded by the PI3 gene in the same chromosome region 20q12–13 as SLPI gene and is composed of three exons spanning about 2 kb. The first exon codes for the 5′ untranslated region, the signal peptide and the first few amino acids of the mature protein; the second exon encodes most of the mature protein, and the third exon encodes the 3′ untranslated region (Molhuizen et al. 1993). Trappin-2 is translated with a signal peptide that is cleaved during secretion and proteolytically processed

to form a ~6-kDa peptide referred to as elafin. Although the antiprotease activity of trappin-2 was initially identified in both the intact 9.9-kDa peptide and its cleaved 6-kDa C-terminus product (elafin), trappin-2 has a reduced protective effect in an in vivo model of elastase-induced lung injury when it is cleaved of its cementoin domain (Tremblay et al. 2002).

2.2 Expression and Binding Interactions

Trappin-2 or its orthologues are also found in other mammals and is expressed both in foetal and adult tissues (Pfundt et al. 1996). Interestingly, the expression trappin-2/elafin has not been demonstrated in rat or mouse tissues (Williams et al. 2006). Trappin-2 inhibits NE, porcine pancreatic elastase and PR-3 with a low degree of reversibility but does not inhibit cathepsin G, trypsin or chymotrypsin and, hence, has a more restricted spectrum of inhibition than SLPI (Williams et al. 2006). The regulation of trappin-2 expression by healthy and inflamed tissues has attracted much attention. Unlike SLPI, low levels of trappin-2 is secreted by bronchial and alveolar epithelial cells as well as keratinocytes in steady state, but its production is significantly increased under the influence of LPS as well as inflammatory cytokines IL-1 and TNF-α (Sallenave et al. 1994). A few signalling pathways, namely, c-jun, p38 mitogen-activated protein (MAP) kinase and NF-κB pathways, are implicated in the trappin-2 response to inflammatory molecules (Pfundt et al. 2000, 2001; Bingle et al. 2001). Likewise, trappin-2/elafin mRNA expression is increased by free NE in bronchial epithelial cells, which is found at high concentrations (μM levels) at inflammatory sites (Reid et al. 1999; van Wetering et al. 2000). In recent years, trappin-2 has increasingly been shown to display functions beyond its protease inhibition [reviewed in (Williams et al. 2006; Roghanian & Sallenave 2008; Sallenave 2010)] such as antimicrobial and mmunomodulatory activities, as will be discussed below.

2.3 Antimicrobial Activity

Our group first ascribed antimicrobial activity to trappin-2 in the late 1990s. Importantly, we demonstrated that trappin-2 was active against two major respiratory pathogens, the Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus* both in vitro and in vivo (Simpson et al. 1999; McMichael et al. 2005a). To this end, overexpression of trappin-2 by adenovirus-mediated gene transfer dramatically increased the local antibacterial defences against *P. aeruginosa* and *S. aureus* infections (Simpson et al. 1999; McMichael et al. 2005a). On the other hand, supernatants of *P. aeruginosa* could induce trappin-2 production in human keratinocytes, and trappin-2 inhibits growth of *P. aeruginosa* in vitro, but not *E. coli* (Meyer-Hoffert et al. 2003; Bellemare et al. 2008). *P. aeruginosa* is an opportunistic pathogen and commonly resistant to conventional antibiotics that is life-threatening

for immunocompromised individuals and for patients suffering from chronic respiratory diseases such as cystic fibrosis (CF). *P. aeruginosa* is also the predominant bacteria associated with nosocomial infections, and acute *P. aeruginosa* infection may result in sepsis and death (Hancock 1998; Erwin & VanDevanter 2002). Similarly, *Staphylococcus aureus* infections are closely associated with pneumonia and sepsis, particularly in nosocomial infections, and its increasing association with antimicrobial resistance has become a major concern for clinicians (Butterly et al. 2010). In addition to the above-mentioned pathogens, trappin-2 has significant bactericidal activity against *Klebsiella pneumoniae*, *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Branhamella catarrhalis* which are also common features of inflammatory lung disorders such as CF and chronic obstructive pulmonary disease (COPD) (Baranger et al. 2008). Furthermore, trappin-2 and its cleaved product elafin possess potent fungicidal activities against pathogenic *Aspergillus fumigatus* and *Candida albicans*, which have preferential tropism for human lungs and other mucosae (Baranger et al. 2008).

Trappin-2/elafin has also been shown to possess anti-human immunodeficiency virus (HIV) activity, although the mechanism(s) of this inhibition is currently unknown. Both trappin-2 and SLPI have been detected in cervicovaginal lavage samples of HIV-negative and HIV-positive women (Moreau et al. 2008; Ghosh et al. 2010a). Reportedly, the 6-kDa elafin was amongst factors that correlated with protective immunity to HIV infection in the genital tract secretions of a group of African women who remain virus-free despite multiple high-risk exposures to HIV infection (Iqbal et al. 2009). Recombinant trappin-2/elafin is able to inhibit both T cell-tropic X4/IIIB and macrophage-tropic R5/BaL HIV-1 in a dose-dependent manner (Ghosh et al. 2010b). This inhibitory activity was observed when virus was incubated with trappin-2/elafin but not when trappin-2/elafin was added to cells either before infection or after infection. This indicates that the inhibitory activity of trappin-2/elafin occurs through a direct interaction with the virus rather than at the level of the target cell surface, for example, through the blocking of receptors.

Collectively, these findings propose that trappin-2/elafin may play an important protective role in vivo against the transmission of HIV from men to women. In the latest attempt to develop preventive anti-HIV therapeutics, engineered commensal bacteria secreting elafin have been utilised that appear to confer protection against HIV infection in vitro (Fahey et al. 2011). These innovative yet unproven approaches are designed to regulate immunity in the female reproductive tract in ways that will potentially reduce HIV infection in women.

2.4 *Unique Roles in Inflammation: Linking Innate and Adaptive Immunity*

The initial interaction between antimicrobial peptides and pathogens is due to electrostatic forces, since the host defence peptide is positively charged and

molecules such as LPS and lipoteichoic acid are negatively charged. Indeed, trappin-2 and its C- and N-terminus peptides are capable of binding both smooth and rough forms of LPS at the lipid A portion of the molecule, with N-terminus binding both forms of LPS more avidly, thus modulating immune responses (McMichael et al. 2005b). Moreover, binding of trappin-2 and cementoin (trappin-2N-terminal domain) to *P. aeruginosa* elicits morphological changes such as wrinkling and blister formation on the cell surface and the presence of pore-like structures (Baranger et al. 2008; Bellemare et al. 2010; Wilkinson et al. 2009). It is commonly assumed that the presence of pore-like structures is indicative of cell lysis. However, several lines of evidence suggest that the membrane disruption properties of cementoin, trappin-2 and elafin are considerably weaker compared to other antimicrobial peptides, such as the amphibian lytic magainin 2 (Bellemare et al. 2010). Moreover, recent evidence indicates that trappin-2 and elafin, but not cementoin, are capable of reducing biofilm development and the secretion of pyoverdine, which correlates with the ability of these peptides to bind DNA in vitro and to accumulate within the bacterial cytosol (Bellemare et al. 2010; Li et al. 2010a). Thus, in addition to bacterial opsonisation and induction of cell lysis, trappin-2 and elafin attenuate the expression of some *P. aeruginosa* virulence factors, possibly through acting on intracellular pathways (Bellemare et al. 2010). Interestingly, it has been suggested that trappin-2 WAP domain also specifically inhibits a *P. aeruginosa*-secreted peptidase with the characteristics of arginyl peptidase (protease IV) and prevents bacterial growth in vitro (Bellemare et al. 2008).

In an effort to further address the mechanisms by which trappin-2 exerts its antimicrobial/immunomodulatory effects in the host, in vitro and in vivo models of the very earliest interactions between *P. aeruginosa* and macrophages were developed by us to mimic the presumed environment encountered in the initial stages of lung infection (Wilkinson et al. 2009). Consequently, subantimicrobial concentrations (nanomolar range) of trappin-2 enhanced clearance of *P. aeruginosa* (strain PA01) by macrophages, which was dependent on prior opsonisation of the bacteria by trappin-2 in vitro. Similarly, wild-type mice receiving an intratracheal dose of trappin-2-opsonised *P. aeruginosa* had significantly decreased bacterial burden compared with mice receiving nonopsonised *P. aeruginosa*. In striking contrast, CD14-deficient mice were resistant to the *P. aeruginosa*-opsonising effects of trappin-2 and were unable to clear the bacteria as effectively (Wilkinson et al. 2009). Hence, CD14, a promiscuous pattern recognition receptor, is the only described receptor involved in mediating the effect of trappin-2 to date. CD14 has a broad ligand specificity allowing it to bind Gram-positive, Gram-negative and viral pathogens (Anas et al. 2010). CD14 can also participate in non-inflammatory or anti-inflammatory responses by acting as a macrophage receptor for engulfment of apoptotic cells (Anas et al. 2010). Furthermore, trappin-2-opsonised *P. aeruginosa* simultaneously promoted a CD14-dependant fivefold increase in CXCL1 compared with nonopsonised bacteria, which led to a rapid recruitment of neutrophils soon after, as previously observed in other experimental models (Simpson et al. 1999; Sallenave et al. 2003; Roghanian et al. 2006). Both CXCL1 and CXCL2 act through the chemokine receptor CXCR2, which has been shown to be essential for host

protection against *P. aeruginosa* pneumonia (Tsai et al. 2000). Thus, in the early stages of infection, trappin-2 simultaneously delivers pathogens to resident alveolar macrophages, while contributing to activation of the neutrophil/CXCR2 axis should the bacterial inoculum appear sufficient to drive significant infection. These recent findings further strengthen the notion that trappin-2 is able to augment clearance of pathogens at early onset of infection, even before recruitment of neutrophils and other effector cells to the inflammatory site.

It is noteworthy to point out that *P. aeruginosa* PAO1-conditioned medium and two purified *Pseudomonas* metalloproteases, pseudolysin (elastase) and aeruginolysin (alkaline protease), are able to cleave recombinant elafin leading to loss of its antiprotease activity and binding to fibronectin following transglutaminase activity, respectively (Ghosh et al. 2010b). Moreover, elafin is cleaved by its cognate enzyme NE, present at excessive concentration at inflammatory milieu, and that *P. aeruginosa* infection promotes this effect (Guyot et al. 2008). Consequently, such cleavages may have repercussions on the innate immune function of elafin.

When secreted locally at mucosal sites, trappin-2 promotes recruitment or priming of innate immunity. Expression of the human trappin-2 gene in the murine lungs results in an increased influx of inflammatory cells in response to infection/inflammation (Wilkinson et al. 2009; Sallenave et al. 2003; Roghanian et al. 2006; Simpson et al. 2001), and the interaction of trappin-2 with LPS results in an augmentation of the LPS-induced TNF-α response in a murine macrophage cell line (McMichael et al. 2005b). Interestingly, transgenic mice expressing human trappin-2 show lower serum-to-bronchoalveolar lavage ratios of proinflammatory cytokines, including TNF-α, macrophage inflammatory protein 2 and monocyte chemoattractant protein 1, than wild-type mice in response to local intratracheal LPS stimulation with a concomitant increase in inflammatory cell influx (Sallenave et al. 2003). Conversely, trappin-2 transgenic mice show lower TNF-α serum levels in response to systemic LPS, indicating that trappin-2 may have a dual function, that is, promoting upregulation of local lung innate immunity while simultaneously downregulating potentially unwanted systemic inflammatory responses in the circulation (e.g. preventing septic shock) (Sallenave et al. 2003).

As discussed above, trappin-2 was first identified as being able to protect tissues from the damaging effects of proteases released during inflammation and was later shown to be functionally active in the regulation of both inflammation and innate immunity (Williams et al. 2006). However, emerging data expand upon the previously described functions for trappin-2/elafin, by showing that the influence of trappin-2 actually extends to include modulation of adaptive immune responses. To this end, using the dual system of trappin-2 expression (either provided as an adenoviral construct or in an elafin-transgenic model), our laboratory provided novel evidence that trappin-2 induces a type 1-biased inflammatory and immunological response (cellular and humoral) in the lungs and spleens of mice overexpressing elafin (Roghanian et al. 2006). The demonstrated Th1 skewing effect of trappin-2 is likely to be mediated through the increase in numbers and/or activation status of lung antigen-presenting cells, as elafin overexpressers exhibited higher numbers of total lung $CD11c^{+high}$ cells and $CD11c^{+high}$ $MHCII^{+high}$ cells (dendritic cells; DCs), expressing

higher levels of the B7 family costimulatory molecules CD80 and CD86 (indicative of activated DCs). In accordance with the increase in the number of activated DCs, increased levels of proinflammatory cytokines IL-12, TNF-α and IFN-γ were observed in BALF of trappin-2 overexpressers (Roghanian et al. 2006). Clinical evidence to support a role for trappin-2 in the augmentation of a Th1 phenotype is also available, for example, increased levels of trappin-2 are found in pathological conditions associated with a type I immune response, such as in the bronchoalveolar lavage of farmer's lung sufferers (Tremblay et al. 1996) and psoriatic skin (Schalkwijk et al. 1993).

More recently, human γδ T cells have been shown to produce trappin-2/elafin (both mRNA and protein) upon stimulation with supernatant of *P. aeruginosa* grown in culture. Between 2 and 5 % of $CD3^+$ T cells in the peripheral blood express the γδ T cell receptor (TCR) instead of the conventional αβ TCR. In contrast to the peripheral blood, γδ T cells represent a major T cell population in other anatomical localisations such as the small intestine where 20–30 % of local T cells are γδ T cells (Kabelitz et al. 2000; Hayday 2000). γδ T cells have the capacity to act as antigen-presenting cells (Brandes et al. 2005) and to secrete antimicrobial effector molecules such as granulysin (Dieli et al. 2001) and the cationic antimicrobial peptide LL37/cathelicidin, which is typically produced by epithelial cells (Agerberth et al. 2000; Selsted and Ouellette 2005). Due to certain features, which γδ T cells share with cells of both the adaptive (e.g. TCR expression) and the innate immune system (e.g. Toll-like receptor expression, antigen-presenting capacity), γδ T cells are thought to bridge innate and adaptive immunity (Hayday 2000). The secretion of elafin by γδ T cells might contribute to the recruitment of neutrophils or the opsonisation of the pathogens in sites of inflammation where access is restricted.

2.5 Key Roles in Mucosal Tissue

2.5.1 Reproductive Tract

In addition to the lung mucosa and skin, trappin-2 expression and regulation has received much interest in the female genital tracts, as it represents a major mucosal site. The mucosal immune system in the female reproductive tract has evolved to meet the unique requirements arising from the need to deal with sexually transmitted bacterial and viral pathogens, allogeneic spermatozoa and the immunologically distinct foetus. In this regard, a wide range of antimicrobial peptides including trappin-2 are expressed throughout the female genital tract [(Nishimura et al. 2008; Tomee et al. 1997), reviewed in (Horne et al. 2008)]. Trappin-2 and SLPI are expressed in the vagina (Draper et al. 2000) and cervix, with high concentrations of SLPI demonstrated in the cervical mucus (12, 57). SLPI is expressed in endometrium from the mid-late secretory phase of the menstrual cycle when it is localised predominantly to the glandular epithelium (King et al. 2000). In contrast,

trappin-2 is expressed primarily in endometrial neutrophils during menstruation (Turpin et al. 1996). Trappin-2 and SLPI are also detectable in the vaginal secretions throughout pregnancy (Shugars et al. 1997). Trappin-2 levels are diminished in bacterial vaginosis, suggesting that it may be an important component of innate immunity in the lower genital tract (Stock et al. 2009). In the Fallopian tube, trappin-2 and SLPI mRNA are upregulated in ectopic pregnancy. In contrast to endometrium, trappin-2 and SLPI are not regulated in a cycle-dependent manner at the mRNA level in the Fallopian tube. The pathology underlying ectopic pregnancy is unclear although previous infection with *Chlamydia trachomatis* is a risk factor. In line with this, the mRNA message for trappin-2 is increased during in vitro chlamydial infection of an oviductal cell line (King et al. 2009).

Natural antimicrobial production is also an important part of the innate immune response of the amnion. Indeed, the primary amnion epithelial cells produce potent natural antimicrobials, including trappin-2 and SLPI, which may help protect the pregnancy from infection (Stock et al. 2007). Taken together, these observations suggest that trappin-2 and SLPI play important roles in the maintenance of the female reproductive tract physiology via regulation of protease activity, wound healing and tissue remodelling. Trappin-2 and SLPI may also be implicated in the event of pathological conditions, such as infection and ectopic implantation (King et al. 2009), and abnormal expression of these peptides may predispose to infection or ectopic pregnancy.

2.5.2 Gastrointestinal Tract

Recent limited studies also point out to the important roles played by antimicrobial peptides, including trappin2/elafin and SLPI, in the gastrointestinal tract and associated pathologies. In a rhesus macaque host–pathogen model, microarray analysis revealed that in *Helicobacter pylori*-infected animals, several innate antimicrobial effector proteins, including elafin and siderocalin, and several novel paralogues of human β-defensin-2 were upregulated, which depended on the presence of the *cag* pathogenicity island (Baqui et al. 1999). In another study, investigating the presence of antimicrobial peptides in biopsies from the healthy oesophagus, stomach and the duodenum, trappin-2 was found to be predominantly expressed in the oesophagus (Hosaka et al. 2008).

Antimicrobial peptide imbalance appears to contribute to aetiology and pathogenesis of inflammatory bowel disease (IBD) (Pillay et al. 2001; Farquhar et al. 2002; Abbinante-Nissen et al. 1993). Interestingly, in biopsies taken from patients with Crohn's disease, the expression of trappin-2 and SLPI was shown to be attenuated upon inflammation, thereby suggesting a disruption of the protease/antiprotease balance in chronic inflammatory status of the gut (Schmid et al. 2007). By taking advantage of the adenoviral construct and two trappin-2 transgenic murine models, we established that restoring this proteolytic imbalance by the expression of the trappin-2 is associated with a strong protective effect against the development of colitis in experimental models (Motta et al. 2011). This

protection appears to be both due to reduced NE/PR-3 and trypsin-like activities and also due to the inhibition of NF-κB proinflammatory pathway by trappin-2, as observed in other models (Velarde et al. 2006; King et al. 2003). Collectively, these results not only provide definitive insights into the importance of the proteolytic balance in gut inflammation but also point to trappin-2 as a possible protective molecule in chronic inflammatory disorders of the gut (Motta et al. 2011).

3 WAP as Therapeutics, Drug Targets or Biomarkers

In vitro and in vivo experimental modelling has identified the activities of SLPI and trappin-2/elafin, but transforming these results into medicines is only just becoming a reality.

Numerous studies in the 1990s reported the effects of giving recombinant SLPI to humans (McElvaney et al. 1993; Bergenfeldt et al. 1990; Stolk et al. 1995; McElvaney et al. 1992) with a view to treating lung disease. These studies confirmed elimination half-lives of 10 min (Bergenfeldt et al. 1990) and 0.2–2.8 h (Stolk et al. 1995) for intravenous administration and inhalation, respectively. Inhaled therapy appears to be the way forward due to increased lung targeting and decreased systemic effects although repeated dosing was necessary to maintain therapeutic levels in CF patients (McElvaney et al. 1993). Analysis of epithelial lining fluid from patients with emphysema has suggested this may be due to SLPI cleavage by cathepsins (Taggart et al. 2001). To improve delivery to the diseased lung, Gibbons and co-workers have developed a dry powder formulation of liposome-encapsulated recombinant SLPI that proved better at retaining a protective function against cathepsin L-induced rSLPI inactivation compared to an aqueous DOPS–rSLPI liposome dispersion and was also more stable under storage (Gibbons et al. 2010).

Improvements have also been made with regard to the production of recombinant protein. Expression of SLPI in bacteria required extensive denaturation and renaturation to refold the disulphide-rich protein into its biologically active form (Lucey et al. 1990). Recently two alternative methods of production have been developed using baculovirus expression (Gray et al. 2002) and the yeast *Pichia pastoris* (Li et al. 2009, 2010b) with purification under non-denaturing conditions. These advances have suggested that SLPI can be produced in an efficient and cost-effective manner for therapeutic purposes. These methods have resulted in a greater yield of protein with improved biological activity. Interestingly Zani and co-workers have produced fusion proteins to create antiproteases with activities overlapping with SLPI and elafin so that elastase, cathepsin G and PR-3 could be inhibited by one molecule (Zani et al. 2009). Such manipulation of these molecules will hopefully result in designer therapeutics directed at lung diseases (e.g. COPD) where protease/antiprotease balance is destructive to the host.

Further advances moving SLPI therapeutics one step closer to reality have been reported recently: firstly, the development of four hybridomas that produce anti-

human SLPI monoclonal antibodies (Chen et al. 2006); secondly, the specificity of serum SLPI levels to differentiate between benign ovarian cysts and malignancies (Tsukishiro et al. 2005); thirdly, the development of cleaved SLPI (cSLPI) as a biomarker of chymase activity in allergic disease (Belkowski et al. 2008); and, finally, the exciting potential of the SLPI promoter as a tissue-specific promoter in the development of ovarian cancer gene therapy (Barker et al. 2003).

4 Concluding Remarks

Recent publications on the WAP SLPI and trappin-2/elafin have dramatically changed our current view of these molecules. They are no longer 'just' antiproteases expressed in lining fluid but major modulators of immunity. Moreover, their actions seem temporally regulated to be expressed during stages of the immune response. They have roles in innate immune priming, which links to the adaptive immune system and also to immune homeostasis and tissue remodelling, suggesting that their plethora of activities are essential throughout the inflammatory response. Understanding how they can produce such varying activities over the course of the inflammatory response is not so well understood and will form the basis for the next generation of literature on these quite extraordinary pleiotropic molecules.

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Histatins: Multifunctional Salivary Antimicrobial Peptides

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Abstract In this chapter, we overview the plethora of properties that have been attributed to histatins including tannin binding, microbicidal activity, immunomodulatory activity, and the recently found stimulation of cell migration. Attention is in particular paid to the molecular mechanisms underlying these properties. We conclude that many properties of histatins can be explained by their physicochemical properties, which allows them to bind a variety of negatively charged molecules and surfaces. For instance, their cationic character is crucial for their membrane-disrupting activity, which forms the basis of their antimicrobial activity. The only function that cannot directly be predicted on the basis of their physicochemical features is the enhancement of wound healing which proceeds via canonical receptor-mediated cell signalling.

1 Introduction

Histatins were among the first antimicrobial peptides that were discovered (MacKay et al. 1984a, b; Pollock et al. 1984). They make up a family of 12 histidine-rich peptides that occur exclusively in the saliva of humans and higher primates (Sabatini et al. 1989; Xu et al. 1990). They are encoded by two genes: *HTN1* and *HTN2* (also referred to as *HTN3* or *HTN5*). The HIS1(1) allele on *HTN1* encodes histatin 1 and the derived histatin 2; the HIS2(1) allele on *HTN3* encodes histatin 3 (Sabatini and Azen 1989), of which all other histatins are derived, presumably by posttranslational proteolytic cleavage (Vanderspek et al. 1990). Of the histatins from human parotid saliva, 85–90 % consists of the histatins 1, 3, and 5 in ratios of 3:1:3 (corresponding to concentrations around 2½ μM, 1 μM, and 4 μM),

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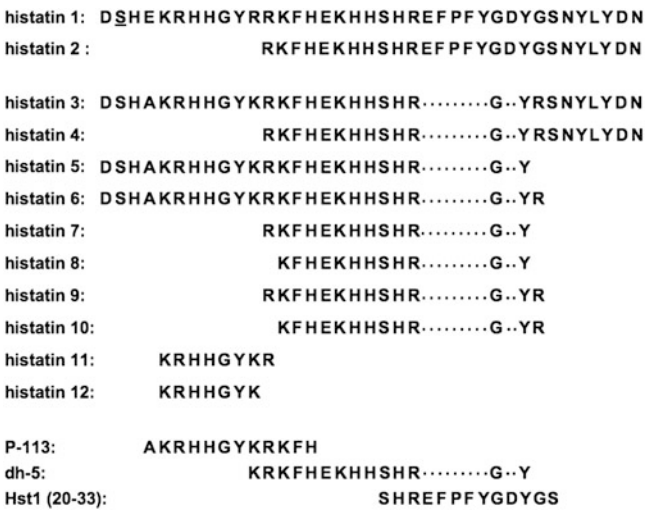

Fig. 1 *Sequence alignment of the histatins and the synthetic variants.* The underscore in the sequence of histatin 1 indicates the presence of a phosphoserine residue at position 2. P-113 and dh-5 are two synthetic minimal-length variants in which the candidacidal potency of the parent peptide, histatin 5, is retained. The large differences between these two peptides, together with the lack of stereospecificity, indicate that the candidacidal activity of histatins is not governed by a proper receptor-binding domain but by a minimal amphipathic stretch. Hst1 (20–33) is the synthetic minimal-length variant in which the epithelial cell-migration-stimulating capacity of the parent peptide, histatin 1, is retained. The corresponding histatin motif is unique for the *HTN1*-derived histatins (1 and 2) and bears neither significant overlap nor structural resemblance with the putative antimicrobial motifs represented by dh-5 and P-113

respectively. Of these, histatin 5 is the most potent peptide, and histatin 1 the least (Oppenheim et al. 1986, 1988). Due to the relatively large contribution of histatin 5, research has primarily focussed on this peptide and its derivatives. Based on activity studies with synthetic truncated variants, two stretches were identified in histatin 5 that can be considered as minimal active domain: residues 11–24 and 4–15, denoted dh-5 (Raj et al. 1990) and P-113 (Rothstein et al. 2001), respectively (Fig. 1).

Over the years, histatins have proved efficient antimicrobial agents in vitro against a broad spectrum of microorganisms. These include Gram-positive bacteria (MacKay et al. 1984a, b), Gram-negative bacteria (Murakami et al. 1992), yeasts and fungi (Brant et al. 1990; Oppenheim et al. 1988; Pollock et al. 1984), and the human protozoan parasite *Leishmania* (Luque-Ortega et al. 2008). In addition, it has become clear that histatins are versatile molecules that exhibit a broad range of properties. In this chapter, we review a number of these properties in the context of human innate immunity.

2 Histatins as Membrane-Disrupting Peptides: Mechanism of Action

The generally accepted cellular target of most antimicrobial peptides is the cytoplasmic membrane. The mechanism of action includes electrostatic attraction of the positively charged peptide to the negatively charged microbial membrane and adoption of an amphipathic peptide structure interacting at the hydrophobic/hydrophilic membrane environment interface, followed by destabilisation and disruption of the membrane leading to lethal efflux of vital cell constituents (Fig. 2). Based on this sequence of events, several mechanistic models have been described, mainly differing from each other in the description of the peptide-induced membrane effects (Van 't Hof et al. 2001). The essential requirement for an antimicrobial peptide is the possession of a positively charged domain that is able to adopt an amphipathic secondary structure. Histatin 5 does not seem to contain a distinct active domain, the fact that dh5 and P-113 are equally active, whereas their sequences only moderately overlap, points to a minimum length of an amphipathic stretch with two positively charged termini (the so-called double wing motif), rather than to a discrete antimicrobial domain. Another observation that supports this non-specific mechanism is that the all-D enantiomers of histatin 5 and P-113 show the same antimicrobial activity as the all-L enantiomers (Rothstein et al. 2001; Ruissen et al. 2003).

Originally, disruption of the microbial membrane was considered as the molecular basis of both their antibacterial (MacKay et al. 1984a) and candidacidal activity (Pollock et al. 1984). However, in the killing of *Candida albicans,* histatins behave differently than canonical antimicrobial peptides. Most salient aspect is that killing of *C. albicans* by histatins proceeds at a much slower rate. Whereas addition of a lethal dose of other antimicrobial peptides almost instantaneously results in complete killing of *C. albicans*, killing by histatins takes 15–60 min to reach completion. Such a slow killing rate was considered incompatible with a membranolytic mechanism of action (Edgerton et al. 1998, 2000; Helmerhorst et al. 2001; Koshlukova et al. 1999; Tsai et al. 1996; Xu et al. 1999). Another argument came from model studies with liposomes as mimics of microbial membranes, showing that histatins showed little, if any, membranolytic activity (Den Hertog et al. 2004; Edgerton et al. 1998; Raj et al. 1994). The putative underlying cause was their poor propensity to adopt an amphipathic conformation. On the one hand, it was reported that histatins do not adopt an amphipathic α-helix (Brewer and Lajoie 2002); on the other hand, it was reported that although histatin 5 and its truncated variants histatin 5(9–24) and histatin 5(11–24) (dh-5, see Fig. 1) were able to adopt α-helical conformations in membrane-mimicking solvents, the resulting hydrophobic moments, a measure of the propensity to adopt an amphipathic structure, were much lower than those of other antimicrobial peptides (Helmerhorst et al. 2001; Raj et al. 1990, 1994, 1998). However, antimicrobial peptides with small hydrophobic moments that display a "conventional" rapid killing are not that unusual (Van 't Hof et al. 2001). Neither does

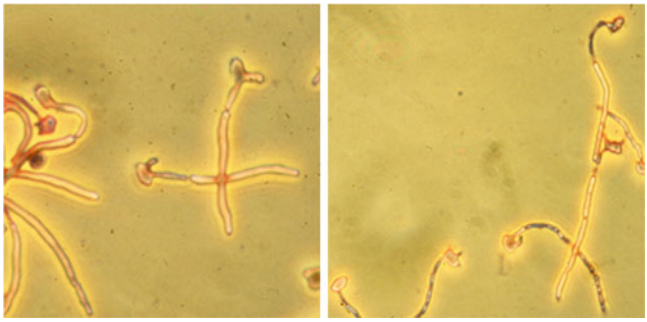

Fig. 2 *Membranolytic effect of histatin 5 on hyphenated C. albicans cells*. Under the microscope, the morphological changes in hyphenated *C. albicans* cells caused by histatin 5 are apparent. In particular, the hyphens appear to have lost their membrane integrity, leading to loss of cell content (*right panel*), compared to untreated viable cells (*left panel*)

the prerequisite of intracellular uptake exclude a membranolytic killing mechanism (Den Hartog et al. 2005; Ruissen et al. 2001).

To evaluate whether histatin 5 acts as a slow pore-former in *C. albicans* cells, we have measured time-dependent membrane depolarisation and permeabilisation as a measure for membrane disruption and correlated these with the degree of cell killing. Contrary to what might be expected from the liposome studies, incubation of *C. albicans* with histatin 5 led to depolarisation of the plasma membrane, as measured using the potential-dependent fluorescent probe $DiSC_3(5)$ (Ruissen et al. 2001). Membrane permeabilisation was monitored using the membrane-integrity probe propidium iodide as well as by measuring the efflux of vital cell components, viz. nucleotides (Den Hertog et al. 2006). Both processes advanced in complete synchronicity with the killing, suggesting an unambiguous relationship between histatin-mediated killing of *C. albicans* and the loss of vital cellular components.

Still, the seemingly absence of membrane disruption by histatins has led to many speculations about alternative mechanisms of action, including energy-dependent receptor-mediated uptake and intracellular targeting (reviewed in: Caldéron-Santiago and Luque de Castro 2009; Isola et al. 2007; Kavanagh and Dowd 2004). Most of these have been proven incorrect (Den Hertog et al. 2006; Isola et al. 2007; Jang et al. 2010; Koshlukova et al. 1999; Ruissen et al. 2001; Veerman et al. 2004, 2007).

In fact, the intracellular uptake of histatins by *C. albicans*, leading to lethal efflux of vital cell components, proceeds more complicated. At lower concentrations, histatin 5 is internalised by receptor-mediated endocytosis and stored in the vacuoles without having any effect on the membrane integrity and viability of the cells (Mochon and Liu 2008). At higher concentrations, histatin 5 crosses the membrane through a mechanism that is dependent on its high cationic charge. Cell sorting experiments demonstrated that the cells with only vacuolar localisation of histatin 5 survived, while none of the cells with cytoplasmic histatin 5 formed colonies. Time-lapse microscopy revealed that histatin 5 induced a single spatially restricted perturbation in the cytoplasmic membrane, which was permeable for small compounds, including histatin 5, propidiumiodide and rhodamine B.

This was accompanied by rapid expansion of the vacuole, by loss of cell volume, and by a rapid efflux of ATP and K^+. The percentages of released ATP and K^+ were proportional to the percentage of killed cells at any time point of the killing curve.

The tendency to form a relatively long-living cluster on the cytoplasmic membrane is a unique feature of histatins that explains the relatively slow killing kinetics. Interestingly, higher salt concentrations do not abrogate the initial interaction of histatins with the cell but arrest the killing process at the formation of these membrane-associated histatin clusters (Mochon and Liu 2008; Xu et al. 1999).

3 Detoxification of Noxious Foodstuffs by Histatins

The propensity of histatins to bind to negatively charged compounds is not limited to microbial membranes. In the oral cavity, many negatively charged molecules occur to which histatins can bind. This may have physiological relevant effects.

Binding of histatins to tannins, polyphenolic compounds from plant origin, has been implicated in detoxification of dietary tannins found in tea, wine, berries, and nuts. Binding of histatin 5 to tannic acid also blocked its inhibition of α-amylase suggesting a protective role of histatins in the enzymatic activity within the digestive tract (Bennick 2002).

4 Inhibition of Dental Plaque Formation by Histatins

Binding of histatins to hydroxyapatite, the principal mineral in dental enamel, may play a role in remineralisation of the teeth after acidic attack (Richardson et al. 1993). Binding to hydroxyapatite also plays a role in the formation of the pellicle, a protective layer of (glyco)proteins glycoproteins that covers the dental surfaces and inhibits the adherence of bacteria. Although the matter whether the histatins retain their antimicrobial activity after binding to hydroxyapatite has been debated (Xu et al. 1993; Yin et al. 2003), results from in vivo studies suggest that hydroxyapatite binding may play a role in the inhibition of plaque formation, either by direct killing or by reduced adherence (Paquette et al. 2002; Van Dyke et al. 2002).

Histatins also retain their candidacidal activity after adsorption onto poly(methyl methacrylate). Modifications of this polymer to improve its histatin 5-adsorbing properties with the long-term goal to produce PMMA-based dentures that are less susceptible to colonisation by *C. albicans* showed promising results in vitro (Edgerton et al. 1995; Yoshinari et al. 2006).

The strong binding of histatins to *Porphyromonas gingivalis*, a Gram-negative bacterium associated with periodontal disease, inhibits hemagglutination of this microorganism, supposedly by blocking adhesins important for adherence to oral surfaces (Murakami et al. 1992). Histatins also significantly reduced co-aggregation of *P. gingivalis* with *Streptococcus mitis*, a cariogenic member of dental plaque (Murakami et al. 1991), thus reducing its adherence to oral surfaces indirectly.

5 Anti-inflammatory Activity of Histatins

The ability of histatins to inhibit the activity of proteolytic enzymes from both microbial and host origins has been implicated in modulation of the immune response to oral infections (Basak et al. 1997; Gusman et al. 2001; Nishikata et al. 1991). However, there has been cast some doubt on whether histatins indeed are *bona fide* protease inhibitors. It has been demonstrated that the inhibitory activity of histatins gradually decreases during incubation with proteinases, because of proteolytic breakdown. In other words, histatins function as a substrate competing for the enzyme with the artificial substrates used for measuring protease activity (O'Brien-Simpson et al. 1998). Furthermore, inhibition of metalloproteinases by histatins has been attributed to their metal-binding properties. It is generally known that histidine-rich peptides have strong affinity for metal ions, because the imidazole group in the side chain of histidine is a chelator of metal ions. It is, therefore, not unexpected that histatins act as inhibitors of metalloproteinases, which require metal ions as cofactor for their enzymatic activity.

Alternatively, histatins have been implicated in immunomodulation by means of their LPS-binding properties, leading to suppression of the inflammatory induction of IL-6 and IL-8 from human gingival fibroblasts in vitro (Imatani et al. 2000; Sugiyama 1993).

6 Enzymatic Activity of Histatins

Serine proteases, esterases, and haloperoxidases each harbour a serine residue in their active site, of which the side-chain hydroxyl group acts as the nucleophile that attacks the substrate. This hydroxyl group is activated via proton abstraction by the imidazole side chain of an adjacent histidine residue, and the resulting imidazolium cation on its turn is stabilised by electrostatic interaction with the negatively charged side chain of a neighbouring aspartate residue (Hofmann et al. 2002). Although histatins do not possess the highly organised structures and specific binding pockets of proper enzymes, they do possess the necessary aspartate, histidine, and serine residues and the structural flexibility to position these in the proper conformation for enzymatic activity, at least in theory. Coordination of metal ions with the histidine side chains may stabilise such conformations and enhance the enzymatic activity considerably.

Enzymatic activity of histatins is poorly documented. We have observed that the use of the non-specific esterase substrate carboxy-DCFDA as membrane-integrity probe resulted in a high background signal due to the esterase activity of histatin 5 and that prolonged storage of highly purified histatin 5 in solution leads to (auto-catalytic?) degradation (unpublished results). Histatins do bind copper and zinc ions very well (Brewer and Lajoie 2000). After binding of copper ions, histatins display nuclease activity (Melino et al. 2006), which has been considered to contribute to their candidacidal activity.

Interestingly, in vitro studies showed that, under physiological concentrations of Cu^{2+}-ions, histatin 5 or histatin 8, and appropriate reductors such as ascorbate or cysteine, a copper-histatin complex was formed that produced hydrogen peroxide with high efficiency (Houghton and Nicholas 2009). By itself, hydrogen peroxide is not very toxic to microorganisms in the mouth, but, in theory, this reaction may well be suited to fuel the lactoperoxidase system, a well-defined antimicrobial system in saliva that uses hydrogen peroxide to enzymatically convert the abundant salivary thiocyanate into the bactericidal hypothiocyanite.

7 The Role of Histatins in Wound Healing

We found that histatins also play a prominent role in wound-healing processes in the mouth (Oudhoff et al. 2008). They achieve this by inducing the stretching and migration of epithelial cells at the edges of the wound, which results in a rapid re-epithelisation towards the centre of the wound. Although histatins are only secreted in saliva, they affect epithelial cells from skin tissue as well; in other words, licking your wounds may accelerate wound closure (Oudhoff et al. 2009).

Many antimicrobial peptides have a high therapeutic index, i.e. a high selectivity to microbial membranes over mammalian (host) membranes. We found that histatin 5, the most potent peptide from the histatin family, has no detectible lytic activity towards human cell membranes (Helmerhorst et al. 1999). This suggests that the interaction of histatins with human cells is governed by other molecular features than their interaction with microbial cells. The first observation confirming this hypothesis is that histatin 5, despite being the most potent antimicrobial histatin, showed little if any activity in the in vivo and ex vivo wound-healing models. On the other hand, histatins 1 and 2, with the lowest antimicrobial activity within the family, were among the most potent wound-healing stimulating histatins. The antimicrobial activity of histatin 5 has been ascribed to a non-specific electrostatic interaction with the microbial membrane caused by a positive charge and the ability to adopt an amphipathic structure. In contrast, in wound closure studies, all-D histatin 2 was completely inactive, and truncation analysis revealed a minimal active domain for stimulation of epithelial cells: SHREFPFYGDYGS (Figs. 1 and 3), residues 20–32 in histatin-1 numbering (Oudhoff et al. 2008, 2009). These two features are suggestive for the involvement of a specific histatin receptor on epithelial cells. There are more observations that point in this direction. Receptor-mediated activation of cell migration is generally accompanied by an active internalisation of the receptor–ligand complex. Indeed, the uptake of fluorescein-labelled histatin 1 by epithelial cells was almost completely abolished by depletion of the energy charge of the cells by lowering the incubation temperature to 4 °C or by pre-incubation with the energy poison sodium azide.

Pretreatment of epithelial cells with trypsin to strip them of membrane-bound surface-exposed proteins rendered them completely insensitive to histatin 1 and prevented the intracellular uptake of the fluorescein-labelled peptide (Oudhoff et al. 2008).

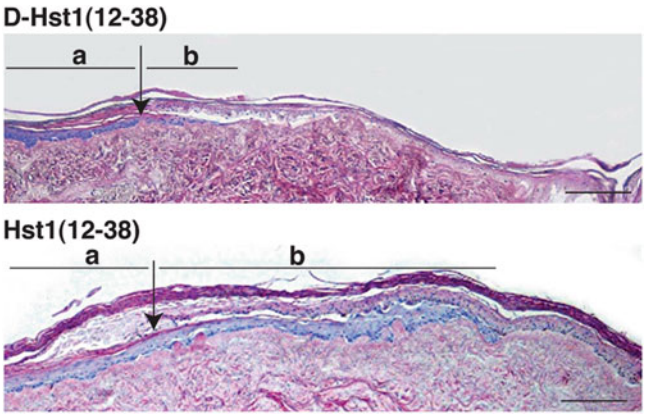

Fig. 3 *Histatin 2-stimulated re-epithelisation of a wound in a human skin model*. Representative micrographs of histatin 2-treated freeze-burn wounds in a validated human skin model, six days after wounding. The *arrows* indicate the boundaries of the wounds: (a) indicates intact epidermis, (b) indicates newly ingrown epidermis, and *bars* represent 250 μm. Epithelial cells are stained blue with hematoxylin and eosin. Histatin 2 (here represented as Hst1(12–38)) enhanced re-epithelisation of the artificial wound considerably (*lower panel*), in contrast to the all-D enantiomer of histatin 2 (here represented as D-Hst1(12–38)) that showed no enhancement of re-epithelisation over spontaneous ingrowth (*upper panel*). The stereospecificity of histatin 2-induced enhancement of re-epithelisation is indicative for the involvement of a receptor-mediated process

Activation of epithelial cells was not only energy and sequence dependent but also conformation dependent. Introduction of a conformational restraint into histatin 1 by head-to-tail cyclisation lead to a 1,000-fold increase in epithelial cell activation in vitro and ex vivo (Oudhoff et al. 2009).

Before we discuss the nature of this histatin receptor in more detail, we must realise that the prominent role of histatins in the wound-healing properties of saliva is unique in the animal kingdom. Histatins are only found in the saliva of humans and higher primates, so the wound-healing properties of saliva from other animals, well established for many years, must be attributed to different effector molecules. A major breakthrough in understanding the wound-healing properties of rodent saliva was reached with the identification of epidermal growth factor (EGF), which plays a crucial role in wound-healing processes such as cell proliferation, cell differentiation, and cell migration (Cohen 2008). EGF is also found in human saliva, and human epithelial cells do possess an EGF receptor (EGFR) at their surface. However, the concentration of EGF in human saliva is ~100,000 times lower than in rodent saliva, too low to play a significant role in human oral wound healing (Oudhoff et al. 2008). Nevertheless, human buccal epithelial cells could be activated by addition of recombinant human EGF, demonstrating that the EGF-driven machinery for activation of epithelial cells and stimulation of wound healing is present in the human mouth, but apparently, histatins have partially taken over this physiological role of EGF in human saliva.

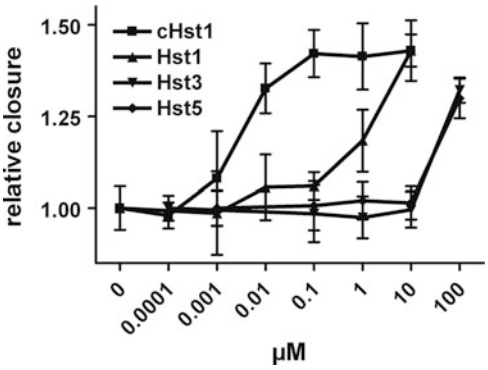

Fig. 4 *Effects of histatins on wound closure in vitro*. The three most abundant histatins in human parotid saliva were tested in scratch assays using confluent layers of human buccal epithelial cells grown on microscope slides as in vitro model for wound closure. Histatin 1 (*filled triangle*) was the most active, with 50 % of the maximal stimulatory effect around a concentration of 1 μM, well within the physiological concentration range in parotid saliva (around 2½ μM). Histatin 3 (*filled inverted triangle*) and histatin 5 (*filled diamond*) were completely inactive up to concentrations of 10 μM, way above their physiological concentrations (around 1 and 4 μM, respectively). Cyclisation of histatin 1 (*filled square*) resulted in a 1,000-fold increase in biological activity, with 50 % of the maximal stimulatory effect around a concentration of 1 nM. Enhancement of activity by cyclisation, i.e. the introduction of a conformational constraint within the peptide structure, is often regarded as an indication for the involvement of a ligand-receptor binding process

Histatins do not use the EGF receptor. Neither the specific EGFR inhibitor AG1478 nor SB203580, a specific inhibitor of the EGFR-linked p38MAPK signalling pathway, had any effect on the activity of histatin 2. On the other hand, pertussis toxin, a specific inhibitor of $G\alpha_1$-linked G-protein-coupled receptors, and U0126, a specific inhibitor of the GPCR-linked ERK1/2 signalling pathway, both completely inhibited the activity of histatin 2, without having any effect on the activity of rhEGF. In this respect, histatins are also unique; other human salivary antimicrobial peptides with wound-healing properties, β-defensins and the cathelicidin peptide LL-37 (even all-D LL-37), act through transactivation of the EGFR. Like EGF, these peptides induce cell proliferation besides cell migration.

Although histatins 3 and 5 activate epithelial cells to a much lesser extent (Fig. 4), they use the same receptor as histatins 1 and 2 for intracellular uptake. Uptake of histatin 5 was inhibited by histatin 2 and vice versa demonstrating that their binding affinities were comparable. Nevertheless, no antagonism between histatin 2 and histatin 5 was observed with respect to cell stimulating activity. This behaviour is not uncharacteristic for G-protein-coupled receptors. For several G-protein-coupled receptors an induced-fit model has been proposed, in which the ligand initially binds loosely to the receptor in its inactive form and subsequently induces the change of both ligand and receptor into the active form, leading to the formation of the ligand-receptor complex. This model explains the apparent discrepancy between the activity and the structural properties of cyclic histatin 1; initially, similar to its linear counterpart, this binds loosely and non-selectively to the inactive conformation of the

receptor. In the binding site then apparently conditions are present which favour the transition to the bioactive conformation of the peptide and, in concert, a transition of the receptor to its active conformation takes place. Peptides such as histatin 5, which are virtually inactive but yet internalised by the cell, can only perform the first step (loose binding to the inactive receptor), but cannot adopt the right conformation required to trigger a conformational change in the receptor to its active form. This initial binding is nevertheless sufficient for endocytosis, ostensibly similar to antagonist activities (Simmons et al. 1997).

8 Histatins: Two Functionally Different Subfamilies

The existence of two separate histatin genes has been enigmatic for many years. With regard to antimicrobial activity, the *HTN1* gene is virtually redundant; the contributions of histatins 1 and 2 to the total antimicrobial activity of the histatins present in human saliva are negligible. From an evolutionary viewpoint, the fact that the *HTN1* gene is nevertheless fully functional therefore must point to another function. Twenty-five years after the discovery of the histatins, this enigma was solved by our discovery that the *HTN1*-encoded histatins 1 and 2 possess high epithelial cell-migration-inducing activity (Oudhoff et al. 2009).

How specific are the histatin genes on a functional level? The antimicrobial activity of the histatin family is governed by the *HTN2*-encoded histatins 3–12, all expressing considerable antimicrobial activity. On molar basis, the *HTN1*-encoded histatins 1 and 2 make up around one third of the total histatin pool present in saliva, but, due to their low potency, their relative contribution to the total histatin antimicrobial activity adds up to less than 10 % (Oppenheim et al. 1986, 1988; Oudhoff et al. 2008). At high concentrations, the *HTN2*-encoded histatins 3 and 5 also show a considerable epithelial cell-migration-inducing activity in vitro; at a 30 μM concentration, histatin 3 reaches the same maximal stimulation as histatins 1 and 2 (Oudhoff et al. 2009). Up to 10 μM concentrations, histatins 3 and 5 were inactive (Oudhoff et al. manuscript in preparation). The physiological concentrations of histatins 3 and 5 in human parotid saliva are well below this threshold (1–4 μM). The *HTN1*-encoded histatins 1 and 2 are active at much lower concentrations: histatin 1 reaches maximal stimulation between 1 μM (50 %) and 10 μM (Oudhoff et al. manuscript in preparation). The physiological concentration of histatin 1 in human parotid saliva lies within this range (~2½ μM). Thus, the wound-healing activity of human parotid saliva can be attributed exclusively to the *HTN1*-encoded histatins, whereas the antimicrobial activity can be attributed almost completely to the *HTN2*-encoded histatins.

Several other properties of histatins can be correlated to either of the encoding genes. Binding of histatins to hydroxyapatite is mainly limited to histatin 1, due to its negatively charged C-terminus containing a phosphoserine residue (Richardson et al. 1993). Histatin 5 is a strong LPS binder and presumably has immunomodulatory properties, whereas histatin 2 shows little if any LPS binding and no immunomodulatory activity (Imatani et al. 2000; Oudhoff et al. 2009).

Obviously, it is beneficial to spread different properties implicated in innate immunity over different peptides, especially as these properties interfere with each other or even are counteractive. In theory, it allows a better fine-tuning of the innate immune response. The activity of a peptide with all these properties combined such as LL-37 is confined to very narrow concentration margins (Oudhoff et al. 2010). For instance, the ability of histatin 2 to induce epithelial cell migration at extreme high concentrations (100 μM) can only be beneficial when accompanied by its loss of cytolytic activity and immunomodulating properties. These are retained in histatin 5, which, in addition, may also assist histatin 2 in the wound-healing process by reducing fibrosis (and subsequent scarification) by inhibition of host collagenases.

Histatin-induced wound healing is a very young area of research, and there are not yet any data supporting the theory that the separation of wound healing and antimicrobial properties within the histatin family leads to cooperation between members of the two functionally different subfamilies. Although the concept is tempting, the slight overlaps in antimicrobial and cell-migration-inducing activities between the subfamilies appear to contradict this theory.

9 Histatins: Multifunctional Salivary Antimicrobial Peptides?

It is amazing how many different functional properties histatins manage to comprise within such small stretches of amino acids. According to many experiments in vitro, histatins may play a pivotal role in innate oral immunity. Their protective activity may cover many different areas, stretching from detoxification of noxious compounds to killing of invading microbes and from acceleration of wound healing to modulation of the immune response. The physiological importance of these various functional properties for an actual role in the innate immunity, however, for the most part still remains unclear. The working environment of the histatins, human saliva, is a very complex fluid containing literally hundreds of chemical compounds: ions, salivary (glyco)proteins and whatever compounds we may fancy to ingest, and hundreds of microbial species. Combined with the multifunctionality of histatins, this leads to a cornucopia of interactions that may interfere with the proposed roles in innate immunity. In principle, these could be beneficial (synergism); however, in most cases, these interactions only reduce the activity of histatins. The most obvious example is the abundance of host and microbial proteases in saliva that lead to a rapid breakdown of the histatins. This makes it extremely difficult to translate the activity of histatins in vitro to the situation in vivo. Several in vivo studies in animal models and patient studies have shown the therapeutic potential of histatins as antimicrobial agents (Paquette et al. 2002; Santarpia et al. 1991; Van Dyke et al. 2002; Welling et al. 2007). Yet, studies in vitro using physiological ionic-strength conditions show almost complete inactivation of histatins due to their high salt sensitivity (Edgerton et al. 1998; Helmerhorst et al. 1997; Xu et al. 1999). Does this mean that the membranolytic

activity of histatins observed in candidacidal assays in vitro plays a minor role in their physiological killing of *C. albicans* and that other antimicrobial properties emerge in vivo? The propensity of histatins, together with copper ions and suitable electron acceptors present in saliva, to generate hydrogen peroxide, which, in its turn, may fuel the antimicrobial lactoperoxidase system, could render such a scenario feasible.

Although histatins have been considered for many years as exceptional antimicrobial peptides, apart from their delayed action on the *C. albicans* membrane, at least in vitro, they behave very much like other membranolytic antimicrobial peptides. Nevertheless, histatins do possess a number of features that justify a special position within the group of antimicrobial peptides. Besides the ability to disturb microbial membranes, histatins display a large number of activities in vitro that have been implicated to innate immunity in humans. However, it is extremely difficult to evaluate whether a more or less artificial activity in vitro corresponds to a physiological function. Virtually, all antimicrobial activities discussed above lack solid evidence of physiologic relevance. Understanding the true role of histatins in the innate immunity of humans remains an important challenge for future research. Another striking difference with other peptides involved in innate immunity is that the interaction of histatins with microorganisms and with host cells is separated over two different subfamilies, each with its own coding gene. Altogether, this makes the histatins a unique group of multifunctional host defence peptides.

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Structure–Function Relationships of Antimicrobial Chemokines

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Abstract The chemokines are a group of small chemotactic cytokines that play an important role in the innate and adaptive immune system. Their main function is related to the recruitment of white blood cells to sites of infection. They bind to specific chemokine receptors, which subsequently triggers signaling pathways in the leukocytes. Recently the discovery of chemokines that possess a direct antimicrobial activity against a broad range of pathogenic bacteria has generated increased interest in the role of these proteins in the innate immune system. Prior studies regarding ligand and receptor binding have already established the structural elements important for chemokine interaction and activation of their receptors. In the same manner, it is important to study the structural features required for the antimicrobial activity of this group of chemokines in order to establish key elements related with this new activity. This review will focus on the structure–function relationships that appear to be related to the direct antimicrobial activity of the chemokines. A close similarity of the C-terminal domain of many chemokines to cationic α-helical antimicrobial peptides suggests that this C-terminal helical region is responsible for the chemokine antimicrobial activity. However, for several chemokines, the antimicrobial activity resides in other parts of the protein, indicating that each chemokine needs to be examined individually. We also discuss the role of dimerization and of linearization of chemokines in their antimicrobial activity.

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Abbreviations

AMPs	Antimicrobial peptides
CA-MRSA	Community-associated methicillin-resistant *Staphylococcus aureus*
CTAP-3	Connective tissue-activating protein-3
DC	Dendritic cells
DOPE	1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOPG	1,2-Dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol)
ENA-78	Epithelial neutrophil-activating protein 78
GCP-2	Granulocyte chemotactic protein-2
GRO	Growth-regulated oncogene
hBD	Human beta-defensin
IL-8	Interleukin-8
IP-10	Interferon-inducible protein-10
I-TAC	Interferon-inducible T-cell alpha chemoattractant
LDL	Low-density lipoprotein
MCP-4	Monocyte chemoattractant protein-4
MEC	Mucosa-associated epithelial chemokine
MIG	Monokine induced IFN-gamma
MIP-3α	Human macrophage inflammatory protein-3α
NAP-2	Neutrophil-activating peptide-2
NMR	Nuclear magnetic resonance
PBP	Platelet basic protein
PF-4	Platelet factor-4
PG-1	Protegrin-1
PGLa	Peptidyl-glycylleucine-carboxyamide
PMP	Platelet microbicidal protein
RANTES	Regulated upon activation normal T-cell expressed and secreted
SDS	Sodium dodecyl sulfate
SIC	Streptococcal inhibitor of complement
SPA	Staphylococcal protein A
SpyCEP	*Streptococcus pyogenes* cell envelope proteinase

1 Chemokines

The chemokines are a family of small chemotactic cytokines, with a size ranging from 8 to 13 kDa (Allen et al. 2007). Their role in the innate and adaptive immunity has been studied since the first chemokines were discovered 28 years ago (Sauder et al. 1984; Yoshimura et al. 1987a). Their primary function concerns the activation and recruitment of specific leukocytes to the site of injury or infection through the creation of a chemokine concentration gradient. The localization and activation of

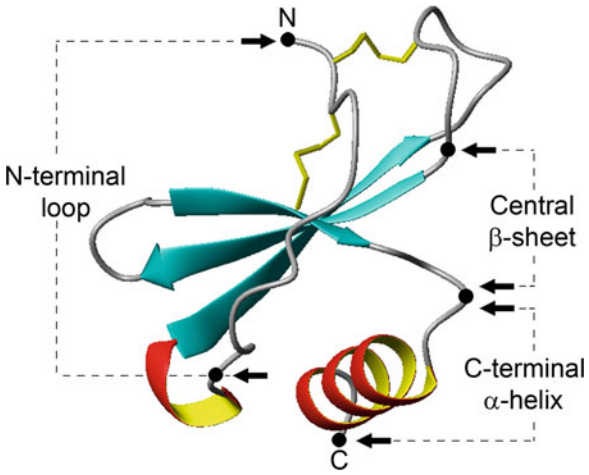

Fig. 1 Stereo ribbon diagram of CCL20 (PDB code 1M8A) showing the highly conserved three-dimensional structure of the chemokine family. The two disulfide bonds are represented in *yellow*, and the N and C termini are labeled. The structural elements normally analyzed for antimicrobial activity, such as the N-terminal loop, the central β-sheet, and the C-terminal α-helix, are indicated between the *arrows*. Figure prepared with MolMol software

the white blood cells is achieved by binding of the chemokines to their cognate chemokine receptors, which are all part of the G-protein-coupled transmembrane receptor family (Proudfoot 2002; Allen et al. 2007). In addition to their chemotactic activity, several chemokines and chemokine receptors are known to play a role in various diseases (Viola and Luster 2008; Proudfoot 2002; Allen et al. 2007). The chemokine family is usually classified into four groups which can be distinguished by the organization of the cysteine residues in the N-terminal region of the protein, i.e., C, CC, CXC, and CX_3C, where X represents any nonconserved amino acid residue (Murphy et al. 2000). The CXC group of chemokines can be further divided into two subgroups, where the presence or absence of the amino acid sequence ELR in the N-terminal region creates the ELR and non-ELR CXC chemokine subfamilies. All chemokines of the CC, CXC, and CX_3C classes form two disulfide bonds, while the C class only possesses one stabilizing disulfide linkage. To date, approximately 50 chemokines and 20 chemokine receptors have been reported (Allen et al. 2007). Although the sequence identity among all the members of the chemokine family is highly variable (20–90 %), their three-dimensional structures display a remarkable conserved topology. Most chemokines are formed by an N-terminal extended loop region, in which the first two cysteine residues are located, a bundle of three antiparallel β-strands, and a C-terminal α-helix (Allen et al. 2007) (Fig. 1). Several structural features are important for the signaling induced by the chemokines upon binding to their receptors. The N-terminal domain has emerged as the main recognition site in these proteins. Deletion of this region inhibits chemokine-induced signaling and in some cases also affects the interaction with the receptor. The N-loop located between the first cysteine residues and the first β-strand is important for the interaction with the receptor and is generally considered as the first docking site during the two-step binding process of the chemokines to their receptors. The loops connecting the β-strands also contribute to the interaction with the receptor. In some cases other residues in the chemokine structure have also been shown to be important for the interactions with the receptors (Allen et al. 2007; Clark-Lewis et al. 1995).

2 Antimicrobial Activity of Chemokines

A different part of the innate immune defense against bacterial infections is formed by a large group of antimicrobial peptides (AMPs) which often have a potent activity against bacteria, fungi, and other organisms, including some viruses. The AMPs are an important part of the host defense system of widely divergent organisms including bacteria, plants, insects, and vertebrates. Antimicrobial peptides act by directly perturbing bacterial membranes or by entering the bacterial cell and interfering with important processes, e.g., DNA transcription (Epand and Vogel 1999; Nguyen et al. 2011). However, recently some antimicrobial peptides have been shown to be involved in the regulation of the immune response as well, by binding to the same receptors as used by the chemokines (Yang et al. 2004). Conversely, it has also been demonstrated that numerous chemokines can have a direct antimicrobial activity against Gram-positive and Gram-negative bacteria, as well as an antifungal activity (see Table 1). Several studies have reported on the antimicrobial activity of individual or small groups of chemokines. Unfortunately, there is no consensus about the methodology and experimental conditions used to determine the antimicrobial activity of chemokines. The liquid-phase antimicrobial and microbicidal assay is a solution-phase test widely used in antimicrobial experiments. In this assay bacteria are incubated with different concentrations of chemokines or antimicrobial agents in liquid media. The assay is based on the microtiter broth dilution assays used to assess minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of antibiotics (Amsterdam 1996). After exposure to the peptide, wells are inspected for growth to assess the minimum inhibitory concentration, and/or serial dilutions are plated on solidified media and colony-forming units are counted after incubation, to assess the microbicidal concentration. Despite the widespread use of this technique, there is considerable variation in test parameters, such as composition of the incubation solution, period of exposure, and test strains used. Hancock and colleagues have attempted to standardize the methodology (Wiegand et al. 2008). Another test to establish the antimicrobial activity of chemokines and antimicrobial peptides is the radial diffusion assay. In this assay the microorganisms are incorporated in buffered agar or agarose-containing media, solidified in plates. Chemokines are added to wells punched in the agar, and after a defined period in which the compounds diffuse into the agar, a nutrient overlay medium is applied to the plates. The plates are then incubated to allow growth of microorganisms, and zones of inhibition are measured (Lehrer et al. 1991). The chemokines antimicrobial activity collected in Table 1 includes antimicrobial information determined by both assays, solution, and/or solid-phase test.

The antimicrobial function of the chemokines draws attention to the complementary roles that these proteins play in immunity. The capacity to exert a direct and potent antimicrobial activity seems to be an additional mechanism for fighting bacteria and other pathogens (Eliasson and Egesten 2008). Recently the group of chemokines with antimicrobial activity have been called “kinocidins” by some

Table 1 Antimicrobial activity of chemokines

	Synonyms	Antimicrobial activity	References
XC chemokine			
XCL1	Lymphotactin α; SCM-1α	*E. coli; S. aureus; S. typhimurium; C. albicans*	Yount et al. (2007), Yang et al. (2003)
XCL2	Lymphotactin β; SCM-1β	ND	
CC chemokines			
CCL1	I-309; (mouse) TCA-3	*E. coli; S. aureus*	Yang et al. (2003)
CCL2	MCP-1; MCAF; (mouse) JE	*S. aureus; S. typhimurium*	Yount et al. (2007)
CCL3	MIP-1α; MIP-1αS	No activity against *E. coli* and *S. aureus*	Cole et al. (2001), Yang et al. (2003)
CCL4	MIP-1β	No activity against *E. coli*	Cole et al. (2001)
CCL5	RANTES	*E. coli; S. aureus; C. albicans; C. neoformans*	Yount et al. (2007), Tang et al. (2002)
CCL6	(mouse) C10; (mouse) MRP-1	*E. coli; S. enterica*	Kotarsky et al. (2009)
CCL7	MCP-3	No activity against *E. coli* and *S. aureus*	Yang et al. (2003)
CCL8	MCP-2	*E. coli*	Yang et al. (2003)
CCL9	(mouse) MRP-2; (mouse) MIP-1γ	ND	
CCL10		ND	
CCL11	Eotaxin	*E. coli; S. aureus*	Yang et al. (2003)
CCL12	(mouse) MCP-5	ND	
CCL13	MCP-4	*E. coli; P. aeruginosa*	Martínez-Becerra et al. (2007), Yang et al. (2003)
CCL14	CC-1; HCC-1; NCC-2; Ckβ1; MCIF	*E. coli*	Kotarsky et al. (2009)
CCL15	CC-2; HCC-2; NCC-3; MIP-5	*E. coli*	Kotarsky et al. (2009)
CCL16	LEC; HCC-4; NCC-4; LCC-1; LMC	No activity against *E. coli* and *S. aureus*	Yang et al. (2003)
CCL17	TARC	*E. coli; S. aureus*	Yang et al. (2003)
CCL18	PARC; DC-CK-1; MIP-4; AMAC-1	*E. coli; S. aureus*	Yang et al. (2003)
CCL19	MIP-3β; ELC; exodus-3; ckβ11	*E. coli*	Yang et al. (2003)
CCL20	MIP-3α; LARC; exodus-1; (mouse) ST38	*E. coli; S. aureus; P. aeruginosa; M. catarrhalis; S. pyogenes; E. faecium; C. albicans; C. neoformans; S. coag. neg.* spp.*; Propionibacterium* ssp.	Hoover et al. (2002), Yang et al. (2003), Maerki et al. (2009)
CCL21	SLC; 6Ckine; exodus-2; TCA4; ckβ9	*E. coli; S. aureus*	Yang et al. (2003)

(continued)

Table 1 (continued)

	Synonyms	Antimicrobial activity	References
CCL22	MDC; (mouse) abcd-1	*E. coli; S. aureus*	Yang et al. (2003)
CCL23	MIP-3; MPIF-1; ckβ8-1	ND	
CCL24	MPIF-2; eotaxin-2; ckβ6	ND	
CCL25	TECK; ckβ15	*E. coli; S. aureus*	Yang et al. (2003)
CCL26	Eotaxin-3; MIP-4α	ND	
CCL27	CTACK; Eskine; ILC (mouse) ALP	*E. coli; C. albicans*	Hieshima et al. (2003), Maerki et al. (2009)
CCL28	MEC	*P. aeruginosa; K. pneumoniae; S. mutants; P. pyogenes; S. aureus; C. albicans*	Hieshima et al. (2003), Liu and Wilson (2010)
ELR CXC chemokines			
CXCL1	GRO-α; MGSA	*E. coli; S. aureus; S. typhimurium; C. albicans*	Yount et al. (2007), Yang et al. (2003)
CXCL2	GRO-β; MIP-2α	*E. coli; S. aureus*	Yang et al. (2003)
CXCL3	GRO-γ; MIP-2β	*E. coli; S. aureus*	Yang et al. (2003)
CXCL5	ENA-78	*S. pyogenes*	Linge et al. (2008b)
CXCL6	GCP-2	*E. coli; S. pyogenes; S. dysgalactiae; S. aureus; P. aeruginosa; N. gonorrhoeae; E. faecalis*	Collin et al. (2008), Linge et al. (2008b)
CXCL7	NAP-2	*S. pyogenes*	Linge et al. (2008b)
CXCL8	IL-8	*E. coli; S. typhimurium; S. aureus; C. albicans*	Yount et al. (2007), Maerki et al. (2009)
Non-ELR CXC chemokines			
CXCL4	Platelet factor-4 (PF-4)	*E. coli; S. aureus; S. typhimurium; B. subtilis; C. albicans; C. neoformans*	Krijgsveld et al. (2000), Yount et al. (2007), Tang et al. (2002), Yeaman et al. (2007)
CXCL9	Mig	*E. coli; S. aureus; S. pyogenes; L. monocytogenes; N. gonorrhoeae; B. anthracis*	Cole et al. (2001), Yang et al. (2003), Egesten et al. (2007), Crawford et al. (2009), Linge et al. (2008a)
CXCL10	gIP-10; (mouse) CRG-2	*E. coli; S. aureus; S. pyogenes; L. monocytogenes; B. anthracis*	Cole et al. (2001), Yang et al. (2003), Egesten et al. (2007), Crawford et al. (2009)
CXCL11	I-TAC; IP9; H174	*E. coli; S. aureus; S. pyogenes; L. monocytogenes; B. anthracis*	Cole et al. (2001), Yang et al. (2003), Egesten et al. (2007), Crawford et al. (2009)
CXCL12	SDF-1α; SDF-1β; PBSF	*E. coli; S. aureus*	Yang et al. (2003)
CXCL13	BCA-1; BLC	*E. coli; S. aureus*	Yang et al. (2003)

(continued)

Table 1 (continued)

	Synonyms	Antimicrobial activity	References
CXCL14	BRAK; bolekine	*E. coli; S. coag. neg.* spp.; *Propionibacterium* spp.; *S. aureus; C. albicans*	Yang et al. (2003), Maerki et al. (2009)
CXCL16[a]	SR-PSOX	*E. coli; S. aureus*	Tohyama et al. (2007)
CXCL17	DMC	ND	
CX_3C chemokines			
CX_3CL1[a]	Fractalkine; (mouse) neurotactin	No activity against *E. coli* and *S. aureus*	Cole et al. (2001), Yang et al. (2003)

ND not determined
[a]Only the chemokine domains of CXCL16 and CX3CL1 were tested for antimicrobial activity

authors (Yount et al. 2004; Yount and Yeaman 2004). One of the structural elements that may relate this group of chemokines to other disulfide-containing antimicrobial peptides is the presence of a multidimensional signature composed by a so-called γ-core motif, perhaps suggesting a common ancestry for previously unrelated groups of antimicrobial agents (Yeaman and Yount 2007; Yount and Yeaman 2004).

The activity of the majority of the antimicrobial chemokines is markedly dependent on the ionic strength of the incubation media as is also observed for antimicrobial peptides. Intriguingly, some of the antimicrobial chemokines, such as CCL20, CCL28, CXCL9, CXCL10, and CXCL11, are expressed in different epithelial cells, and it is noteworthy that they are secreted into fluids with a relatively low salt concentration which allows them to exhibit their full antimicrobial potency (Moser et al. 2006; Fujiie et al. 2001; Nakayama et al. 2001; Shirane et al. 2004; Starner et al. 2003; Sauty et al. 1999).

The structural requirements for chemokines with antimicrobial activity are not clearly identified, but some studies have tried to uncover the main features involved. A comprehensive study of a group of 30 chemokines has established that almost 80 % of the chemokines can exhibit antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* at neutral pH (Yang et al. 2003). An initial analysis of the biochemical characteristics of the antimicrobial chemokines showed that normally the chemokines with pI values higher than 9.0 possess antibacterial activity, which indicates the importance of cationicity as a major factor for the antimicrobial activity. However, unlike what is observed with many antimicrobial peptides, the potency of this activity was not correlated with the pI value of the chemokines. Other factors, such as the cationicity of the N-terminal tail, and the hydrophobicity of the surface of the chemokines were analyzed, but again there was no direct correlation between these features and the antimicrobial activity. However, the three-dimensional structures of the chemokines and in particular the electrostatic potential surface distribution of the positive charges revealed a common theme. The presence of a large three-dimensional positively charged surface patch in the proteins was a characteristic shared by all the antimicrobial chemokines. Chemokines without antimicrobial activity either do not have such a surface or have negatively charged residues interfering with the cationic

surface patches. Nonetheless, the potency of the antimicrobial activity seemed not related with the size of the positively charged surface patches (Yang et al. 2003). In general, dissecting the structural elements that are responsible for the antimicrobial activity of the chemokines can be performed by studying peptides resembling particular domains of the chemokines (Fig. 1). These domains are normally selected on the basis of biochemical properties, such as cationicity and amphipathicity, which are important properties for many AMPs (Haney et al. 2009a; Epand and Vogel 1999). A limitation to this approach obviously is that conformation-dependent positively charged surface domains of the full-length proteins cannot be mimicked. Rather, these peptides represent parts of the linearized molecules which would arise from reduction of their disulfides. Still, a considerable number of such peptides with antimicrobial activity have been identified.

Interestingly, despite the evidence related to the antimicrobial activity of chemokines, recently it had been reported that antimicrobial chemokines also induce the release of the virulence factor protein A (SPA) by a community-associated methicillin-resistant *S. aureus* (CA-MRSA) (Yung et al. 2011). It is possible that this is an example of how bacteria may utilize host defense system signals (e.g., chemokines) in order to evade the immune response. In addition, the binding of chemokines to the *S. aureus* membrane, which had been reported for CXCL9 and CXCL10, may also contribute to avoid the immune reaction of the host by restricting the amount of free chemokines available for recruitment and activation of the immune cells (Yung et al. 2011).

3 Antimicrobial Activity of CC Chemokines

3.1 Monocyte Chemoattractant Protein-4 (MCP-4)/CCL13

The monocyte chemoattractant protein-4 (MCP-4)/CCL13 contains 75 amino acid residues (Uguccioni et al. 1996). It interacts with the chemokine receptors CCR2, CCR3, and CCR5 (Leach et al. 2007; Uguccioni et al. 1996) and induces the migration of monocytes, T lymphocytes, and eosinophils (Garcia-Zepeda et al. 1996; Uguccioni et al. 1996). This particular chemokine has been shown to be involved in several inflammatory diseases including arthritis and asthma (Rojas-Ramos et al. 2003; Iwamoto et al. 2006; Kalayci et al. 2004). Although the sequence identity for the members of the monocyte chemoattractant protein (MCP) subfamily of CC chemokines (composed of CCL2, CCL7, CCL8, CCL12, and CCL13) is around 60 %, CCL13 is the only chemokine from this subfamily to exhibit antimicrobial activity against Gram-positive and Gram-negative bacteria (Martínez-Becerra et al. 2007). Studies on 19-mer peptides using CCL13 sequence as a template showed that the C-terminal peptide $CCL13_{57–75}$, called CDAP-4, had similar activity as full-length CCL13, against *E. coli* (Martínez-Becerra et al. 2007). The antimicrobial activity of this peptide was further studied against *Pseudomonas aeruginosa* and significant morphological changes were observed by transmission electron microscopy when lethal concentrations were used (Martínez-Becerra et al. 2007). The

antibacterial activity of CDAP-4 was susceptible to the ionic strength of the media, being greatly reduced at NaCl concentrations higher than 100 mM (Martínez-Becerra et al. 2007). The stability of the three-dimensional structure of the CDAP-4 peptide was studied by molecular dynamics simulations. The peptide forms a short amphipathic α-helix with a net positive charge (+5) and a high pI (10.58), characteristics that are normally observed for α-helical AMPs (Martínez-Becerra et al. 2007). In addition, an electrostatic potential analysis revealed a large positively charged surface on the peptide, which may account for its antimicrobial activity (Martínez-Becerra et al. 2007).

The structure of the full-length CCL13 shows the typical central three-stranded antiparallel β-sheet flanked by an extended loop in the N-terminal region and an α-helix in the C-terminal region, which includes residues 57–67, which are part of the CDAP-4 peptide (Barinka et al. 2008). The crystal structure suggests the formation of CCL13 dimers (Barinka et al. 2008), although solution experiments in 100 mM NH_4OAc at pH 6.8 show that CCL13 is a monomer or that it forms heterodimers when combined with other members of the same chemokine family (Crown et al. 2006). Taken together, all these results indicate that the C-terminal region of CCL13 is important for the antibacterial activity of this chemokine and that the full-length molecule may form dimers which can possibly contribute to the antimicrobial activity.

3.2 Human Macrophage Inflammatory Protein-3α (MIP-3α)/CCL20

The human macrophage inflammatory protein-3α (MIP-3α)/CCL20 is made up of 70 amino acid residues (8 kDa) and bears some resemblance with the antimicrobial β-defensin peptides (Hoover et al. 2002). This chemokine plays a role in diseases such as cancer and rheumatoid arthritis, among others (Kleeff et al. 1999; Schutyser et al. 2003; Matsui et al. 2001). CCL20 is responsible for the migration of immature dendritic cells (DC), effector/memory T cells, and B cells, upon interaction with the chemokine receptor CCR6 (Schutyser et al. 2003). The structure of CCL20 resembles that of all CC chemokines (Fig. 1). The N-terminal region, containing the two first cysteine residues, is fairly flexible, and it is connected by a 3.10 helical turn to the central region made up of a three-stranded antiparallel β-sheet, which is followed by the usual C-terminal α-helix (Chan et al. 2008; Hoover et al. 2002; Malik and Tack 2006). The available crystal structures of CCL20 show the protein forming a dimer structure (Hoover et al. 2002; Malik and Tack 2006). Diffusion NMR and pH titration studies established that the dimerization of CCL20 is markedly pH dependent, CCL20 being a dimer at neutral pH and a monomer at lower pH (Chan et al. 2008). The CCR6 receptor only interacts with CCL20, while CCR6 is also the only chemokine receptor for CCL20. This specificity between chemokine ligand and receptor is an interesting characteristic shared with eight more chemokine receptor-ligand couples (Viola and Luster 2008). However, the human β-defensin antimicrobial peptides (hBD 1–3) can also induce the migration

of cells that express either CCR6 or CCR2 (Rohrl et al. 2010a, b; Yang et al. 1999). Like the β-defensins, CCL20 has a direct antimicrobial activity against Gram-positive and Gram-negative bacteria (Hoover et al. 2002; Yang et al. 2003), as well as antifungal (Yang et al. 2003) and antiviral activity (Ghosh et al. 2009; Kim et al. 2007). The activity of CCL20 against *E. coli* is actually higher than the activity of human β-defensin 2 (hBD-2) (Hoover et al. 2002). Structurally, there are no obvious similarities between CCL20 and the β-defensins which could account for the antimicrobial activity of CCL20. The presence of a relatively high number of positive charges in the N-terminal region of CCL20 was initially thought to contribute to its antimicrobial activity, but the existence of chemokines with positively charged N-terminal regions and without antimicrobial activity indicates that other aspects should be also considered. The overlap in antimicrobial activity may arise from the presence of similarly localized positively charged patches on the surfaces of CCL20 and hBD-2 (Fig. 2). These regions are located in the turns between the N-terminal loop and the β1 strand and the turn between the β2 and β3 strands (Hoover et al. 2002). Another important structural characteristic for antimicrobial activity, contributing to possible pore formation in bacterial membranes, is the amphipathicity of the protein. CCL20 lacks a large hydrophobic surface, and the region surrounding Leu-8 is the only recognizable hydrophobic patch on the protein surface (Hoover et al. 2002).

In addition to the antimicrobial activity of the full-length CCL20, antimicrobial activity is also found in some peptides derived from CCL20. During cancer progression, the protease cathepsin D generates a C-terminal 12-residue peptide from CCL20 ($CCL20_{59–70}$) with antimicrobial activity against *E. coli*, which is significantly reduced compared to the activity of intact CCL20 (Hasan et al. 2006). Further analysis of the CCL20 structure revealed that the C-terminal α-helix starts after the Pro-51 residue. It is possible that this segment of the protein is also important for the antimicrobial activity, because of its overall cationicity and amphipathicity. The antimicrobial activity of a synthetic C-terminal CCL20 peptide composed of the last 20 amino acids ($CCL20_{51–70}$) was 60 times higher against *S. aureus* and *E. coli*, and 30 times higher against *Bacillus subtilis*, than the activity of the naturally occurring C-terminal peptide $CCL20_{59–70}$ (Chan et al. 2008; Nguyen et al. 2010). The $CCL20_{51–70}$ peptide has a large number of positive charges and forms an amphipathic α-helix upon binding to membrane mimetics such as SDS micelles (Chan et al. 2008; Nguyen et al. 2010). The shorter 12-residue $CCL20_{59–70}$ peptide has a lower positive net charge and does not form a stable α-helix in the presence of SDS micelles. Additionally its amphipathicity is perpendicular to the length of the peptide instead of parallel, as was found for $CCL20_{51–70}$ (Nguyen et al. 2010). These structural differences account for the large difference in antimicrobial activity, as a high net positive charge and amphipathicity are shared characteristics for most members of the α-helical class of antimicrobial peptides such as the magainins, cecropins, and lactoferrampin (Epand and Vogel 1999; Haney et al. 2007, 2009b). The lack of antimicrobial activity for the N-terminal fragments $CCL20_{1–52}$ and $CCL20_{1–55}$ further indicates that the C-terminal region of CCL20 is mostly responsible for the antimicrobial activity of this chemokine (Chan et al. 2008; Hasan et al. 2006).

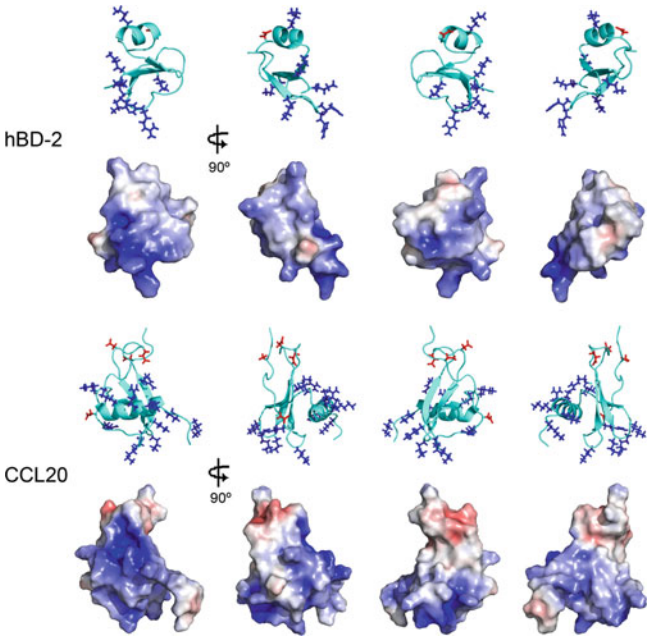

Fig. 2 *Ribbon* representation and electrostatic potential surface of human β-defensin 2 (hBD-2) (PDB code 1FD3) and chemokine CCL20 (PDB code 2JYO), with positively charged side chains in *blue* and negatively charged side chains in *red*. Each image is rotated 90° from the vertical axis. Electrostatic potential surfaces were calculated by adaptive Poisson–Boltzmann Solver (APBS) using PDB2PQR software and depicted by PyMOL software

3.3 Mucosa-Associated Epithelial Chemokine MEC/CCL28

The mucosa-associated epithelial chemokine (MEC)/CCL28 is a protein of 108 amino acids residues (12.3 kDa) that regulates the migration and activation of specific leukocytes, such as cutaneous lymphocytes, antigen-positive (Ag^+) memory T cells, and eosinophils. It interacts with the chemokine receptors CCR10 and CCR3 (Pan et al. 2000). CCL28 is constitutively expressed in human and mouse epithelial cells of tissues such as the mammary glands, the respiratory tract, the colon, and the salivary glands (Pan et al. 2000; Wang et al. 2000). The C-terminal region of CCL28 shares a high sequence identity (53 %) with the antimicrobial peptide histatin-5, which has a potent antifungal activity against *Candida albicans* (Hieshima et al. 2003). This unique feature of CCL28 focused early attention on its possible antimicrobial activity. Human CCL28 (hCCL28) exerts a direct antimicrobial activity against Gram-negative and Gram-positive bacteria, in addition to possessing an antifungal activity (see Table 1). The antimicrobial activity against *C. albicans* and *P. aeruginosa* was more potent at lower salt concentrations (Hieshima et al. 2003; Liu and Wilson 2010). The most closely related chemokine, CCL27 (sequence identity 40 %) (Pan et al. 2000), which lacks the extended C-terminal region of CCL28, does not have bactericidal activity and

only low activity against *C. albicans* at high concentrations (Hieshima et al. 2003; Yang et al. 2003). In line with this, the first two positively charged residues in the 85–89 region (RKDRK) of murine CCL28 (mCCL28) are highly conserved among CCL28 homologues in all studied mammalian species and are essential for the antibacterial activity (Liu and Wilson 2010). These results indicate that the direct antimicrobial and antifungal activities of CCL28 rely on the C-terminal histatin-like region. A 28-residue peptide corresponding to this C-terminal region of the hCCL28 was even more active than histatin-5 against *C. albicans* but showed a low antibacterial activity (Hieshima et al. 2003). In a similar fashion, the 52-residue peptide corresponding to the C-terminal region of mCCL28 had a reduced antimicrobial activity against *S. aureus* and *P. aeruginosa*, while a 56-residue peptide resembling the N-terminal region of mCCL28 had no antibacterial activity (Liu and Wilson 2010). The broader antimicrobial activity of CCL28 compared to its C-terminal CCL28 peptide established that not only the C-terminal region is required for killing the bacteria but in addition the interaction with the rest of the protein is essential for the expression of the full antimicrobial potential, a notion that was further supported by elegant studies with protein chimeras (Liu and Wilson 2010). In addition, the influence of the disulfide bridges that normally stabilize the chemokine structure of CCL28 was studied, showing that the tertiary structure is not required for antimicrobial activity but is essential for its chemotactic activity (Liu and Wilson 2010). Similar results have been obtained for human β-defensin 3 (hBD-3) and tachyplesin I, showing that the cysteine bridges are not mandatory for their antimicrobial activity (Hoover et al. 2003; Wu et al. 2003; Ramamoorthy et al. 2006) but in the case of hBD-3 are again required for the chemotactic function (Hoover et al. 2003; Wu et al. 2003).

In addition to the above-mentioned CC chemokines, other members of this family have been shown to possess antimicrobial activity (see Table 1). Further studies need to be done in order to establish the structural characteristics that confer the antimicrobial activity to these chemokines.

4 Antimicrobial Activity of ELR CXC Chemokines

4.1 Granulocyte Chemotactic Protein-2 (GCP-2)/CXCL6

Granulocyte chemotactic protein-2 (GCP-2)/CXCL6 is a chemokine of the ELR CXC family, made up of 77 amino acids residue (Proost et al. 1993a, b; Froyen et al. 1997; Van Damme et al. 1997). It is mainly involved in chemoattracting neutrophils (Froyen et al. 1997; Wuyts et al. 1997; Viola and Luster 2008) due to its interactions with the chemokine receptors CXCR1 and CXCR2 which are also expressed in monocytes and mast cells (Wuyts et al. 1997, 1998; Wolf et al. 1998). CXCL6 is expressed by epithelial cells, macrophages and mesenchymal cells (Fillmore et al. 2003; Collin et al. 2008; Mine et al. 2003; Prause et al. 2003; Gijsbers et al. 2004;

Wuyts et al. 2003). CXCL6 has been shown to possess an important NaCl-sensitive antimicrobial activity (Collin et al. 2008), which is comparable with the activity of LL-37 (Linge et al. 2008b), a well-known human antimicrobial peptide of the cathelicidin family (Zanetti 2004). Gram-positive and Gram-negative bacteria that are normally involved in infections of dermis and mucosal surfaces are susceptible to the bactericidal action of CXCL6 (see Table 1), which appears to be related to the disruption of the bacterial membranes (Linge et al. 2008b). Interestingly, previous studies did not detect any antimicrobial activity of CXCL6 against *E. coli* and *S. aureus* (Yang et al. 2003). The discrepancies in the antibacterial activity can likely be attributed to the differences in the incubation media used in the different studies. Egesten and coworkers showed that under the same conditions, the antimicrobial activity of CXCL5/ENA-78 and CXCL7/NAP-2 against *Streptococcus pyogenes* was 30 times less than the activity of CXCL6 (Linge et al. 2008b). The structure of CXCL6 has not been determined yet, but a structural model can be predicted based on the known structures of other members of the CXC chemokine family, which showed that the structure resembles the general fold of chemokines. The N-terminal region is devoid of regular secondary structure and it contains two cysteine residues located at positions 12 and 14, which form disulfide bonds with the Cys residues at positions 38 and 54. The central region is formed by three antiparallel β-strands and the C-terminal region is a short α-helix. The cationic charge and amphipathicity of the C-terminal α-helix resembles the biochemical properties and secondary structure of some antimicrobial peptides, pinpointing this region of the chemokine as being possibly responsible for the antibacterial activity. Comparisons of the antimicrobial activity of the full-length CXCL6 protein and peptides resembling the N-terminal or C-terminal region of CXCL6 established that full-length CXCL6 is more active than either of these peptides. Interestingly, the bactericidal activity of the peptide encompassing the N-terminal region was higher than the activity of the C-terminal α-helix peptide, indicating that the C-terminal region alone is not the major determinant for the antimicrobial activity. Instead, the N-terminal region seems to be more relevant for the bactericidal activity in this case. These results also correlate with the higher leakage induced in DOPE/DOPG liposomes by CXCL6 and its N-terminal region peptide. Circular dichroism experiments showed that CXCL6 and its C-terminal region share up to 25 % of helical content in the structure, while the N-terminal region only contains 6 % upon interaction with PGPE liposomes. These results reveal that helical content is not directly correlated with the antimicrobial activity of CXCL6-derived peptides. When the net charge of the CXCL6-derived peptides is compared, the presence of an extra positive charge in the N-terminal peptide appeared related with the higher antibacterial activity (Linge et al. 2008b). It is known that the secreted *Streptococcus pyogenes* cell envelope proteinase (SpyCEP) cleaves CXCL6 in a position affecting the chemokine-induced neutrophil activation (Zingaretti et al. 2010; Sumby et al. 2008). Additionally, in vivo studies showed that the non-SpyCEP expressing bacterial strains gave rise to larger lesions in infected mice (Sumby et al. 2008). Although studies of the direct antimicrobial activity of the CXCL6-derived peptides

resulting from SpyCEP digestion have not yet been reported, it is possible that the action of this protease also impairs the antimicrobial activity of CXCL6 thereby contributing to the large lesions observed in the in vivo studies.

4.2 Neutrophil-Activating Peptide-2 (NAP-2)/CXCL7 and CTAP-3

Neutrophil-activating peptide-2 (NAP-2)/CXCL7 is a 70 amino acid residue chemokine of the ELR CXC family (Brandt et al. 2000). Together with connective tissue-activating protein-3 (CTAP-3), CXCL4/PF-4, and CCL5/RANTES, it forms the major portion of the platelet-derived chemokines (Flad and Brandt 2010). CXCL7 is derived from the β-thromboglobulins which represent a group of homologous α-granule-stored proteins (Brandt et al. 2000). The primary sequences of these proteins are identical except for their N-termini (Fig. 3a). The two main β-thromboglobulins found in human platelets are the platelet basic protein (PBP) and CTAP-3 (Brandt et al. 2000). Although both of these CXCL7 precursors contain the full sequence of the active CXC chemokine (CXCL7) including the ELR motif, they lack detectable chemotactic activity (Walz et al. 1989). PBP and CTAP-3 have minor antimicrobial activity against *E. coli*, *S. aureus*, and *Cryptococcus neoformans* under slightly acidic conditions (Tang et al. 2002). Proteases such as cathepsin G, which is membrane-associated or released from neutrophils and monocytes, can cleave both precursors between a specific Tyr and an Ala residue in the N-terminal region, thereby releasing the active CXCL7 chemokine (Car et al. 1991; Walz and Baggiolini 1990; Cohen et al. 1992; Brandt et al. 1991; Harter et al. 1994). CXCL7 exhibits important chemoattracting activity for neutrophils (Brandt et al. 2000), but its antimicrobial activity is almost negligible (Linge et al. 2008b; Krijgsveld et al. 2000). In addition to the N-terminally extended precursors of CXCL7, C-terminally truncated variants of CXCL7 have been described (Fig. 3a). Proteolytic processing of chemokine proteins occurs quite frequently in vivo and can have a major impact on the biological activity of the proteins (Wolf et al. 2008). C-terminally truncated CXCL7 has increased chemotactic activity, which is attributed to the removal of negatively charged Asp residues that are present in the C-terminal end of the full-length CXCL7 protein (Ehlert et al. 1995, 1998; Brandt et al. 1993, 2000). Regarding the antimicrobial activity of C-terminally truncated CXCL7 variants, a protein with high microbicidal activity, termed thrombocidin-1 (TC-1), has been isolated from human platelets following antimicrobial activity-guided purification. It possesses the same primary sequence as CXCL7, but the last two residues of the C-terminal region have been cleaved off, which causes an important increase in the microbicidal activity of the chemokine, which due to the truncation becomes highly active against *E. coli*, *B. subtilis, S. aureus, Lactococcus lactis*, *and C. neoformans* (Krijgsveld et al. 2000). Additionally, TC-1 is an important factor in the defense against infective endocarditis induced by viridans streptococci (Dankert et al. 2001). The TC-1 protein may

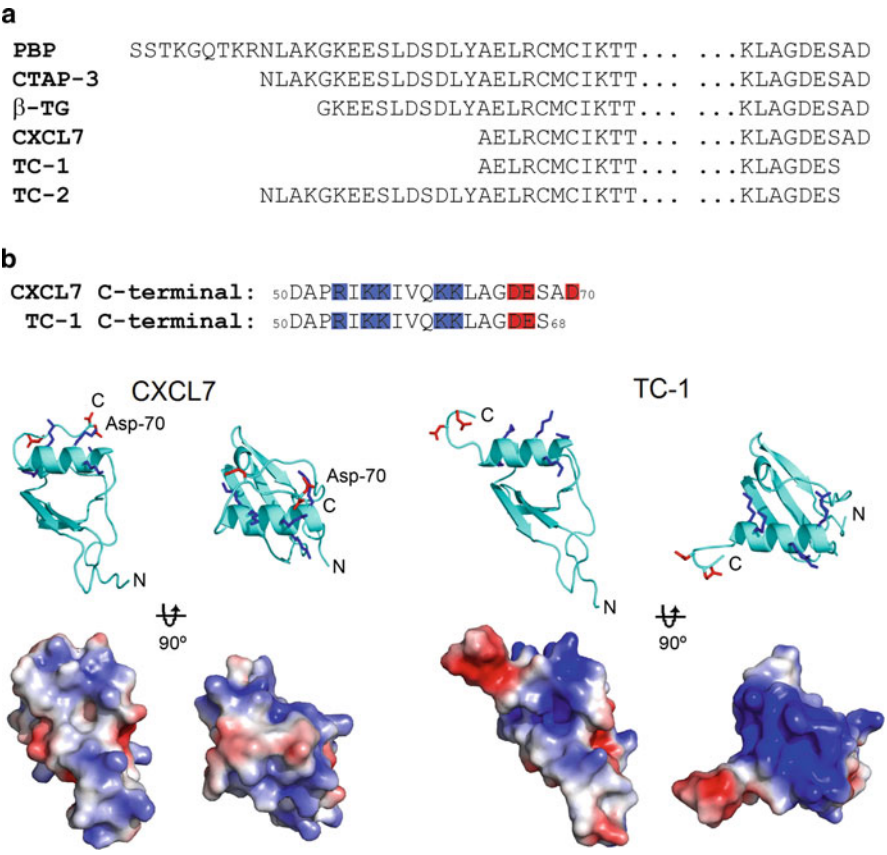

Fig. 3 (**a**) N- and C-terminal primary sequences of platelet basic protein (PBP) and derived ß-thromboglobulin (ß-TG) family members. (**b**) NAP-2/CXCL7 and TC-1 3D structures and electrostatic potential surfaces. Full-length CXCL7 and TC-1 structures were created by Nguyen et al. (2011) based on the combination of CXCL7 crystal structure (PDB code 1NAP) and C-terminal peptides (*upper sequences*) NMR structures. Negatively and positively charged residues are colored in *red* and *blue* respectively. Electrostatic potential surface were calculated by Adaptive Poisson–Boltzmann Solver (APBS) using PDB2QR software and depicted by PyMOL software

not act through membrane perturbation, as indicated by its inability to dissipate the transmembrane potential of *L. lactis* bacteria and of liposomes composed of *E. coli* lipids (Krijgsveld et al. 2000). The structure of CXCL7 has been solved by NMR spectroscopy (Mayo et al. 1994; Yang et al. 1994) and X-ray crystallography (Young et al. 1999). The structures are in agreement and show the classic CXC chemokine topology of an N-terminal loop, a three-stranded antiparallel β-sheet, followed by a C-terminal α-helix which includes residues Arg54-Asp66 (Fig. 3b). Disordered electron density for the last four residues of the crystal structure suggest a high degree of flexibility for the extreme C-terminal region of this chemokine (Young et al. 1999). Recent NMR studies have shown that the overall fold of TC-1 closely resembles that of CXCL7 (Nguyen et al. 2011). One of the truncated

residues in TC-1 is the negatively charged Asp-70, the removal of which seems to account for the emergence of the antibacterial activity. Studies of synthetic C-terminal α-helical peptides of both chemokines (CXCL7 and TC-1) were carried out in order to identify the influence of these parts of the proteins on the antimicrobial activity. Neither of the peptides was antimicrobial, and they showed a helical structure with a low degree of hydrophobicity and amphipathicity. The structure of the CXCL7 C-terminal peptide showed that the side chain of the C-terminal residue Asp-70 actually folds back and interacts with the positively charged residue Arg-61 located in the α-helical region (Nguyen et al. 2010). Subsequent NMR relaxation studies, which studied the flexibility of the protein backbone in CXCL7 and TC1, showed that the Asp-70 residue of CXCL7 is motionally restricted and interacts with the positive surface region of the protein (Fig. 3b). In contrast, in TC-1 the last few residues are highly flexible, and the positively charged surface region is exposed (Fig. 3b), being able to directly interact with negatively charged bacterial membranes to either perturb the membrane or enter the cell (Nguyen et al. 2011), explaining the difference in microbicidal activity with full-length CXCL7.

In the activity-guided isolation of human platelet antimicrobial proteins, a second protein, thrombocidin-2 (TC-2) was identified (Krijgsveld et al. 2000). TC-2 is identical to CTAP-3 except for a truncation of the two C-terminal residues. CTAP-3 has been putatively identified as one of the antimicrobial-active PBP derivatives in monocytes after detection in gel overlay assays with a highly AMP-susceptible *S. typhimurium phoP* mutant strain as the target organism (Schaffner et al. 2004). As mentioned before, the activity as such of CTAP-3 is not high, with only minor activity reported against wild-type strains of *S. aureus*, *E. coli*, and *C. neoformans* in slightly acidic conditions (Tang et al. 2002), and no bactericidal activity against *Bacillus subtilis*, *E. coli*, or *S. aureus* when tested up to 30 μM. A C-terminal two amino acid truncation, yielding TC-2, however strongly increases the microbicidal activity (Krijgsveld et al. 2000). Of note, the truncation is identical to that generating TC-1 from CXCL7 (Fig. 3), suggesting that this is a more general step in generating antimicrobially active derivatives from members of the PBP protein family.

4.3 Interleukin-8 (IL-8)/CXCL8

Interleukin-8 (IL-8)/CXCL8 was one of the first chemokines discovered (Schroder et al. 1987; Walz et al. 1987; Yoshimura et al. 1987b). It contains 72 amino acid residues (8 kDa) and upon stimulation CXCL8 can be produced by a wide variety of cells, including fibroblasts, epithelial and endothelial cells, hepatocytes and monocytes, among others (Baggiolini et al. 1989). CXCL8 is important for the activation and attraction of neutrophils to sites of inflammation, but other cells are also susceptible to its chemotactic activity (Baggiolini et al. 1989). As expected, the chemotactic activity arises through interactions with chemokine receptors, in this case CXCR1 and CXCR2 (Wu et al. 1996; Holmes et al. 1991; Murphy and Tiffany 1991). CXCR1 and CXCR2 and their associated ligands have been related to a large number

of pathologies (Bizzarri et al. 2006). CXCL8 is also important for the promotion of angiogenesis (Li et al. 2005; Matsuo et al. 2009). As described above for CXCL7, a C-terminally truncated variant of CXCL8 lacking the last three residues exhibits higher chemotactic activity than the full-length chemokine (Clark-Lewis et al. 1991). The structure of CXCL8 has been resolved by both NMR spectroscopy and X-ray crystallography (Baldwin et al. 1991; Clore et al. 1989, 1990). These structures are in agreement and show an architecture composed of an extended loop followed by a 3.10 helical turn (involving residues 19–22) and the antiparallel stranded β-sheet connected to a C-terminal α-helix (corresponding to residues 57–72). In solution CXCL8 behaves as a dimer, where the monomers are connected by six backbone hydrogen bonds between residues 25, 27, and 29 (Clore et al. 1989, 1990). The antibacterial activity of CXCL8 has been somewhat controversial. Bylund and colleagues, using a modified inhibition zone assay, were not able to observe any antimicrobial activity for full-length CXCL8 against *E. coli* (Bjorstad et al. 2005). Similarly, no antimicrobial activity for CXCL8 against *E. coli* and *S. aureus* (Cole et al. 2001; Yang et al. 2003) was found using standard colony-forming unit assays (Harder et al. 2001) or radial diffusion assays (Steinberg and Lehrer 1997). In contrast, Yeaman and colleagues observed different levels of pH-dependent antimicrobial activity of CXCL8 against *Salmonella typhimurium* and *S. aureus* in solid-phase assays, but they did not record bactericidal activity in solution-phase assay. Their results suggest that the growth inhibition observed in the solid-phase assay is due to bacteriostatic effects. Additionally, an antifungal activity against *C. albicans* was detected in both solid- and solution-phase assays (Yount et al. 2007). The γ-core motif of CXCL8 (IL-8γ), located in the central β-sheet region, was tested as a separate peptide for antimicrobial and antifungal activity. The peptide showed no activity against *S. typhimurium, S. aureus* or *C. albicans*, in neither the solid-phase nor the solution-phase assay (Yount et al. 2007).

Another interesting structural element of CXCL8 which may exert antimicrobial activity is the C-terminal α-helix. A 19-residue C-terminal peptide, termed IL-8α, proved to be active against *S. typhimurium* and *C. albicans* in solid-phase assays and in solution-phase assays against *C. albicans*. In human blood matrices, the IL-8α 19-mer peptide exerts anti-*E. coli* activity (Yount et al. 2007). Acid hydrolysis of CXCL8 in vitro can generate a 20-residue IL-8α peptide with an extra Pro residue at the N-terminal region compared to the 19-mer peptide (Bjorstad et al. 2005). Antimicrobial assays performed with this IL-8α 20-mer peptide showed high activity against *E. coli* and moderate activity against *Salmonella enterica, Klebsiella pneumoniae*, and *S. pyogenes*, as established by inhibition zone assays (Bjorstad et al. 2005). The same peptide was tested for antibacterial activity in blood agar plates and moderate activity was found for *B. subtilis* and almost no activity could be detected with *S. aureus* and *E. coli* (Nguyen et al. 2010). The differences in the antimicrobial activity for IL-8α and CXCL8 in these studies may have been due to differences in media and incubation conditions. When comparing the SDS-micelle-bound structure of IL-8α 19-mer peptide and the C-terminal α-helix in the full-length CXCL8, there are no significant differences (Bourbigot et al. 2009). The structure of the IL-8α 20-mer peptide, dissolved in a chloform–ethanol–water (4:4:1) solvent mixture (Nguyen et al. 2010), is also in

agreement with the structures of the IL-8α peptide (Bourbigot et al. 2009) and that of the C-terminal α-helix in intact CXCL8 (Baldwin et al. 1991; Clore et al. 1989, 1990). In summary, the C-terminal region of CXCL8 seems to be important for the antimicrobial activity of this particular chemokine. As mentioned before, the *S. pyogenes* SpyCEP protease can cleave ELR CXC chemokines, including human CXCL8 (Edwards et al. 2005; Zinkernagel et al. 2008; Zingaretti et al. 2010; Hidalgo-Grass et al. 2006). The action of SpyCEP on CXCL8 releases a C-terminal peptide composed of residues 60–72 (Edwards et al. 2005). The direct antimicrobial activity of this peptide has not yet been tested in vitro, but in vivo and in vitro studies did show that the SpyCEP protease prevents eradication by neutrophils of the SpyCEP-expressing bacteria and other bacteria at the site of infection (Zinkernagel et al. 2008; Hidalgo-Grass et al. 2006). This effect has been attributed to diminished neutrophil recruitment and migration (Edwards et al. 2005; Zinkernagel et al. 2008) in addition to an inhibition of the formation of neutrophil extracellular traps (NETs) (Zinkernagel et al. 2008), due to digestion of CXCL8.

4.4 Growth-Regulated Oncogene α (GROα)/CXCL1, β (GROβ)/CXCL2 and γ (GROγ)/CXCL3

Among the members of the ELR CXC chemokine family with antimicrobial activity, there are three chemokines with similar characteristics: CXCL1/GROα, CXCL2/GROβ, and CXCL3/GROγ, closely related chemokines that are produced mainly by macrophages (Becker et al. 1994). These proteins have the capacity to attract neutrophils (Clark-Lewis et al. 1995) and monocytes (Smith et al. 2005), through interactions with the chemokine receptor CXCR2 (Katancik et al. 2000). All three chemokines have antibacterial activity against *E. coli* and *S. aureus* (Yang et al. 2003).

In addition to the above-mentioned ELR CXC chemokines, CXCL5 also exhibits antimicrobial activity against *S. pyogenes*. In summary, all members of the ELR CXC chemokine family exhibit a direct antimicrobial activity (see Table 1).

5 Antimicrobial Activity of Non-ELR CXC Chemokines

5.1 Platelet Factor-4 (PF-4)/CXCL4

Although the platelet factor-4 (PF-4)/CXCL4 is the earliest discovered member of the CXC chemokine family (Deuel et al. 1977), its biological function was not known to be related to the chemoattraction of specific cells until recent years.

CXCL4 is expressed mainly in the megakaryocytes and in platelets (Slungaard 2005). While other members of the non-ELR CXC chemokines, e.g., CXCL9–11, induce the migration of lymphocytes (Bonecchi et al. 1998; Sallusto et al. 1998), CXCL4 was initially thought to be devoid of such an activity (Clark-Lewis et al. 1993). A spliced variant of CXCR3 (named CXCR3-B) was described early on as the receptor for CXCL4. However, this receptor did not mediate a chemotactic response but induced an increase in the intracellular cyclic AMP levels instead (Lasagni et al. 2003; Slungaard 2005). A recent study suggests that migration of activated T lymphocytes can be induced by CXCL4 and that this is mediated by the chemokine receptor CXCR3 (Mueller et al. 2008), but these results have not been confirmed by other groups (Flad and Brandt 2010). In addition, the biological activity of CXCL4 is broad and includes roles in coagulation and functions such as inhibition of angiogenesis and hematopoiesis, promotion of neutrophil adhesion and activation, enhancement of oxy-LDL binding to the LDL receptor, and stimulation of anticoagulant activated protein C generation by the thrombomodulin/protein C system (Slungaard 2005). The generation of a CXCL4 knockout mouse has provided support for the role of this chemokine in platelet-dependent thrombosis (Slungaard 2005). CXCL4 also exhibits direct antimicrobial activity under slightly acidic conditions against Gram-positive and Gram-negative bacteria, in addition to antifungal activity (Tang et al. 2002) (see Table 1). The structure of human CXCL4 was solved as a tetramer by X-ray crystallography, showing a three-dimensional structure in agreement with the overall chemokine topology formed by the usual N-terminal loop, three-stranded antiparallel β-sheet, and C-terminal α-helix (Zhang et al. 1994). Based on phylogenetic relatedness, similar sequence motif, and predicted three-dimensional structures, platelet microbicidal protein-1 (PMP-1) was identified as a rabbit analogue of the human CXCL4 (Yount et al. 2004). In earlier studies, prior to their sequence identification, PMP-1 and its variant tPMP-1 (secreted by platelets after thrombin stimulation) were shown to exhibit a pH-dependent antimicrobial activity against Gram- positive and Gram-negative bacteria and fungi (Yeaman et al. 1997). The antimicrobial activities of PMP-1 and human CXCL4 exhibit similar specificities and efficacy (Yeaman et al. 2007). In the case of *S. aureus*, PMP-1 activity has been linked with an inhibition of intracellular macromolecular synthesis (Xiong et al. 2002). A synthetic peptide corresponding to the last 13 residues of the CXCL4 C-terminal region, which constitute an α-helix, is active against *E. coli* in the presence of a sub-MIC concentration of cefepime (a β-lactam antibiotic) in serum bactericidal assays (Darveau et al. 1992). RP-1 and RP-11 are peptides designed based on the α-helical C-terminal region of platelet proteins PMP-1, tPMP-1, and CXCL4, which have antimicrobial activity in in vitro biological matrices (Yeaman et al. 2002). The effects of RP-1 on *S. aureus* resemble the effects induced by tPMP-1, indicating that the antimicrobial mechanism of certain proteins can be reproduced by the synthetic peptides modeled upon relevant structural domains (Xiong et al. 2006). Comparison of the sequences for CXCL4, PMP-1, and their respective variants led to the creation of a consensus PMP sequence (cPMP), which was used to

generate a peptide library constituting of 15 amino acid long peptides, overlapping by 3 residues each, and larger peptides comprising the N or C-terminal half of the cPMP (Yeaman et al. 2007). Among the library, the peptides with the highest antimicrobial activity were $cPMP_{38–74}$, $cPMP_{49–64}$, and $cPMP_{60–74}$, which represent the C-terminal region of the protein (Yeaman et al. 2007). Most of the peptides corresponding to the N-terminal and the β-sheet regions did not exhibit considerable antimicrobial activity (Yeaman et al. 2007). These results suggest that the C-terminal region of CXCL4 is important for the overall antimicrobial activity of this chemokine.

5.2 *Monokine Induced by IFN-γ (MIG)/CXCL9*

The human monokine induced by IFN-γ (MIG)/CXCL9 is a protein of 103 residues of the CXC chemokine family (Liao et al. 1995; Farber 1993). The protein is chemotactic for monocytes, activated T cells, and NK cells (Liao et al. 1995; Lazzeri and Romagnani 2005; Loetscher et al. 1996). The chemotactic activity involves binding to the chemokine receptors CXCR3A and CXCR3B (Loetscher et al. 1996; Lazzeri and Romagnani 2005). The long C-terminal region of CXCL9 undergoes proteolytic processing by monocytes. This truncation has a profound effect on its biological activity (Liao et al. 1995; Farber 1997). Interferon-inducible protein-10 (IP-10)/CXCL10 and interferon-inducible T-cell alpha chemoattractant (I-TAC)/CXCL11 are closely related to CXCL9/MIG, sharing the same receptor and subsequently expressing similar biological activity (Farber 1997; Cole et al. 1998). Full-length CXCL9 displays a broad antimicrobial activity against organisms including *E. coli*, *Listeria monocytogenes, S. aureus* (Cole et al. 2001; Yang et al. 2003), *S. pyogenes* (Egesten et al. 2007), *Neisseria gonorrhoeae* (Linge et al. 2008a), and *Bacillus anthracis* spores and bacilli (Crawford et al. 2009). This antibacterial activity was shown to be inhibited by an increase in the ionic strength of the media in the case of *E. coli, L. monocytogenes*, and *S. pyogenes*. Although the three-dimensional structure of CXCL9 has not yet been solved, a model structure was created using the NMR structure of a truncated CXCL2/GROβ (Qian et al. 1999) as a template (Egesten et al. 2007). Due to the presence of a high number of positive charges in both the N-terminal and C-terminal regions of CXCL9, the antimicrobial activity might be embedded in either of these two sections of the protein. Consequently, peptides resembling the cationic regions of the N-terminal and C-terminal sections of CXCL9 were tested against *S. pyogenes*, and the activity of the C-terminal peptide was similar to the antibacterial activity of the full-length CXCL9. The N-terminal peptide did not have any anti-*S. pyogenes* activity, indicating that the antimicrobial activity of CXCL9 resides in its C-terminal region (Egesten et al. 2007). Streptococcal inhibitor of complement (SIC) is a protein secreted by *S. pyogenes* strains of the M1 serotype, which in addition to interfering with complement function inhibits the activity of human antimicrobial peptides,

such as LL-37 and β-defensins (Frick et al. 2003). Additionally SIC interferes with the antibacterial activity of CXCL9, without disturbing its chemotactic activity (Egesten et al. 2007), suggesting that SIC interacts with the antimicrobial C-terminal region of CXCL9. Although CXCL9, CXCL10, and CXCL11 are interferon-γ-inducible related chemokines that interact with the same CXCR3 receptor and have a similar antibacterial spectrum (Cole et al. 2001; Egesten et al. 2007; Yang et al. 2003; Crawford et al. 2009), the antimicrobial activity of CXCL10 and CXCL11 against *E. coli*, *L. monocytogenes*, and *S. pyogenes* is tenfold less than that of CXCL9 (Cole et al. 2001; Egesten et al. 2007). Analysis of the structures of CXCL10/IP-10 (Swaminathan et al. 2003), IP-10 mutant (NMeLeu27) (Booth et al. 2002), and CXCL11 (Booth et al. 2004) has shown that their C-terminal α-helices are smaller than the predicted C-terminal α-helix in CXCL9, which may account for the difference in the antimicrobial activities (Egesten et al. 2007; Eliasson and Egesten 2008). SpeB from *S. pyogenes* is a cysteine protease that cleaves and inactivates the antimicrobial peptide LL-37 (Schmidtchen et al. 2002). It also inactivates several human chemokines with antibacterial activity (Egesten et al. 2009). SpeB fully degrades CXCL10 and CXCL11, completely abolishing their chemotactic as well as antimicrobial activity. In contrast CXCL9 is only partially digested by SpeB, with cleavage sites in the N-terminal and C-terminal regions, releasing a smaller version of CXCL9 comprising the residues 18–73. This truncated version of CXCL9 has lost its chemotactic activity but retains its antibacterial activity against *S. pyogenes*. The synthetic peptide $CXCL9_{57–83}$ and SpeB-produced $CXCL9_{18–73}$ showed similar antimicrobial activities as the full-length CXCL9 (Egesten et al. 2007, 2009), indicating that not all the residues in the α-helical C-terminal peptide are responsible for the antimicrobial activity. Similarly, the truncation of CXCL10 by the furin protein generates a CXCL10 with four C-terminal residues removed. This variant is as antimicrobial as its precursor, CXCL10, against *E. coli* and *L. monocytogenes* (Hensbergen et al. 2004).

Among the group of non-ELR CXC chemokines, most members exert antimicrobial activity (see Table 1). Although structural elements as the C-terminal α-helical region have emerged as possibly being responsible for the antimicrobial activity in these chemokines, more studies are required.

6 Chemokine Dimerization and Antimicrobial Activity

Although many of the chemokines have the ability to form dimers or even higher-order oligomers in solution or after binding to glycosaminoglycans (Allen et al. 2007; Salanga and Handel 2011), the relationship of these oligomers with the antimicrobial activity of the bactericidal chemokines has not yet been studied much. Oligomerization has been postulated as one of the key steps in the antimicrobial activity mechanism of pore-forming antimicrobial peptides (Shai 1999; Mihajlovic and Lazaridis 2010; Mani et al. 2006). Oligomerization can allow the

formation of transmembrane pores leading to bacterial cell death (Shai 1999; Matsuzaki 1998). For some antimicrobial peptides, such as LLP1 and modified versions of magainin-2, the formation of disulfide-linked dimers has been shown to play an important role in the antimicrobial activity against Gram-positive and Gram-negative bacteria (Tencza et al. 1999; Dempsey et al. 2002). The formation of dimers prior to the interaction with the bacterial membranes increases the attraction for negatively charged membranes, probably due to the increase in the net positive charge of these peptides (Dempsey et al. 2002), generating peptides with 4–8 times higher activity, compared with the monomeric forms (Tencza et al. 1999). Not only the formation of covalently bound dimers can be related with an increase in antimicrobial activity. Protegrin-1 (PG-1) is a potent antimicrobial peptide for which the mechanism of action has been related to the formation of transmembrane pores (Bolintineanu et al. 2010; Langham et al. 2008). Using NMR, the dimerization of PG-1 was established as an essential requirement for the formation of pores in anionic membranes (Mani et al. 2006; Roumestand et al. 1998). Molecular dynamic simulations have confirmed the tendency of PG-1 to form dimers in the aqueous phase, on a membrane surface, or in the membrane core (Vivcharuk and Kaznessis 2010b). It was also established that the PG-1 dimer rather than the PG-1 monomer has a favorable energy for interactions with the membrane (Vivcharuk and Kaznessis 2010a). Other examples of this are seen in the β-defensin family of antimicrobial peptides. The tendency of human β-defensin 3 (hBD-3) to form non-covalent dimers may explain the large difference in the antimicrobial activity among the known human β-defensins (Schibli et al. 2002). When compared with other human β-defensins (hDB-1 and hDB-2), hBD-3 has been reported to possess a more potent and salt-resistant antimicrobial activity against a broader spectrum of pathogens (Pazgier et al. 2006; Taylor et al. 2008). It has been proposed that this increase may be related to the formation of dimers which exhibit a considerable increase in the size of the positively charged surface, thereby increasing the attraction for negatively charged membranes (Schibli et al. 2002). Although crystallography studies of the hDB-2 structure in addition to molecular dynamic studies had shown the possibility of dimerization for this protein (Suresh and Verma 2006; Hoover et al. 2000), static and dynamic light scattering experiments in 0.1 M Tris and pH 8.0 showed that only hDB-3 exists as a dimer in solution (Schibli et al. 2002). Some of the antimicrobial chemokines also have the ability to form non-covalent dimers in solution, which in addition to enhancing the net positive charge of the protein also increases the size of the positively charged surface. The configuration of the chemokines dimers can be divided into two groups. CC-chemokines normally form dimers by interactions between the residues of the first β-strand, forming a two-stranded antiparallel β-sheet with the corresponding β-strand from the other monomer, resulting in a more elongated dimer structure. In contrast, CXC chemokines normally form dimers by interactions between the β-strands forming a continuous six-stranded β-sheet, resulting in a more compact dimer structure (Allen et al. 2007) (Fig. 4a). The chemokine CCL20 is an exception to the previously described dimerization scheme for CC chemokines, as it follows the scheme for CXC chemokines. The CCL20

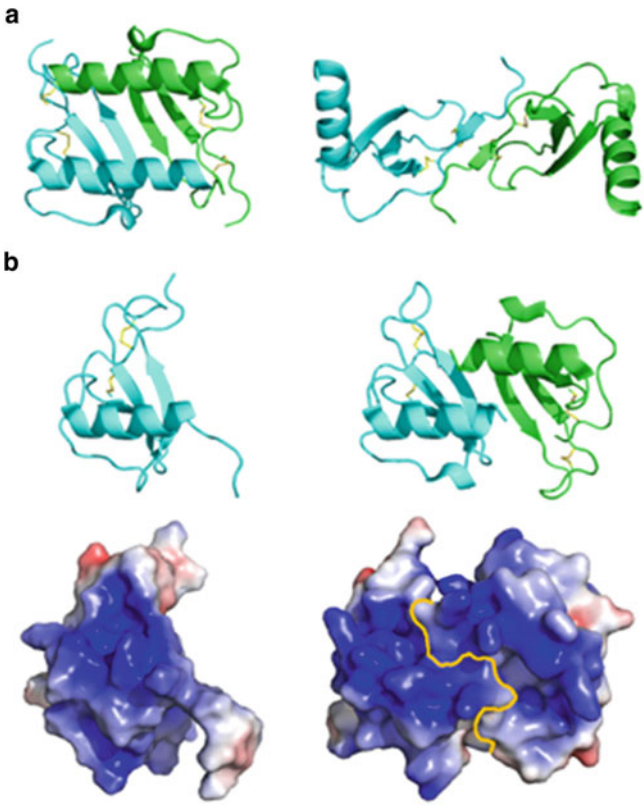

Fig. 4 (**a**) Dimerization schemes for chemokines. CXCL8 chemokines dimer structure (PDB code IL8) (*left*). CCL3 chemokine dimer structure (PDB code 2X69) (*right*). Each monomer is depicted in a different color and the disulfide bonds are represented in *yellow*. (**b**) Chemokine CCL20 (PDB code 2JYO) and dimer (PDB code 1M8A) structure (*upper*) with the electrostatic potential surface distribution (*lower*). The disulfide bonds are represented in *yellow* (*upper*) and the monomer interface is depicted by a *yellow line* (*lower, right*). Electrostatic potential surfaces were calculated by Adaptative Poisson–Boltzmann Solver (APBS) using PDB2PQR software and depicted by PyMOL software

dimer structure characteristics may explain the high antimicrobial activity of this particular chemokine. In a monomeric form, CCL20 already exhibits a large positively charged surface. This is even increased considerably when the dimer is formed by the generation of the six-stranded β-sheet with both C-terminal α-helices located on top, establishing a large contiguous positively charged surface (Fig. 4b). The dimerization of CCL20 is dependent on the protonation state of the His-40 sidechain. At neutral pH, His-40 is unprotonated, allowing dimer formation of CCL20; at lower pH, the two His-40 residues become positively charged, and charge repulsion between these residues prevents the dimer formation (Chan et al. 2008). The formation of an extended positively charged surface under physiological conditions may explain the strong antimicrobial activity of CCL20,

and the same could apply to other chemokines. In a recent study, it was also suggested that the differences in the dimerization behavior of CXCL7 and its truncated TC-1 derivative contributed to the drastically increased antimicrobial activity of the latter (Nguyen et al. 2011). Clearly detailed studies addressing the influence of dimerization on the antimicrobial activity of chemokines are warranted in order to definitively establish its possible role in the activity of these proteins.

In addition to the formation of homodimers, several chemokines are also able to form heterodimers. This has been shown to modulate their biological activities (Allen et al. 2007). For example, the chemotaxis of specific cells has been significantly affected by the formation of heterodimers as observed for the CXCL8-CXCL4 and CXCL4-CCL5 dimers (Nesmelova et al. 2005; von Hundelshausen et al. 2005). Heterodimers are also observed for other members of the innate immune system, such as AMPs. Some AMPs can undergo heterodimerization thereby modifying their antimicrobial activity, membrane permeabilization activity, and/or their resistance to protease digestion. Distinctin is an example of a covalently bound heterodimer antimicrobial peptide isolated from the tree frog *Phyllomedusa distincta* (Batista et al. 2001; Dalla Serra et al. 2008; Raimondo et al. 2005; Resende et al. 2009). Not only covalently bound dimers are observed among AMPs, but it is believed that the peptides magainin-2 and peptidyl-glycylleucine-carboxyamide (PGLa) form a non-covalent dimer upon interaction with membranes increasing the antimicrobial activity and membrane permeabilization activity but also increasing their cytotoxicity (Matsuzaki 1998; Hara et al. 2001). Similar to AMPs, the ability of chemokines to form heterodimers could be related not only with the modulation of their chemotactic function, but it could also regulate their microbicidal activity. Further studies are required in order to establish the effects of chemokine heterodimerization on their direct antimicrobial activity. Finally, it is known that several chemokines can form larger oligomeric structures (Salanga and Handel 2011). Such aggregated structures could markedly influence the antimicrobial activity. The formation of the oligomers is usually promoted by binding of glycosaminoglycans. In a recent study the structure of an oligomeric form of CCL5/RANTES was reported (Wang et al. 2011). This work sets the stage for future studies with other chemokines. It will be interesting to see if the binding of negatively charged glycosaminoglycans promotes or competes with the antimicrobial activities of the chemokines.

7 Activity of Unfolded Chemokines and of Derived Peptides

The antimicrobial chemokines share a large conformational, positively charged surface patch (Yang et al. 2003). In thrombocidin-1 (TC-1), disruption of this positive patch by substitution of the central cationic Lys-17 with an alanine residue substantially reduced the antimicrobial potency (Kwakman et al. 2011), providing direct evidence for its importance. Indeed, lysine substitution of negative or neutral residues bordering the positive patch enhanced the activity. Interestingly, reduced

TC-1 had equal antimicrobial activity as the folded protein, even though the positive patch was disrupted (Kwakman et al. 2011). The structural elements of unfolded TC-1 were likely localized in the N-terminal region of the protein, since linear 15-mer synthetic peptides corresponding to the N-terminal part of TC-1 had very potent antimicrobial activity. This implies that the structural elements involved in antimicrobial activity of native folded TC-1 strongly differ from those of the reduced, unfolded protein.

Similar to TC-1, several cationic antimicrobial peptides with positive patches retain their antimicrobial activity after linearization (Klüver et al. 2006; Liu and Wilson 2010; Mandal et al. 2002; Wu et al. 2003). For instance, variants of hBD-3 lacking disulfides are still antimicrobial (Hoover et al. 2003; Wu et al. 2003) despite the loss of most structural elements of the native protein. A variant of the antimicrobial chemokine CCL28, lacking both cysteines of the CC sequence, has similar antimicrobial activity as the native, folded protein (Liu and Wilson 2010). As CCL28-derived peptides have antimicrobial activity equivalent to the intact, folded protein (Liu and Wilson 2010), the activity of linear CCL28 may well be due to peptide regions in the unfolded protein, similar to the case of linearized TC-1.

The strongest example of the influence of unfolding on antimicrobial activity probably is the case of human β-defensin 1 (hDB-1). This protein is abundantly expressed by all human epithelia but was considered a low-activity AMP. However, recent research showed that reduction and unfolding of hBD-1 turns this protein into a potent antimicrobial (Schroeder et al. 2011). Reduced hBD-1 colocalizes with thioredoxin in several human tissues, suggesting that this enzyme system may be involved in hBD-1 reduction in vivo (Schroeder et al. 2011).

Assuming that linearization of chemokines and antimicrobial proteins does indeed occur in vivo, one may speculate that in addition to the known truncation peptides from folded proteins, additional linear peptides may be generated by proteolytic degradation from the now linearized parts, potentially further contributing to the already impressive role of this class of molecules to host defense.

8 Concluding Remarks

The study of the antimicrobial activity of chemokines is still a relatively unexplored field, which is not surprising as this “moonlighting” activity of chemokines was only first reported a little more than 10 years ago. To date, the activity of a number of CC and CXC chemokines has been dissected in detail, as described in this contribution. While in quite a few cases the characteristic C-terminal α-helix of the chemokines plays a role in the antimicrobial activity, this is certainly not universal, as for selected chemokines the N-terminal region of the proteins seems to be more important. In some instances extensions beyond the usual ~70 residues of the typical chemokine structure play a role in the activity, as in the case of the histatin-like extension of CCL28 and for the 30-residue C-terminal extension of CXCL9. The latter activities seem to be grafted onto the normal chemokine structure. A positively charged patch is essential for the activity of a number of

chemokines in their folded form. In addition, we have drawn attention to the potential effects of proteolysis and protein dimerization, which can further modulate the activity. A new topic is the structure-activity relationship of reduced, unfolded chemokines, with the reduction-activated hBD-1 as a recent revelation. Obviously much work remains to be done, and clearly more surprises will be found in this intriguing family of antimicrobial host defense proteins.

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Mechanisms and Significance of Bacterial Resistance to Human Cationic Antimicrobial Peptides

Maira Goytia, Justin L. Kandler, and William M. Shafer

Abstract Cationic antimicrobial peptides (CAMPs) are essential compounds of the innate immunity system possessed by humans. CAMPs protect the host by exerting bactericidal activity, molecular signaling, modulating the immune response, and facilitating the communication between innate and acquired immunity. Over the millennia, bacteria have developed mechanisms to circumvent the antimicrobial activity of CAMPs, thereby promoting their survival during infection. In this chapter, we focus on the mechanisms used by various bacterial pathogens to resist the antibiotic-like action of CAMPs and the consequences of such resistance.

1 Introduction

Regardless of the host, signs of infection caused by a bacterial pathogen are typically noticed after damage to the host has occurred and symptoms are manifested. These symptoms of infection can arise from toxins produced by the pathogen or host inflammatory processes triggered when the host recognizes pathogenic bacteria or their associated virulence factors. Early during infection, mediators of innate immunity are brought to the front line of defense to combat the invader and protect the host. The efficacy of this response can determine the duration, spread, and severity of

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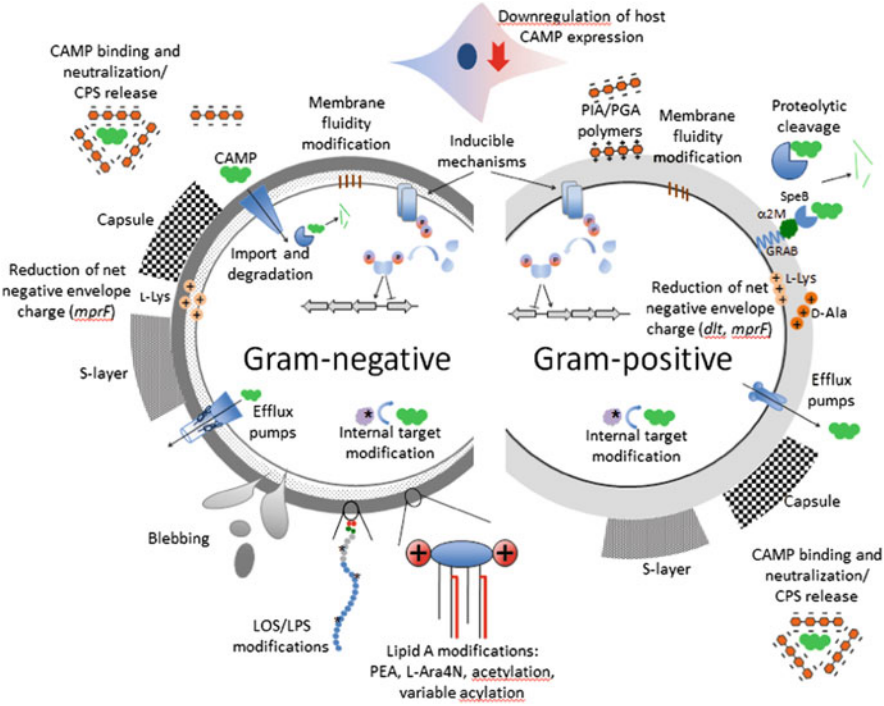

Fig. 1 Schematic representation of the mechanisms of resistance to cationic antimicrobial peptides (CAMP) used by Gram-negative and Gram-positive bacteria. Key: *CAMP* cationic antimicrobial peptide, *CPS* capsule polysaccharide, *D-Ala* D-alanine, *L-Ara4N* 4-amino-4-deoxy-L-arabinose, *L-Lys* L-lysine, *LOS* lipooligosaccharide, *LPS* lipopolysaccharide, *PEA* phosphoethanolamine. These examples are of specific mechanisms of CAMP resistance expressed by Gram-positive and Gram-negative bacteria and should not to be considered exhaustive

disease. Cationic antimicrobial peptides (CAMPs), also appropriately called "host defense peptides" (Brown and Hancock 2006), are important in this response as they can directly or indirectly exert antibacterial activity. Any successful pathogen must find ways to evade the direct action of CAMPs or risk having their numbers severely reduced or even eliminated.

Although thousands of CAMPs exist in nature, humans confront bacteria with three main classes: the α- and β-defensins, the sole human cathelicidin termed LL-37, and peptides derived from protease digestion of proteins that perform important roles in other host response processes (e.g., cathepsin G). Unless otherwise stated, we focus this review on the mechanisms employed by bacterial pathogens to escape the action of gene-encoded CAMPs. It is important to note that CAMPs are, in essence, antibiotics. It is therefore not surprising that many of the general mechanisms developed by bacteria to resist classical antibiotics are in concept also used by bacteria to resist CAMPs. These mechanisms are summarized in Fig. 1.

Studies on mechanisms of bacterial resistance to CAMPs have been facilitated by the ability to construct and use isogenic strains that differ by a single, defined mutation or the presence of a gene that impacts levels of bacterial susceptibility to CAMPs. To assess the significance of such differences, it is first important to have reproducible antibacterial assays, which are discussed below. It is difficult to use purified human CAMPs or synthetic versions, due to availability of materials and cost constraints, to select genetic variants, but these problems can be circumvented by the use of recombinantly produced CAMPs or commercially available compounds that mimic the bactericidal action of CAMPs; the cationic antimicrobial peptide polymyxin B has been a reliable CAMP substitute used by many investigators.

Understanding how bacteria can develop CAMP resistance requires the use of in vitro antimicrobial assays that are reproducible and relatively easy to use. Typically, these assays involve an assessment of direct colony forming unit reduction when bacteria are incubated in liquid media with purified CAMPs. Other standardized assays for determining minimal inhibitory concentrations (MIC) or minimal bactericidal concentrations (MBC) of CAMPs are also routinely employed (Institute Clinical and Laboratory Standard 2009). Typically, actively growing bacteria are diluted and incubated with CAMPs in broth or phosphate buffer-based solutions. These liquid media can be altered in pH and ionic strength to assess the impact of changes in these conditions on the killing efficacy of CAMPs. The radial diffusion agar overlay/underlay assay developed in the Lehrer laboratory (Qu et al. 1996) is also particularly useful and can provide quantitative results as well as allowing one to test how changes in ionic strength, presence of divalent cations, and pH impact CAMP activity. While these assays can collectively provide important information, laboratory growth media have little resemblance to the natural environments in which CAMPs must function in vivo. For instance, it is rare that the presence of other host compounds is taken into account, but these can either have positive or negative actions on the susceptibility to CAMPs. The presence of lysozyme or phospholipase A2 can enhance bacterial killing in CAMP assays. In our own studies with *Neisseria gonorrhoeae*, which typically (and often) infects the human genital tract, it was found that physiologically relevant levels of polyamines (e.g., spermine and spermidine) can decrease bacterial susceptibility to LL-37 (Goytia and Shafer 2010). Additionally, Dorschner and colleagues deduced that physiologically relevant levels of carbonate (CO_3^{2-}) in laboratory media greatly increase the killing potential of numerous and varied CAMPs, but not of the anionic skin-derived antimicrobial peptide (AMP) termed dermcidin (Dorschner et al. 2006). They further found that *Staphylococcus aureus* responds to CO_3^{2-} through a dynamic transcriptional response, thinning its peptidoglycan via repression of the alternative sigma factor, *sigB*, which may explain the increased susceptibility of this pathogen to the tested peptides. Interestingly, others have shown that LL-37 adopts an α-helical (active) conformation in the presence of carbonate (Johansson et al. 1998), which may have played some part in the enhanced killing activity seen in the Dorschner study. Thus, CAMP behavior in the lab setting may not always reflect CAMP-bacteria interactions in vivo.

Environmental conditions (e.g., limitation of iron, anaerobiosis, local pH, ionic strength, and presence of divalent cations) can also significantly impact AMP activity against bacteria. The recent report (Schroeder et al. 2011) that anaerobic conditions can potentiate the antibacterial action of human β-defensin 1 (hBD-1) against commensal gut bacteria nicely illustrates this point. In this instance, reduction of the intramolecular disulfide bonds by the thioredoxin system significantly enhanced HBD-1 activity against anaerobic commensal bacteria, and this was proposed by the authors as a means used by the host to prevent their overgrowth. Interestingly, Nuding et al. found that similar anaerobic conditions can actually weaken the antibacterial action of the related peptide human β-defensin 3 (HBD-3) (Nuding et al. 2009). Such diversity of CAMP function may be a way for the body to fine-tune its gut microbiota population. Alternatively, conditions of hypoxia may induce, in the absence of a surrogate electron acceptor, a state of bacteriostasis for some bacteria. This has been observed in *N. gonorrhoeae*, resulting in gonococcal resistance to antimicrobial proteins and CAMPs (Casey et al. 1985). Bacteria also face iron-starvation conditions imposed by host iron-binding proteins and have a variety of response mechanisms to acquire iron (Ganz 2009). As an example, this environmental stress can influence the level of susceptibility of *Streptococcus pyogenes* to LL-37 (Froehlich et al. 2009).

Ex vivo and in vivo infection models have also been employed to gain insights regarding the significance of mechanisms of bacterial resistance to CAMPs. Perhaps the most widely used ex vivo model is that of polymorphonuclear leukocytes (PMNs). These professional phagocytic cells are used in monolayers or in suspension to evaluate changes in the intraleukocytic microbicidal activity that may be due to AMP resistance mechanisms. CAMPs (e.g., neutrophil defensins termed HNP 1–4 and LL-37) are, along with antimicrobial proteins such as the *b*actericidal/*p*ermeability-*i*ncreasing (BPI) protein (Marra et al. 1990), lactoferrin, cathepsin G, CAP37, and lysozyme, important mediators of nonoxidative killing by PMNs (Sorrell et al. 1978; Spitznagel and Shafer 1985). These antimicrobial compounds are stored within the cytoplasmic specific and azurophilic granules and are delivered into the phagocytic vacuole after granule fusion and degranulation. Bacteria that inhibit phagosome-lysosome fusion can resist CAMPs and antimicrobial proteins (Scott et al. 2003). For instance, *Salmonella* presents a well-studied example where a protein (SipC) belonging to the *Salmonella p*athogenicity *i*sland-2 (SPI-2) is essential to prevent the fusion of *Salmonella*-containing vacuoles with host cell lysosomes (Uchiya et al. 1999). A recent study suggests that *Salmonella* recruits host proteins to the vacuole to inhibit the fusion (Madan et al. 2012). However, once CAMPs are delivered into the developing phagolysosome, they rapidly coat the bacterial surface and can achieve mg/mL concentrations, making it remarkable that any bacteria can survive inside the phagolysosome at all (Lehrer et al. 1988). With PMN models, it is possible to test inferences made regarding the significance of bacterial mutations that alter the susceptibility to isolated, PMN-derived CAMPs; specific examples are described below.

To test the idea that CAMP resistance mechanisms can enhance bacterial survival during infection, numerous studies have employed whole animal models. Mouse models of infection have been particularly useful in this respect, allowing investigators to readily test inferences they have drawn from in vitro and ex vivo tests. In this regard, mouse strains bearing knockout mutations in genes similar to human genes [e.g., CRAMP encoding the murine version of LL-37 (Nizet and Gallo 2002)] or knocked-in human CAMP genes [e.g., the HBD-5 used by N. Salzman and colleagues in their studies dealing with *Salmonella typhimurium* Salzman et al. (2003), Wehkamp et al. (2005)] have been used to test both the significance of CAMPs in host defense and if bacterial resistance mechanisms are important in promoting microbial survival during infection. Briefly, CRAMP knockout mice were more susceptible to invasive group A streptococcal infection than their $CRAMP^{+/+}$ counterparts, while expression of HBD-5 provided mice with increased resistance to a lethal *S. typhimurium* infection.

To date, only a single report has appeared in the literature describing a human bacterial infection model for the purpose of studying the significance of a CAMP resistance mechanism. In this instance, Bauer et al. (Bauer et al. 2006; Bauer and Spinola 2000) utilized the forearm skin puncture model (Spinola et al. 2003) to study host responses to infection by *Haemophilus ducreyi*, the causative agent of the sexually transmitted infection chancroid. *H. ducreyi* is intrinsically resistant to CAMPs and uses two transport systems, the Mtr efflux and Sap importer systems, for this purpose; these systems are described in greater detail below. Loss of these systems significantly decreased the survival of *H. ducreyi* and lesion pathology in this model (Rinker et al. 2011; Mount et al. 2010), indicating that CAMP resistance is important for its ability to cause disease.

2 Mechanisms of Bacterial Resistance to CAMPs

How do we define CAMP resistance? This is not an easy question to answer, as "breakpoints" typically used to differentiate antibiotic-sensitive from antibiotic-resistant strains are seldom considered for CAMP studies undertaken by research laboratories. This matter is complicated by a number of issues: CAMPs can achieve very different concentrations depending on their location; local environmental conditions can be antagonistic or agonistic; inducible resistance can be displayed in the presence of sublethal levels of CAMPs, yet lost under normal conditions; and CAMPs can exert multiple mechanisms of killing which, for a given peptide, might differ depending on the target bacteria. The precise mechanism by which CAMPs kill bacteria is a matter of some controversy, and no unifying mechanism has been readily accepted by the CAMP research community. Certainly, CAMPs must first bind to the microbial surface and traverse the cell envelope. The events occurring post binding that result in bacterial death are where controversy exists, and it has not always been easy to separate direct killing from postmortem events. For instance, changes in membrane integrity and potential, inhibition of cell wall

biosynthesis, and interaction of CAMPs with nucleic acids have been invoked as bactericidal events for certain CAMPs acting on a given bacterial target. Our purpose below is not to review how CAMPs kill bacteria, but rather describe how bacteria use constitutive and inducible mechanisms to circumvent their action.

In order for CAMPs to efficiently kill bacteria, they must reach their target in extracellular fluids or within intracellular compartments avoiding the action of peptidases/proteases, navigate past hydrophilic surface structures such as capsules and *O*-antigen chains of lipopolysaccharide (LPS), interact with negatively charged surface structures, insert into the cell envelope, reach the cytoplasmic membrane, and, in some instances, enter the cytosol. All of these steps provide opportunities for bacterial interference, which can decrease the susceptibility of the target microbe to CAMPs. Briefly, pathogens have evolved several strategies to circumvent the attack by CAMPs: (1) modulate CAMP gene expression, (2) degrade CAMPs by extracellular or intracellular peptidases/proteases, (3) trap CAMPs, (4) reduce binding of CAMPs to the cell surface, (5) export CAMPs by efflux pumps, and (6) alter intracellular targets. CAMP resistance mechanisms are typically expressed constitutively, but many are also under control of regulators of gene expression that respond to environmental cues. Below, we review examples of these strategies from several medically relevant pathogens. Table 1 summarizes specific examples from various pathogens, while Fig. 1 summarizes the different strategies described in this section. We review this subject by beginning with examples of downregulation of CAMP production and then follow CAMPs as they bind and enter target bacteria, providing descriptions of the various systems employed by different bacteria to avoid the killing action of these important peptides.

2.1 Bacterial Modulation of CAMP Gene Expression

Bacteria have developed novel and diverse strategies to modulate the availability of CAMPs in extracellular fluids and within phagolysosomes of phagocytes; the latter subject has been extensively reviewed elsewhere (Ray et al. 2009; Flannagan et al. 2009), and we will concentrate on studies dealing with bacterial modulation of CAMP production. It is important to note that modulation of CAMP production can have profound downstream effects on the overall host immune system, which can facilitate bacterial growth and dissemination during infection. Bacterial products can directly or indirectly modulate CAMP expression and activation of immune responses. For instance, *Escherichia coli* lipopolysaccharide (LPS) can increase mRNA production of human β-defensin 2 (HBD-2) via CD14-activation of neutrophils (Becker et al. 2000). Tada et al. showed that proteases from *Porphyromonas gingivalis* can cleave the macrophage CD14 outer-membrane receptor. CD14 recognizes pathogen-associated molecular patterns (PAMPs), and cleavage of this protein rendered macrophages unresponsive to the presence of this pathogen and prevented CAMP production (Tada et al. 2002). Additionally, uropathogenic *E. coli* (UPEC), collected from patients with urinary tract infections (UTI), express the so-called "curli" fimbriae

Table 1 Examples of CAMP resistance mechanisms expressed by bacteria

Product name/gene	Organism	References
CAMP binding/inactivation		
Staphylokinase	*S. aureus*	Braff et al. (2007), Jin et al. (2004)
SIC	*S. pyogenes*	Akesson et al. (1996)
M1 surface protein	*S. pyogenes*	Lauth et al. (2009)
CAMP proteolytic cleavage		
V8 protease	*S. aureus*	Sieprawska-Lupa et al. (2004)
Aureolysin	*S. aureus*	Sieprawska-Lupa et al. (2004)
ZapA	*P. mirabilis*	Schmidtchen et al. (2002), Belas et al. (2004)
LasA	*P. aeruginosa*	Park et al. (2001), Schmidtchen et al. (2002)
Elastase LasB	*P. aeruginosa*	Schmidtchen et al. (2002)
Gelatinase GelE	*E. faecalis*	Schmidtchen et al. (2002)
Surface protease PgtE	*S. enterica*	Guina et al. (2000)
Metalloproteinase SepA	*S. epidermidis*	Lai et al. (2007)
Metalloprotease DegP	*E. coli*	Ulvatne et al. (2002)
Cysteine protease SpeB	*S. pyogenes*	Nyberg et al. (2004), Schmidtchen et al. (2002), Johansson et al. (2008)
Production of capsular polysaccharides		
PIA, PGA	*S. epidermidis*	Vuong et al. (2004), Kocianova et al. (2005)
Capsule-synthesis gene cluster		
cps cluster	*N. meningitidis*	Frosch et al. (1989)
	K. pneumoniae	Campos et al. (2004)
Mycolic acid synthesis		
kasB	*M. marinum*	Slayden and Barry (2002), Gao et al. (2003)
D-Ala modification of teichoic acids		
dltABCD	*S. pyogenes*	Kristian et al. (2005)
	L. monocytogenes	Abachin et al. (2002)
	S. aureus	Peschel et al. (1999)
Modification of membrane phospholipids with amino acids		
mprF, lysS	*S. aureus*	Fields et al. (1989), Miller et al. (1990), Peschel et al. (2001)
lysX	*M. tuberculosis*	Maloney et al. (2009)
mprF1 and *mprF2*	*C. perfringens*	Roy and Ibba (2008)
Lipid A modifications		
Aminoarabinose addition: *pmrAB, pmrE, pmrFHIJKL, pmrC*	*S. enterica* ser. Typhimurium	Gunn et al. (1998), Lee et al. (2004), Gunn (2008)
Acetylation: *O*-acetyltransferase	*P. mirabilis*	McCoy et al. (2001)
PEA addition: LptA	*N. gonorrhoeae* *N. meningitidis*	Lewis et al. (2009), Tzeng et al. (2005)
Phosphorylcholine substitution: GlpQ	*H. influenzae*	Fan et al. (2001), Lysenko et al. (2000)
Decreased membrane fluidity		
htrB	*H. influenzae*	Starner et al. (2002)

(continued)

Table 1 (continued)

Product name/gene	Organism	References
Production of carotenoids		
crtOPQMN	*S. aureus*	Mishra et al. (2011)
Plasmid-encoded efflux pump		
QacA	*S. aureus*	Kupferwasser et al. (1999)
CAMP expulsion		
MtrCDE	*N. gonorrhoeae*	Shafer et al. (1998)
	N. meningitidis	Tzeng et al. (2005)
MtrCD-GlmU	*H. ducreyi*	Rinker et al. (2011)
AcrAB-TolC	*K. pneumoniae*	Padilla et al. (2010)
EpiFEG	*S. aureus*	Otto et al. (1998)
MefE/Mel	*S. pneumoniae*	Zähner et al. (2010)
Controlled import leading to degradation		
Sap	*S. enterica*	Parra-Lopez et al. (1993)
Sap	*H. influenzae*	Mason et al. (2006)
SapA	*H. ducreyi*	Mount et al. (2010)
Induction mechanisms		
phoP/*phoQ*	*S. enterica*	Groisman et al. (1992), Groisman (2001)
phoP/*phoQ*	*P. aeruginosa*	Macfarlane et al. (1999)
pmrA/*pmrB*	*S. enterica*	Roland et al. (1993)
pmrA/*pmrB*	*P. aeruginosa*	McPhee et al. (2003)
misR/*misS*	*N. meningitidis*	Tzeng et al. (2004), Johnson et al. (2001)
covR/*covS*	*S. pyogenes*	Froehlich et al. (2009)
agr, sarA, aps/*graRSX*	*S. aureus*, *S. epidermidis*	Lai et al. (2007), Kraus et al. (2008)
Biofilm formation		
icaB	*S. aureus*	Vuong et al. (2004)
Bacterial regulation of host AMP expression		
Plasmid DNA-mediated decrease in LL-37 and HBD-1 expression	*S. dysenteriae, S. flexneri*	Islam et al. (2001)
Decreased LL-37 expression	*N. gonorrhoeae*	Bergman et al. (2005)
Diminished membrane potential		
Small colony variants	*Sn aureus*	Vesga et al. (1996), Yeaman and Yount (2003)

that modulate the immune system of the host and provide resistance to LL-37. Curli, an amyloid-like fiber expressed in biofilms, promotes cell adherence, increases induction of IL-8 (a human proinflammatory cytokine), binds to LL-37 inhibiting its killing activity, and increases bacterial virulence in a mouse model (Kai-Larsen et al. 2010). Furthermore, Islam et al. showed that *Shigella flexneri* and *S. dysenteriae* downregulate and prevent expression of CAMPs such as LL-37 and HBD-1 by the host. Even though the molecular mechanism was not completely elucidated, the authors suggested a role for plasmid DNA from the bacteria (Islam et al. 2001). Zughaier et al. reported that Neisseria meningitidis capsular polysaccharide (CPS) can

bind LL-37 and, as a consequence, dampen the host immune response against this strict human pathogen (Zughaier et al. 2010). Finally, *N. gonorrhoeae* can impair expression of LL-37 in cervical epithelial cell line ME180 (Bergman et al. 2005). This effect was observed with live bacteria, but not with dead gonococci nor with live commensal *Neisseria* species considered avirulent in a normal host. They concluded that a specific interaction took place between *N. gonorrhoeae* and the ME180 epithelial cell that suppressed expression of LL-37. The gonococcal structures responsible for this suppression of LL-37 production remain to be discovered.

2.2 Degradation of CAMPs

Bacteria can degrade CAMPs by proteolytic cleavage before they reach or pass the bacterial surface. CAMP degradation can occur extracellularly, by secreted and membrane-associated proteases, or intracellularly; the latter is facilitated by importers that deliver CAMPs to the bacterial cytosol, where they are degraded by peptidases and cytosolic proteases.

Studies with *Salmonella enterica* and *Staphylococcus aureus* have contributed significantly to our understanding of the role of bacterial peptidases/proteases in CAMP resistance. *S. enterica* expresses an outer-membrane protease, PgtE, which cleaves LL-37 and other linear CAMPs, and results in increased resistance to LL-37 in vitro and in vivo (Guina et al. 2000). PgtE is similar to the OmpT protein produced by *E. coli*, which cleaves protamine. *E. coli ompT* mutants are more susceptible to human protamine (Stumpe et al. 1998). *S. aureus* produces many proteases, and evidence has been presented that the action of aureolysin, a metalloprotease, and V8, a serine endopeptidase, can enhance its resistance to CAMPs. V8 cleaves and inactivates LL-37 (Sieprawska-Lupa et al. 2004) as well as complement proteins C3a and C4a, which have antimicrobial action (Zipfel and Reuter 2009). Aureolysin cleaves complement protein C3 at a nonphysiological site, rendering its cleavage products inactive (Laarman et al. 2011). Aureolysin-cleaved C3 protein is further degraded by host mechanisms, thus blocking the complement cascade, inactivating the antimicrobial activity of C3a, and preventing the targeting of *S. aureus* by the host immune response (Laarman et al. 2011). Schmidtchen et al. have published a series of elegant papers that collectively emphasize the role of proteases produced by medically important pathogens (and other relevant microorganisms), highlighting their importance for resistance to CAMPs and antimicrobial proteins (Schmidtchen et al. 2001, 2002). Briefly, these studies showed that elastase and alkaline protease from *Pseudomonas aeruginosa*, gelatinase from *Enterococcus faecalis*, a secreted cysteine proteinase (SpeB) from *Streptococcus pyogenes,* and a 50-kDa proteinase from *Proteus mirabilis*, possibly ZapA (Belas et al. 2004), can degrade LL-37 (Schmidtchen et al. 2001). As a secondary effect, these enzymes also degrade proteoglycans from the host's extracellular matrix, releasing negatively charged dermatan and/or heparan sulfate, which significantly inhibits the antimicrobial activity of the α-defensin human neutrophil peptide 1 (HNP-1) (Schmidtchen et al.

2001), and of bactenecin-5 and bactenecin-7 (Park et al. 2001). An additional mechanism developed by *S. pyogenes* involves the bacterial membrane-associated protein GRAB, which binds α-2-macroglobulin (α2M), a host-derived protease inhibitor. α2M, in turn, binds the secreted bacterial protease SpeB, which maintains its proteolytic activity against CAMPs, preventing the action of CAMPs at their target site on the bacterial surface (Nyberg et al. 2004). Thus, *S. pyogenes* binding of host α2M appears to serve two purposes: (1) facilitate cleavage of CAMPs before they reach their target and (2) promote immunological mimicry by presenting self-antigens at the bacterial surface.

Bacteria can also degrade CAMPs intracellularly through the combined action of an importer, which delivers CAMPs to the cytosol, and intracellular peptidases normally used to cleave bacterial peptides and increase the available pool of amino acids. For example, non-typeable *H. influenzae* (NTHi), a commensal Gram-negative bacterium that can cause conjunctivitis, sinusitis, acute and chronic otitis media, and bronchitis (Erwin and Smith 2007), expresses the Sap (*s*ensitivity to *a*ntimicrobial *p*eptides) ABC transporter. This transporter, first identified and characterized in *Salmonella* (Groisman et al. 1992; Parra-Lopez et al. 1993), can increase bacterial resistance to CAMPs by 8-fold and is required for virulence of NTHi in a chinchilla model of otitis media (Mason et al. 2005). SapA binds chinchilla BD-1 as well as human CAMPs (e.g., LL-37, HBD-2 and HBD-3, and HNP-1) (Mason et al. 2011). The binding of CAMPs to SapA upregulates expression of the *sap* operon (*sapABCDFZ*) (Mason et al. 2005), promoting transfer of the CAMPs into the cytosol where they are degraded (Mason et al. 2006). Further research by this group proposed a mechanism where CAMPs are taken up by the periplasmic binding protein SapA then transferred to the cytoplasm through the SapBCDF transporter and SapZ accessory protein (Shelton et al. 2011). Interestingly, this mechanism might increase the intracellular levels of nutrients, since the amino acids from the degraded CAMPs could be recycled. SapA of *H. ducreyi* also increases bacterial resistance to LL-37, but not to α- and β-defensins. However, the most important effect of SapA in *H. ducreyi* was observed in the human forearm model of chancroid in that SapA production was found to increase virulence, probably by promoting resistance to the higher concentrations of LL-37 that are secreted at infection sites in the dermis (Mount et al. 2010).

2.3 Hindering CAMP Localization to the Bacterial Surface

When bacteria find themselves in environments rich in CAMPs, they have strategies other than proteolysis to neutralize or repulse CAMPs, thereby reducing their susceptibility to these antimicrobials. Non-proteolytic mechanisms of CAMP resistance include (1) the presence of CAMP-binding agents, (2) expression of hydrophilic bacterial biopolymers to retard the passage of amphipathic CAMPs in

the electronegative bacterial surface, and (3) architectural constraints imposed by biofilms.

Binding or repulsion of CAMPs by extracellular compounds reduces their capacity to interact with negatively charged target sites on the bacterial surface. Several CAMP-binding compounds have been described. For example, staphylokinase (Sak) and the *s*treptococcal *i*nhibitor of *c*omplement (SIC) bind to and neutralize CAMPs such as HNP 1–3 and LL-37 in the extracellular milieu, effectively decreasing the local CAMP activity as much as 80 % (Jin et al. 2004; Pence et al. 2010). The production of extracellular polymers shields bacteria with an extra layer of protection against CAMPs. Polysaccharide intercellular adhesin (PIA) and poly-γ-glutamic acid (PGA) polymers, produced by staphylococci, inhibit HBD-3 and LL-37 activity (Kocianova et al. 2005; Vuong et al. 2004). It is thought that the charges present on these polymers are a triple threat as they are able to repulse similarly charged antimicrobials, neutralize and sequester oppositely charged antimicrobials, and behave as a mechanical barrier to their entry. Alginic acid produced by *P. aeruginosa* inhibits CAMPs in a similar fashion (Friedrich et al. 1999).

The production of capsule, or glycocalyx (literally, "sugar coat"), is a common defense mechanism also utilized by other human bacterial pathogens, including *Neisseria meningitidis*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Streptococcus pneumoniae*, *S. aureus*, and *Bacillus anthracis*. It is notable that many of these pathogens are the cause of mucosal and respiratory tract infections that may progress to the bloodstream, an environment where encapsulated organisms are at a distinct advantage over other bacteria (Yeaman and Yount 2003). However, not all prokaryotic glycocalyces are produced by the pathogen itself and can sometimes be stolen from the host (see pathogenic *Neisseria* below). In the environment, several species of both *Eubacteria* and *Archaea* produce S-layers, which is a glycoprotein shroud that completely surrounds the prokaryotic cell. Interestingly, some eubacterial S-layers can be glycosylated with up to 150 carbohydrate moieties per protein unit (Messner et al. 2008). S-layers may be another protective mechanism against CAMPs in the highly competitive soil and water microbiome.

As with proteolysis, biopolymer sequestration of CAMPs can still function even when physically separated from the cell. Certain Gram-negative bacteria can release membrane vesicles rich in CAMP-binding sites called "blebs," and the act of blebbing could provide an extracellular sink for CAMPs. Some bacteria may also release negatively charged *c*apsular *p*oly*s*accharides (CPS) that titrate CAMPs by electrostatic interactions. Llobet et al. have described this mechanism of resistance in several clinically relevant pathogens, such as *E. coli*, *K. pneumoniae*, *P. aeruginosa,* and *S. pneumoniae* (Llobet et al. 2008). They show that CPS from different bacteria at concentrations as low as 1 μg/mL (*E. coli*, *K. pneumoniae*, *P. aeruginosa*) can increase the MIC of HNP-1 between 5- and 30-fold, regardless of the CPS source. However, this mechanism can be rendered inadequate by the presence of polycations that preferentially bind to CPS, freeing CAMPs to react with the bacterial cell wall and kill the targeted cell (Llobet et al. 2008). Additionally, Campos et al. showed that a *K. pneumoniae* mutant lacking CPS is more sensitive to CAMPs such as HNP-1, HBD-1, protamine sulfate, and polymyxin B, compared to the wild-type strain

expressing CPS (Campos et al. 2004). They also showed that the CPS mutant binds more polymyxin B than the wild-type strain, suggesting that CPS protects the bacteria, either by mechanically shielding the bacteria or by titrating the CAMP. Moranta et al. injected wild-type or CPS mutant *K. pneumoniae* into mice and showed decreased levels of β-defensins produced in response to the wild-type strain, as compared to higher levels produced for the CPS mutant isogenic strain (Moranta et al. 2010). This suggested that CPS not only prevented the action of CAMPs at the surface of the bacteria, but also prevented signaling to the host immune system that would normally increase levels of β-defensins. In *N. meningitidis*, Jones et al. observed that expression of LOS and capsule is directly linked to increased resistance to LL-37 compared to the LOS-deficient and capsule-deficient mutants (Jones et al. 2009). Furthermore, they show that incubation of the wild-type bacteria with sublethal concentrations of LL-37 induced the expression of the capsule-associated genes *siaC* and *siaD*, which results in upregulation of capsule biosynthesis (Jones et al. 2009).

CAMPs are typically amphipathic molecules whose hydrophobic domain allows membrane insertion, an event required for killing the target microbe. Accordingly, the presence of bulky hydrophilic structures on the bacterial surface may hinder the migration of CAMPs from the extracellular milieu to the negatively charged bacterial surface structures that form the gateway to hydrophobic lipid bilayers. This may partly explain why the presence of the hydrophilic *O*-antigen in the LPS of Gram-negative enterics endows them with greater CAMP resistance than rough mutants lacking this hydrophilic glycopolymer. Indeed, early work with *S. typhimurium* (Rest et al. 1977) showed that when the *O*-antigen is lost and the inner core sugar chain is progressively truncated, bacterial susceptibility to granule extracts from human neutrophils increases proportionally. These granules are rich in defensins and cationic antimicrobial proteins (Spitznagel 1990). Although these "deep-rough" mutants have increased exposure of their negatively charged lipid A phosphate groups, which are important for CAMP binding, their surface is generally more hydrophobic. This characteristic is conducive for CAMP/membrane interactions and enhances the likelihood of membrane insertion.

The above mechanisms have been defined with planktonic bacteria. However, it is now recognized that many bacterial species form a specialized, highly organized community termed a biofilm. Due to their ultrastructural organization, bacteria within biofilms can exhibit increased resistance to antimicrobials, including CAMPs, compared to their planktonic counterparts (Anderl et al. 2000). Biofilms were correlated with persistent bacterial infections, such as chronic lung infection in cystic fibrosis (Singh et al. 2000). Antimicrobial resistance displayed by bacteria within biofilms appears to be due to mechanisms different from those typically observed in free-floating cells. Since biofilms are multicellular communities of bacteria encased in a hydrated matrix of polysaccharide, proteins, and/or nucleic acids, the capacity of antimicrobials to interact with all members of the community is reduced. Biofilms can also trap and inactivate CAMPs in the complex matrix imposed by its structure. Leid et al. demonstrated that biofilms from *S. aureus* can be penetrated by leukocytes that are active and secrete antimicrobial compounds (including CAMPs). However,

though these leukocytes were able to phagocytose planktonic *S. aureus*, they could not engulf sessile cells (Leid et al. 2002). The authors suggested that the structure of the biofilm is more of a porous hydrogel than a fixed impenetrable structure. Three main hypotheses have been advanced to explain the increased antimicrobial resistance displayed by bacteria in biofilms (Stewart and Costerton 2001). One hypothesis invokes a less permeable and less diffusible environment created by the biofilm with negatively charged compounds (i.e., nucleic acid, polysaccharide) in the matrix that could retard CAMP diffusion. The second hypothesis emphasizes the ultrastructural architecture of the biofilm, where microenvironments might present unfavorable conditions of pH, salt, and anaerobiosis that render CAMPs inactive or inefficient. The third hypothesis speculates that bacteria go through a cell-differentiation process and reach a spore-like metabolic state while in the biofilm, which allows some of them to be resilient to higher concentrations of antibiotics. These hypotheses are not mutually exclusive, and other defense mechanisms described throughout this chapter could contribute to biofilm-mediated CAMP resistance. Another possible mechanism of resistance that needs to be considered is that biofilm formation may trigger an alternate gene expression profile that modulates resistance phenotypes.

Biofilm formation is a dynamic process and can be influenced by environmental conditions, including the presence of certain CAMPs, other host compounds, and bacterial gene products. In the first instance, LL-37 and lactoferrin can prevent biofilm formation by *P. aeruginosa* by promoting bacterial motility (Dean et al. 2011; Overhage et al. 2008). Due to its structural organization, the availability of molecular oxygen may differ at sites within the biofilm complex, and this could influence the antimicrobial action of CAMPs. Accordingly, Schroeder et al. found that hBD-1 was highly efficient against various human flora and select pathogens in its reduced form but lacked efficient antimicrobial activity in its oxidized form (Schroeder et al. 2011). This suggests that an oxidative environment (perhaps present at different degrees within biofilms) could preclude some CAMPs from their killing activity. Finally, a number of bacterial products directly enhance survival of CAMP attack in sessile cells. For example, inducible resistance can be controlled by two-component regulatory (TCR) systems (Mulcahy et al. 2008; Amer et al. 2010) and stand-alone regulators (Warner et al. 2007, 2008; Shafer et al. 2010); the hairlike surface appendage termed curli promotes formation of biofilm structures in UPEC strains of *E. coli* (Kai-Larsen et al. 2010); and periplasmic glucans may bind to CAMPs on their way to the cytoplasmic membrane and sequester them (Mah et al. 2003).

2.4 *Envelope Modifications That Decrease CAMP Binding and Permeability*

Nearly 50 years ago, Spitznagel and coworkers found that the antimicrobial, arginine-rich, cationic peptides present in neutrophil granules (now known as

defensins) rapidly coat the surface of ingested bacteria (Spitznagel 1961; Zeya and Spitznagel 1966a, b). This electrostatic interaction between CAMPs and bacterial surface structures, and how bacteria modify these structures to inhibit said interaction, is perhaps the most studied mechanism of CAMP resistance (the reader is also directed to several excellent reviews especially those by Peschel 2002; Brogden 2005). In general, eukaryotic membranes are zwitterionic and have low affinity for CAMPs, which may provide them with some immunity to the lytic activity of these peptides. Prokaryotic cell surfaces, on the other hand, are typically negatively charged and so have a higher affinity for CAMPs. In order to prevent the deadly consequences of this attraction, many bacteria have evolved ways to decrease the net negative charge of their exteriors and modify the permeability of their membrane(s). Importantly, these mechanisms are not always specific to CAMPs and may provide protection against a broad spectrum of host and pharmacological cationic antimicrobials, including myeloperoxidase, phospholipase A2, lysozyme, vancomycin, moenomycin, and daptomycin (Peschel 2002).

Though both groups are negatively charged on their exteriors, Gram-negative bacteria are generally more resistant to CAMPs than Gram-positive bacteria, due to the presence of an outer membrane that can retard the passage of CAMPs to the inner membrane and cytoplasm. This is perhaps facilitated by LPS molecules that are held tightly together by (1) van der Waals interactions that exist between acyl chains and (2) salt bridges formed by divalent cations between neighboring carbohydrate chains and between lipid A phosphates. Early studies with deep-rough LPS mutants of *S. typhimurium* (Rest et al. 1977), and the *pmrA* mutants tested by Vaara and coworkers (Vaara et al. 1981; Helander et al. 1994) and Shafer et al. (1984), as well as Farley et al. (1987, 1988), support the concept that the availability of exposed, unsubstituted lipid A phosphate groups are critical to the ionic (and hydrophobic) interactions between CAMPs and the bacterial surface. More recent studies also support this model. For instance, a knockout insertion of a putative LPS synthesis gene *galU* in *Campylobacter jejuni*, a leading food-borne pathogen, decreased the length of LPS and reduced bacterial resistance to polymyxin B (Lin et al. 2009). Similarly, *Bordetella bronchiseptica*, an upper respiratory tract pathogen and close relative of *B. pertussis* (the etiologic agent of whooping cough), appears to require the addition of a negatively charged trisaccharide to LPS by the *wlbA* and *wlbL* genes for full resistance to several phylogenetically diverse CAMPs. It is thought that the uronic acid sugar moieties present in this trisaccharide shield the membrane from antimicrobial attack, perhaps by sequestering the peptides or providing a bulky barrier to entry (Banemann et al. 1998). Phosphorylcholine is produced by *H. influenzae* (Lysenko et al. 2000) and can increase the membrane fraction of zwitterionic phospholipids in the bacterial inner membrane. This would decrease the net negative charge normally present at the exoplasmic leaflet of the cytoplasmic membrane and slow the rate of CAMP self-promoted uptake (see below). Importantly, the investigators also observed modification of *H. influenzae* lipooligosaccharide (LOS) with phosphorylcholine. Such a modification is hypothesized to mimic host membranes (which contain phosphatidylcholine) and further reduce LL-37 binding. The viscosity of the periplasm may also contribute, since this space is densely packed with hydrophilic proteins that may nonspecifically hinder

CAMPs on their way to the inner membrane and cytoplasm, similar to the nonspecific binding of drugs by plasma proteins in the human body (Silhavy et al. 2010).

Gram-negative bacteria can also decrease the net negative charge of their exterior by decorating LPS/LOS lipid A with positively charged small molecules. In *N. meningitidis*, *N. gonorrhoeae*, *Helicobacter pylori*, *E. coli*, and *S. enterica* serovar Typhimurium, the addition of phosphoethanolamine (PEA) not only removes the negative charge once provided by free lipid A phosphate, but also adds a positive charge, thereby decreasing the net negative charge of the outer membrane and perhaps membrane permeability as well (Lewis et al. 2009; Lee et al. 2004; Beceiro et al. 2011). In *Campylobacter jejuni*, PEA can be added to lipid A and to a flagellar protein serving two purposes: (1) increase CAMP resistance and (2) promote bacterial motility (Cullen et al. 2012). Alternatively, 4-amino-4-deoxy-L-arabinose (L-Ara4N) may be added to the same phosphates in some Gram-negative bacteria and provides resistance to CAMPs in a similar manner to phosphoethanolamine (Trent 2004). Bacteria may also use the LpxE lipid A phosphatase to simply remove phosphate from lipid A and reduce negative charge, a phenomenon seen in the plant symbiont *Rhizobium leguminosarum*, as well as the human pathogen *H. pylori*. LpxE orthologues are present in *Francisella tularensis*, *Brucella melitensis*, and *Legionella pneumophila* (Karbarz et al. 2003; Trent 2004). These modifications are not only important for bacterial survival, but also impact the immune response to LOS/LPS or "endotoxin," one of the most potent inducers of septic shock (Peschel 2002). The regulation of these modifications has been very thoroughly studied in *S. enterica* and is controlled by combined efforts of the PhoP/PhoQ and PmrA/PmrB TCR systems (Lee et al. 2004; Gunn et al. 1998); these regulatory systems are discussed in more detail below.

In Gram-positive organisms, polyalditol, polyglycerol, or polyribitol phosphate polymers [teichoic acids (TA)] create a "continuum of negative charge" (Neuhaus and Baddiley 2003) with deprotonated phosphate residues present along each chain. The cationic antimicrobial lysosomal protein cathepsin G appears to use TA as a binding site on *S. aureus* (Shafer and Onunka 1989). Seminal work by Andreas Peschel and coworkers first characterized the CAMP repulsive effect caused by the D-alanylation of TA in *S. aureus*, an ability that endows this pathogen (and others) with decreased susceptibility to diverse CAMPs from different sources (Peschel et al. 1999). The enzymatic pathway encoded by the *dltABCD* operon processes the esterification of D-alanine to TA alditol residues and transforms TA into partly zwitterionic polymers, reducing the net negative charge at Gram-positive surfaces. The expression of the *dlt* operon in *S. aureus* is under the control of the Aps/GraRSX regulatory system (Li et al. 2007a, b). This appears to be a widespread defense mechanism present in other Firmicutes such as *Bacillus, Enterococcus*, and *Streptococcus*. It is also important to mention that *S. aureus* mutants lacking a functional *dlt* operon are more susceptible to the glycopeptide antibiotic vancomycin (Peschel et al. 2000), and in the future it may be possible to counter vancomycin-resistant *S. aureus* (VRSA) with drugs that block D-alanylation of TA.

Changes in membrane rigidity can also influence levels of CAMP resistance. PhoP/PhoQ control of *pagP*, which encodes a palmitoyltransferase that hepta-

acylates *S. enterica* lipid A in response to stresses typically found in a phagosomal environment, can influence membrane rigidity and the capacity of CAMPs to productively insert into bacterial membranes. These stresses include low pH, varying Ca^{2+} and Mg^{2+} ionic strength, and high concentrations of CAMPs (Guo et al. 1998; Prost and Miller 2008). The MsbB protein in *Vibrio cholerae* plays an analogous role by adding an acyl chain to the same position as PagP. This modification was found to greatly enhance resistance to polymyxin B, LL-37 (and its mouse homolog CRAMP), and magainin 2 (Matson et al. 2010). Interestingly, *msbB* deletion mutants were unable to induce a TLR4 response in human embryonic kidney cells, which suggests that efficient recognition and binding of bacterial endotoxin is largely due to lipid A structure. This finding also supports the notion that CAMP resistance mechanisms may protect not only directly, but indirectly through modulating the host immune response. The actions of both PagP and MsbB ultimately increase the stability and hydrophobicity of the outer membrane and decrease its permeability, thus enhancing the fortitude of an already formidable barrier to CAMP entry (Peschel 2002). In *S. aureus*, pigment production through the *crtOPQMN* operon performs a similar function in CAMP resistance by increasing membrane rigidity (Mishra et al. 2011). The cold shock system of *S. aureus* may also be important in resistance as mutants lacking CspA (Katzif et al. 2003) and CspB (Duval et al. 2010) have reduced pigment levels and altered susceptibilities to certain antimicrobials, including cathepsin G-derived CAMPs.

Conversely, decreased membrane rigidity may also provide CAMP resistance. *S. aureus* tPMP (*t*hrombin-induced *p*latelet *m*icrobicidal *p*rotein)-resistant strains were consistently found to have greater membrane fluidity than their tPMP susceptible counterparts, caused by a preponderance of longer chain, unsaturated fatty acids (Bayer et al. 2000). It has also long been known that *S. aureus* membranes contain unsaturated sexa-, hepta-, and octa-isoprenoid menaquinones (Nahaie et al. 1984). Apart from their function as redox molecules in the electron transport chain, they also increase membrane fluidity. Thus, it has been hypothesized that large fluctuations in membrane fluidity to either extreme may distance membrane order from the "sweet spot" required for optimum CAMP bactericidal activity (Mishra et al. 2011; Yeaman and Yount 2003).

One of the most nonspecific and ubiquitous mechanisms of CAMP defense in bacteria is mediated by the MprF (*m*ultiple *p*eptide *r*esistance *f*actor) protein (Peschel et al. 2001). Originally described by Peschel and colleagues (2001), it soon became clear that MprF provides significantly enhanced bacterial resistance to neutrophils and several evolutionarily distinct CAMPs. MprF is present in a wide variety of Gram-positive, Gram-negative, and acid-fast bacteria and is also present in the *Archaea*. This CAMP resistance mechanism is remarkable in that it only requires substrates that are abundant in the bacterial cell—charged tRNAs and membrane phospholipids—and is somewhat indiscriminant when recognizing tRNA donor and phospholipid acceptor molecules. This is thought to be why the MprF mechanism is so widespread (Roy and Ibba 2008). MprF is an integral cytoplasmic membrane protein and may add the positively charged L-lysine, L-alanine, and perhaps, in *Mycobacterium tuberculosis*, L-ornithine (Ernst and Peschel 2011; Khuller and

Subrahmanyam 1970) amino acids to phosphatidylglycerol and cardiolipin (diphosphatidylglycerol). *S. aureus* MprF consists of (1) a transesterase domain which adds the amino acid residue to phosphatidylglycerol (PG) on the cytoplasmic leaflet of the membrane and (2) a "flippase" domain that flips the nascent lysyl-PG to the exoplasmic leaflet of the membrane where it can serve to repulse CAMPs by reducing the net negative charge of the membrane's outer surface. It is noteworthy that MprF is the first flippase to be discovered in prokaryotes (Ernst and Peschel 2011). In *M. tuberculosis*, the *lysX* gene is actually a fusion of *mprF* and *lysU*, a lysyl-tRNA synthase. Here, lysyl-tRNA can be made at the cytoplasmic membrane level by the LysU domain and then shuttled into the MprF reactions described above (Maloney et al. 2009). *Clostridium perfringens* produces two MprF proteins, 1 and 2, which produce alanyl-PG and lysyl-PG, respectively (Roy and Ibba 2008). It is probable that, like other CAMP resistance mechanisms, the activities of MprF are under the control of two- or three-component regulatory systems; indeed, MprF appears to be under the control of the VirR protein of the VirRS TCR in *Listeria monocytogenes* (Mandin et al. 2005) and the Aps/GraRSX TCR in *S. aureus* (Li et al. 2007a, b; Otto 2009).

Intriguingly, it seems that CAMPs themselves are not the only stimuli that can induce CAMP resistance mechanisms in bacteria. A brief but elegant study by Dorrer and Teuber in the 1970s (Dorrer and Teuber 1977) demonstrated that phosphate starvation induced polymyxin B resistance in *Pseudomonas fluorescens* by increasing the membrane fraction of ornithylated lipids, which decreases the net negative charge of the bacterial envelope. Notably, it was later discovered that survival inside of macrophages induced the expression of phosphate importers 9.4-fold in *Salmonella typhimurium* (Valdivia and Falkow 1997). This might indicate that (1) low phosphate levels preclude the use of phosphate on membrane lipids and require that other groups (e.g., ornithine) provide the hydrophilic portion of the membrane lipid to maintain a stable bilayer structure and (2) the host environment may unwittingly hinder its own efforts to kill with CAMPs by inducing the production of these cationic lipid species.

Though the mechanisms described above allow the microorganism to change the envelope structure without dire consequences for growth, there are other CAMP resistance strategies that come at great fitness cost to the bacterium. In *S. aureus*, small colony variants (SCVs) are typically deficient in electron transport and have a diminished membrane potential ($\Delta\psi$) (Yeaman and Yount 2003). They also arise much more readily (10,000-fold) in the host than in laboratory culture (Vesga et al. 1996). This suggests that slowing cell growth may represent a "niche-specific" defense mechanism, triggered by growth in a host, that allows bacteria to depolarize their membrane and decrease the rate of "self-promoted uptake" by host CAMPs (Peschel 2002) (see (Hancock 1997) for a description of this phenomenon). Interestingly, the *S. aureus cspB* mutant studied by Duval et al. described above, exhibited many of the characteristics of SCVs (Duval et al. 2010). In Gram-negative bacteria, reduced growth rates may also help stave off death by CAMP by reducing the occurrence of nascent septa that are a critical component during bacterial binary fission. Sochacki and colleagues used real-time fluorescence microvideography to

show that rhodamine-labeled LL-37 consistently binds *E. coli* cells at their nascent septa first, then proceeds outward in a continuous "circumferential band" towards the distal poles of each developing daughter cell (Sochacki et al. 2011), though LL-37 was still able to bind to nonseptated cells.

2.5 Export of CAMPs

Even if CAMPs successfully traverse the formidable barriers described above, bacteria can still circumvent their action by the use of drug efflux pumps that capture and export structurally diverse antimicrobials after they breach the cell envelope. Drug efflux pumps are grouped into superfamilies based on their component stoichiometry, number of transmembrane regions in the transporter, energy source, and type of substrates recognized. Five superfamilies of efflux pumps are known: the *r*esistance-*n*odulation-*d*ivision (RND) superfamily, the *m*ajor *f*acilitator (MFS) *s*uperfamily, the *m*ultidrug *a*nd *t*oxin *e*xtrusion (MATE) superfamily, the *A*TP-*b*inding *c*assette (ABC) transporter superfamily, and the *s*mall *m*ultidrug *r*esistance (SMR) superfamily (see (Piddock 2006) for an excellent review of bacterial efflux pumps). The MtrCDE efflux pump of *N. gonorrhoeae* is a member of the RND superfamily and was the first efflux pump shown to export CAMPs to the extracellular milieu (Shafer et al. 1998). This pump has been studied in detail and will be described later (see below); the analogous pump in *N. meningitidis* also can export CAMPs (Tzeng et al. 2005). Other Gram-negative pathogens have been found to use efflux pumps to resist CAMPs. *Yersinia enterocolitica* can protect itself from CAMP activity by expressing the RosAB efflux pump, which is induced upon growth at 37 °C. The RosA pump activity is powered by the potassium antiporter RosB and is thought to provide resistance through (1) efflux of CAMPs and (2) acidification of the cytoplasm (Bengoechea and Skurnik 2000). *K. pneumoniae* expresses the AcrAB-TolC efflux pump that mediates resistance to human antimicrobial peptides, as an AcrB mutant was more sensitive to HBD-1 and HBD-2 (Padilla et al. 2010). The homologous efflux pump in *E. coli*, AcrAB-TolC, is arguably the most structurally characterized efflux pump to date (Murakami et al. 2006; Husain and Nikaido 2010; Symmons et al. 2009) and will likely become an invaluable tool for the design and testing of an emerging class of antibiotics, the efflux pump inhibitors (EPIs) (Lomovskaya and Bostian 2006).

Efflux pumps also function in Gram-positive bacteria for CAMP resistance. For instance, the EpiFEG efflux pump of *S. epidermidis* exports and increases staphylococcal resistance to various CAMPs. EpiFEG is an ABC transporter that is known to export bacterial-derived CAMPs, such as gallidermin, nisin, and epidermin (Otto et al. 1998). The MefE/Mel efflux pump possessed by certain strains of *S. pneumoniae* is a mechanism used by this pathogen to develop resistance to macrolides. Expression of this pump was found (Zähner et al. 2010) to be inducible by 14- and 15-membered macrolides as well as LL-37/CRAMP and that such induction enhanced pneumococcal resistance to macrolides and LL-37. Maximal

constitutive and inducible cathelicidin resistance expressed by pneumococci required a functional MefE/Mel pump system, although it is yet to be determined if these CAMPs are actual pump substrates. It also appears that some efflux pumps might actually enhance CAMP resistance independently of their efflux capacity. In *S. aureus*, QacA (a plasmid-encoded multidrug MFS efflux pump) mediates resistance to tPMP, but does not affect levels of resistance to HNP-1 or protegrin-1 (Kupferwasser et al. 1999). Curiously, later studies found that QacA-mediated resistance to tPMP was not due to efflux activity and that membrane fluidity seemed to diminish slightly in strains bearing the *qacA* gene (Bayer et al. 2006), but the exact mechanism of how QacA expression protects against tPMP remains to be determined.

Of note is a very intriguing pattern that has emerged among the Gram-positive bacteria that teams ABC-transporter efflux pumps and TCR systems into very close functional associations called "resistance modules." Each component of the module is dependent on the other for resistance against antimicrobial peptides. Extensive phylogenetic analysis suggests a coevolution of efflux pumps and TCR systems in the phylum *Firmicutes* (over 250 resistance modules are estimated). In this partnership, sensor domain-deficient inner membrane histidine kinases (IMHKs) still relay signals through their cognate response regulators, but recognition of the environmental stimulus is carried out by a neighboring permease/transporter protein in the membrane (Dintner et al. 2011). Well-characterized examples of such TCR/efflux pump couplings include the BceRS TCR and BceAB pump in *Bacillus subtilis* and *Streptococcus mutans* (Bernard et al. 2007; Ouyang et al. 2010) and the BraRS TCR and BraED pump in *S. aureus* (Hiron et al. 2011). The VraED pump also appears to play a role in *S. aureus* resistance, but is required only for efflux and not for sensing.

2.6 *Modification of Internal Targets*

Since the antimicrobial action of most CAMPs is independent of stereochemistry, it has generally been thought that they do not recognize targets with a chiral center (Bessalle et al. 1990; Wade et al. 1990). Furthermore, their killing activity has been linked to processes such as loss of membrane integrity and depolarization. Due to these broad-spectrum killing mechanisms, CAMPs have been likened to "dirty bombs" in contrast to the "smart bomb"-like action of many antibiotic drugs (Peschel and Sahl 2006). This analogy may not be completely correct as it is now clear that CAMPs may also kill bacteria by interfering with internal cellular functions like DNA/RNA/protein synthesis, protein folding, peptidoglycan polymerization, and septum formation (Hale and Hancock 2007; Cudic and Otvos 2002). Just as the bacterial envelope can accumulate changes to hinder CAMP attack, antimicrobial stress selects for mutants containing modified and thus less CAMP-accessible cytoplasmic targets. One example of this is a mutation in the *gyrB* gene in *E. coli*. *gyrB* encodes DNA gyrase, a type II topoisomerase that maintains a level of DNA supercoiling necessary for replication, transcription,

and recombination. GyrB is a target of the well-known class of antibiotics called quinolones, and also the bacteriocin microcin B17, which is produced by *E. coli*. del Castillo and colleagues (2001) found that mutation of residue W751 to hydrophilic amino acids like lysine or arginine imparts a great deal of resistance to microcin B17. The authors hypothesize that this residue may be located on the entry gate through which the intact DNA can be transported (T-segment) and that microcin B17 normally binds and inhibits GyrB activity, leading to cell death (del Castillo et al. 2001).

2.7 Inducible Mechanisms of CAMP Resistance

Inducible mechanisms of CAMP resistance allow bacteria to promptly respond to stressful changes in their environments. TCR systems sense potentially harmful changes, orchestrate a response to the imposed stress, and adapt gene expression to the new context. TCR systems consist of a sensor histidine kinase on the inner membrane and a cytoplasmic regulatory protein. Typically, the sensor kinase detects a signal in the environment, becomes autophosphorylated, and in turn phosphorylates the cognate intracellular regulator, activating it. The activated regulator binds to DNA and alters the expression of different genes. Numerous and functionally distinct TCR systems are found in bacteria. The PhoP/PhoQ TCR system was initially studied in *S. typhimurium* (Fields et al. 1989; Groisman et al. 1989; Miller et al. 1989), and homologs were subsequently found and studied in *P. aeruginosa* (Macfarlane et al. 1999), in various *Enterobacteriaceae*, and in *Neisseria*, where it is named MisR/MisS (Tzeng et al. 2004).

The PhoP/PhoQ and the PmrA/PmrB are well-studied examples of TCRs that have important roles in CAMP resistance. Under favorable conditions, PhoQ is bound by divalent cations such as Mg^{2+} and Ca^{2+} in the environment and is not active. At low concentrations of divalent cations, PhoQ phosphorylates PhoP, which in turn regulates many genes involved in AMP resistance, such as *pagP*, *pgtE*, *slyA,* and *pmrD* (Roland et al. 1994). PmrD is required for activation of the PmrA/PmrB TCR system (Otto 2009; Guina et al. 2000; Macfarlane et al. 2000, 1999; Navarre et al. 2005). When CAMPs are present in the media, it is thought that they displace divalent cations bound to an acidic patch of PhoQ and induce activation of the TCR system (Prost and Miller 2008). In *S. enterica*, PhoP/PhoQ is activated by CAMPs and upregulates genes related to CAMP resistance such as *pagP*, *pagL*, and *lpxO* (Bader et al. 2005; Hancock and McPhee 2005). Using a mutant strain of *S. enterica* showing increased resistance to polymyxin B, azurocidin, and CAP57 (Shafer et al. 1984) due to a *pmrA* mutation, Roland et al. identified the TCR system PmrA/PmrB (Roland et al. 1993). This TCR regulates expression of genes involved in CAMP resistance such as *pmrHFIJKLM* (or *pbg* operon), *cld,* and *cptA*, which are responsible for LPS modifications (Gunn 2008). In *P. aeruginosa*, PmrA/PmrB is induced by low concentrations of Mg^{2+} and by LL-37, which promotes expression of genes, such as *pbgP*, *pbgE,* and *ugd,* involved

in LPS modification and resistance to polymyxin B (Gunn and Miller 1996; McPhee et al. 2003; Moskowitz et al. 2004). It was shown that mutating *pmrAB* in *P. aeruginosa* rendered the bacteria hypersusceptible to killing by LL-37 or by other CAMPs such as polymyxin B (Lewenza et al. 2005).

Li et al. uncovered a novel regulatory system in *S. epidermidis* that they named *aps* for *a*ntimicrobial *p*eptide *s*ensor (Li et al. 2007a, b), also observed in *S. aureus* and named *gra* for *g*lycopeptide *r*esistance *a*ssociated genes (Kuroda et al. 2000; Cui et al. 2005). The system consists of a TCR system with a sensor kinase (*apsS*) and a regulator (*apsR*) and a third protein with unknown yet essential function (*apsX*) (Li et al. 2007a, b). Their research showed that deletion of any or all of these components led to downregulation of the *dlt* and *mprF* genes, which modify cell surface structures and enhance resistance to CAMPs (Li et al. 2007b). Furthermore, Lai et al. have described *agr* and *sarA* in *S. aureus* and *S. epidermidis* as major regulators that are induced in the presence of the anionic AMP dermcidin. These gene regulators increase expression and proteolytic activity of the SepA metalloprotease in presence of dermcidin (Lai et al. 2007).

3 CAMP Resistance in Clinically Relevant Pathogens

In the above sections, we reviewed major mechanisms that bacteria have evolved to resist CAMPs. In order to highlight how such CAMP resistance systems can influence the efficacy of host resistance to infection and bacterial pathogenesis, we discuss them in the context of three major clinically relevant pathogens of public health concern. In this respect, we focus on the obligate human Gram-negative pathogens *N. gonorrhoeae* and *N. meningitidis* and on the Gram-positive bacteria *S. aureus*, particularly the methicillin-resistant strains (MRSA).

3.1 N. gonorrhoeae *and* N. meningitidis

N. gonorrhoeae and *N. meningitidis* are Gram-negative diplococci and strict human pathogens (Shafer et al. 2010). Gonococci (GC) cause the sexually transmitted infection gonorrhea. In contrast, meningococci (MC) are present as commensals in 8–25 % of the human population, but can cause bacterial meningitis and fulminant septicemia (Stephens 2009). Gonorrhea is the second most reported infection in the United States, though many cases are asymptomatic, and can enhance HIV transmission (Klotman et al. 2008; McNabb et al. 2008). Furthermore, although there are vaccines available for many MC serogroups that cause disease, a protective vaccine for serogroup B MC is still under development; no vaccine has been developed that blocks GC infection. Both of these pathogens are also becoming increasingly resistant to antibiotics (Shafer et al. 2010). Worryingly, a recent report described a strain of gonorrhea that is resistant to the last remaining first-line antibiotic used

in empirical treatment, ceftriaxone (Ohnishi et al. 2011). Thus, the pathogenic *Neisseria* represent a significant threat to global health.

As strictly human pathogens, GC and MC have evolved remarkable and redundant mechanisms to defend themselves against host CAMPs. These include capsule production by MC (Jones et al. 2009; Spinosa et al. 2007); host-molecule "cloaking" using the highly anionic polymers heparin/heparan sulfate and short, cationic polyamines (Goytia and Shafer 2010; Jones et al. 2009; Seib et al. 2009); MC sequestration of LL-37 in the bacterial cytosol (Frigimelica et al. 2008); downregulation of host LL-37 production (Bergman et al. 2005); export of CAMPs by the MtrCDE efflux pump (Shafer et al. 1998); decoration of lipid A with PEA (Cox et al. 2003; Tzeng et al. 2005; Lewis et al. 2009); and hexa-acylation of lipid A (Tzeng et al. 2005). Some of these mechanisms have been shown to be under the control of the MisR/MisS TCR system, named for its regulation of *m*eningococcal LOS *i*nner core *s*tructure, which itself is necessary for resistance to CAMPs (Johnson et al. 2001; Newcombe et al. 2004; Tzeng et al. 2004). Other mechanisms may be induced by different inputs, e.g., upregulation of *mtrCDE* expression in GC upon exposure to hydrophobic pump substrates typically present at infection sites (Rouquette et al. 1999).

Is there evidence that any of these resistance mechanisms influence bacterial survival during infection? Briefly, yes: in support of this idea, elegant experiments performed in the laboratory of A. Jerse have shown that loss of the MtrC–MtrD–MtrE efflux pump due to its genetic inactivation decreased the ability of GC to survive in an experimental model of lower genital tract infection in female mice (Jerse et al. 2003). Further work by her group (Warner et al. 2007, 2008) showed that overexpression of MtrC–MtrD–MtrE increases fitness of GC during infection by nearly three orders of magnitude. In contrast, loss of the ability to activate transcription of *mtrCDE* decreased fitness in vivo by 500-fold. Finally, *lptA* mutants of GC that are unable to decorate their lipid A with PEA are more susceptible to CAMPs and less fit in vivo than the parental wild-type strain (Jerse, personal communication, 2011).

3.2 Staphylococcus aureus

S. aureus is a Gram-positive bacterium that has evolved to survive in a commensal capacity on the human host. It can be found on the skin and in the nares in 20 % of the population, but when staphylococci breach host defenses, they can cause many different illnesses, including skin infections, abscesses, and life-threatening diseases such as endocarditis, pneumonia, meningitis, toxic shock syndrome, and sepsis. Importantly, *S. aureus* is one of the most frequent causes of hospital- and community-acquired infections. The incidence of multiple antibiotic-resistant strains of *S. aureus* continues to increase, restricting the options for treatment. *S. aureus* has become one of the most difficult bacterial infections to treat as multidrug-resistant strains have emerged; a typical example is *m*ethicillin-*r*esistant *S. aureus*, or MRSA. MRSA colonizes 2 % of the population, many of whom are immunocompromised due to age (e.g., the elderly and young children) or medical

condition (e.g., pregnant women, HIV-positive, and cancer patients) (Kowalski et al. 2005). MRSA can cause life-threatening infections such as pneumonia, septicemia, and infections following surgery. MRSA resist most β-lactam antibiotics (penicillins and cephalosporins) including penicillin, methicillin, and amoxicillin. Furthermore, it is quite common to see resistance develop when MRSA infection is treated with macrolides and/or fluoroquinolones. Importantly, as of 2007, MRSA infections caused more deaths (>17,000) in the United States than HIV/AIDS (Klevens et al. 2007).

S. aureus has found strategies to impair all of the events associated with CAMP killing activity and sometimes in more than one way. For instance, *S. aureus* secretes proteolytic enzymes, V8 and aureolysin, which are able to degrade and inactivate CAMPs such as LL-37 (Sieprawska-Lupa et al. 2004). It was suggested that loss of these enzymes by molecular modification could render *S. aureus* more susceptible to CAMPs in vitro, ex vivo, and in vivo (Sieprawska-Lupa et al. 2004). Staphylokinase (Sak) is a secreted protein that sequesters LL-37 and increases virulence in vivo (Braff et al. 2007). Burlak et al. demonstrated that *S. aureus* express Sak in vivo, since injection of *S. aureus* in mouse elicited the production of specific antibodies against Sak (Burlak et al. 2007). *S. aureus* produces positively charged polysaccharide intercellular adhesin (PIA) and negatively charged poly-γ-glutamic acid (PGA) at its surface, which increases the net positive charge of the cell surface and, as it was described for *S. epidermidis*, could impair binding of positively charged CAMPs by electrostatic repulsion; however, other mechanisms might be involved as well, since PIA also protected *S. aureus* from the negatively charged AMP dermcidin (Vuong et al. 2004; Kocianova et al. 2005). Moreover, PIA, which is produced by the intercellular adhesion *ica* locus, has been involved in biofilm formation in *S. aureus* (Cramton et al. 1999). *S. aureus* can also express D-Ala and L-Lys at its surface, modifying the net charge, through the *dlt* operon and *mprF* gene, respectively (Peschel et al. 1999, 2001; Staubitz et al. 2004; Collins et al. 2002; Nishi et al. 2004). These mechanisms are also efficient against other CAMPs, such as those derived from lactoferrin and phospholipase A2 (Koprivnjak et al. 2002).

S. aureus regulates these genes and many others involved in CAMP resistance with Aps/GraRSX, an inducible system that is activated in presence of CAMPs (Li et al. 2007a; Kraus et al. 2008). Li et al. showed that a mutant with a deletion of *aspS* was less virulent in an intraperitoneal mouse infection model than the wild-type strain (Li et al. 2007a). Other inducible mechanisms described in *S. aureus* and implicated in CAMP resistance involve Agr and SarA (Huang 2006). Modifications of the membrane involve carotenoid production by the *crtOPQMN* operon, which can suppress nonoxidative host defenses mediated by CAMPs (Mishra et al. 2011). *S. aureus* is also able to prevent CAMP activity by expressing efflux pumps such as the plasmid-encoded QacA and the ABC transporter EpiFEG (though QacA-mediated resistance is independent of efflux activity). Finally, *S. aureus* is able to form biofilms, which are ultrastructures that promote bacterial resistance to AMPs and other killing agents. Internal targets are probable in *S. aureus*, since CAMPs

are able to kill *S. aureus* without significant depolarization or disruption of the membrane (Koo et al. 2001).

4 Conclusions and Perspectives

Bacteria have constantly evolved novel mechanisms to overcome attacks by CAMPs. It seems that for every way CAMPs kill, bacteria have developed a resistance mechanism(s) in response. As mentioned above, the mechanisms of CAMP resistance are for all purposes similar to those developed by bacteria to resist classical antibiotics. At first glance, mechanisms of bacterial resistance to CAMPs would seem to favor the microbe and not the host. However, this view may be overly simplistic; most of the bacteria we interact with on a daily basis are not normally pathogenic, and many are associated with good health. If such commensally carried, helpful bacteria were to be reduced or eliminated in the presence of CAMPs, how would this impact our health? Perhaps CAMP resistance mechanisms evolved not as a way for pathogens to avoid elimination, but rather as a way for the helpful commensals to survive.

CAMPs have been promoted as a new class of therapeutic antimicrobials for treating multi-antibiotic-resistant pathogens, some of which cause infections that are becoming untreatable. Studies exploring various characteristics of CAMPs (charge, amphipathicity, hydrophobicity, etc.) (see Shprung et al. 2012) will help gain insight in the design of ever more efficient synthetic CAMPs. Alternatively, further research focusing on specific bacterial metabolic states could prevent formation of structures such as biofilms that are extremely hard to destroy and that increase the risk of chronic infections and antibiotic resistance development. In this respect, work in the Hancock laboratory on the ability of CAMPs to prevent formation of biofilms is especially important and could be exploited by attaching these peptides to medical devices (de la Fuente-Núñez et al. 2012). The continued advancement of these peptides as therapeutics will require additional studies to further analyze their potential short- and long-term toxic effects, their specificity, their pharmacokinetics, the appearance of resistance patterns, and immunomodulatory/immunostimulatory secondary effects.

Continued studies on mechanisms of CAMP resistance are also warranted. As therapeutic antimicrobial peptides pass through clinical trials, we can use the knowledge gained from such experiments to predict how bacteria will respond to their presence during treatment (which will likely be at higher levels than what occurs naturally) and if resistance (especially broad spectrum) will develop. We must, however, be cognizant of the possibility that resistance to administered CAMPs may negatively impact innate host defenses mediated by the natural CAMPs that function at different sites in the human body. How this might influence decisions to move forward with the therapeutic application of CAMPs is a matter for future consideration.

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Antimicrobial Peptides and Inflammatory Bowel Disease

Simon Jäger, Eduard F. Stange, and Jan Wehkamp

Abstract The pathogenesis of inflammatory bowel disease (IBD) is a complex and multifactorial process. In the last decades, IBD was thought to originate from dysregulation of adaptive immunity networks and classified as autoimmune disorder. Recent years have witnessed a revision of this dogma, and spurred by the findings of genome-wide association studies, defects in the mucosal barrier are now regarded as central to IBD pathogenesis. The major components of the barrier are the epithelial cell lining, the mucus layer, commensal bacteria, and an arsenal of antimicrobial peptides (AMPs). The expression pattern of these antimicrobials (e.g., defensins or cathelicidin-LL-37) in the human intestine has been elucidated over the last years, and numerous alterations were detected in IBD, especially in Crohn's disease of the ileum. The α-defensin HD-5 is secreted in large quantities by Paneth cells, which are small epithelial cells at the bases of intestinal crypts. Variations in genes which show the highest linkage to Crohn's disease affect proteins involved in Paneth cell degranulation. In addition to an outline of the strong associations between defective AMP secretion and ileal Crohn's disease, defensin expression and function in colonic Crohn's disease and ulcerative colitis will be covered in this chapter.

1 Introduction

The pathogenesis of inflammatory bowel disease (IBD) is a complex and multifactorial process. In a genetically susceptible individual, a variety of mechanisms lead to a breakdown of the mucosal barrier that facilitates the advent of intestinal

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inflammation. Today, there is a general agreement that the composition of intestinal microbiota and environmental factors in concert with malfunctioning of innate and adaptive immunity lead to the manifestation of disease (Jager et al. 2010). Long since, the implication of a role for bacterial pathogens in IBD etiology, e.g., *Mycobacterium avium paratuberculosis* (Sartor 2005), has led to the exploration of empiric antibiotic treatments in both forms of IBD, Crohn's disease, (CD) and ulcerative colitis (UC). In many clinical trials, antibiotics do have reproducible albeit moderate beneficial effects (recently reviewed by Khan et al. 2011), and superiority to placebo could be demonstrated for induction of remission in active CD and UC. In addition, antibiotics are also used as an effective standard treatment in case of perianal fistulas in CD. Importantly, human epithelial surfaces themselves are known to secrete an arsenal of antimicrobial peptides (AMPs), and their expression pattern in the human intestine has been thoroughly characterized over the last years (Tollin et al. 2003; Wehkamp et al. 2002, 2006).

The intriguing question whether a reduced expression or a functional impairment of these AMPs might contribute to the pathogenesis of IBD has been the focus of many research efforts over the last years. In the following pages, the reader is presented an overview of intestinal AMPs and their respective roles in inflammatory bowel disease.

2 Anatomic Distribution of IBD

The vast majority of chronic inflammation of the intestine can be classified as CD or UC, while a small proportion of patients showing colonic inflammation which defies a clear cut distinction between the entities termed indeterminate colitis (Guindi and Riddell 2004). Based on the Montreal classification's categories—age of onset, localization of inflamed tissue, extent of disease, and type of inflammation (stenosis/scarring or fistulas)—a concise description of the subtype of IBD can be made, and rational decisions about treatment avenues should be based on this information (Satsangi et al. 2006). UC is limited to the large intestine, whereas CD can exclusively affect the small or the large intestine, but in about 50 % of cases involve both sections. A small percentage of patients have concomitant disease activity in the upper gastrointestinal tract. In addition to the gut, there can be various extraintestinal manifestations of disease, but due to complexity, this will not be further discussed here. Importantly, and paralleling these clinical observations of intestinal disease location, distinct profiles of antimicrobial peptides are produced at the different intestinal sections. The most consistent associations between AMPs and IBD are found for the class of α-defensins in small intestinal CD. Yet, defensin expression and function in colonic disease merits a close look as well.

3 General Properties of Intestinal Antimicrobial Peptides

Gastrointestinal AMPs include defensins, molecules with defensin-like antimicrobial activity (Sallenave 2002) [e.g., elafin or secretory leukocyte protease inhibitor (SLPI)], cathelicidin-LL37, and others, which are of epithelial and of leukocyte origin. The most prominent group are the defensins, which function as endogenous antibiotics with microbicidal activity against Gram-negative and Gram-positive bacteria, fungi, viruses, and protozoa (Zasloff 2002). Defensins have a low molecular mass from 3 to 6 kDa, and structurally, three intramolecular disulfide bonds are a characteristic element. The pattern of linkage between the cysteine residues allows the classification into two major groups, the α-defensins and the β-defensins. The former show disulfide bridges between cysteines 1–6, 2–4, and 3–5, and in the latter, cysteines 1–5, 2–4, and 3–6 are linked. Their three-dimensional structure is similar, though (Ganz 2003). The total of six α-defensins includes human neutrophil peptides 1–4 (HNP1-4) produced by granulocytes and human defensin 5 and 6 (HD-5 and HD-6), which are produced by Paneth cells (PC), a specialized epithelial cell linage of the small intestine. It should be noted that these PC defensins are stored as pro-peptides and require cleavage by trypsin, which is stored inside of PC granules as well (Ghosh et al. 2000, 2002).

β-defensins are produced by a large number of human epithelial cells (Zhao et al. 1996). Three subtypes, designated hBD-1 to hBD-3, have been characterized in depth and identified on the protein level in human mucosa so far. In total, more than 40 potential coding regions for β-defensins are known, but the in vivo function and relevance of many of those remain unclear to date. The exact mechanism by which defensins exert their bactericidal effect has still not been clarified either (Pazgier et al. 2007). In vitro testing reported sensitivity to a high salt concentration, most notable in hBD-1 and hBD-2. These are potent antimicrobials against Gram-negative bacteria. hBD-3 in turn shows enhanced activity against Gram-negative species and less sensitivity to salts (Ganz 2003). Furthermore, a very interesting observation from our laboratory showed that breaking up the tertiary structure of hBD-1 by reduction of its characteristic disulfide bonds yields a linearized peptide with enhanced antimicrobial activity (Schroeder et al. 2011), the mechanistic details are still under investigation though.

The cationic charge of defensins, which allows them to bind to negatively charged phospholipid groups on microbial surfaces, has been identified as crucial for their antimicrobial effect. The model devised by Shai, Matsuzaki, and Huangh proposes that after integration of defensins into a cell membrane, its outer layer expands and strains the inner leaflet of the bilayer, leading to disruption or formation of toroidal pores (Papo and Shai 2003). Investigations on hBD-3 revealed that it can additionally inhibit steps in the biosynthesis of the bacterial cell wall (Sass et al. 2010).

Besides their well-known antimicrobial activity, defensins display chemotactic properties. The chemoattractant effect on immature dendritic cells and CD4+ T cells has been shown to act through the chemokine receptor CCR6 (Yang et al.

1999). In addition, chemoattraction of macrophages and monocytes is mediated through CCR2 (Rohrl et al. 2010). Other investigations have shown that hBD-2 induces the migration of mast cells by activating G-protein-phospholipase C coupled receptors and is a specific chemoattractant for human neutrophils (Niyonsaba et al. 2002, 2004). Also, HD-5 may influence the intestinal inflammatory response by binding to the cell membrane of intestinal epithelial cells. A subsequent induction of interleukin (IL)-8 was observed in a concentration- and structure-dependent fashion (de et al. 2009; Kotarsky et al. 2010). Defensins thus figure as an important link between innate and adaptive immunity by attracting immature dendritic cells, helping in their maturation and promoting the subsequent activation of T cells (Peyrin-Biroulet and Chamaillard 2007). Yang et al. assumed the perspective of CLL20/macrophage-inflammatory-protein-3α and an additional 17 chemokines and verified that they have antimicrobial activity on their own (Yang et al. 2003).

Cathelicidins are another major group of antimicrobial peptides in mammals, but only a single cathelicidin has been identified in humans. It is termed LL-37/hCAP18. While a signal peptide called "cathelin prosequence" can be found at the N terminus, the C-terminal part is formed by a cationic region that harbors the antimicrobial activity. LL-37 is constitutively expressed in various immune cells, epithelia of respiratory, digestive and reproductive tracts, and in keratinocytes and intestinal cells expression is inducible. LL-37 is chemotactic to blood cells, can elicit histamine release from mast cells or induce angiogenesis (Zanetti 2005).

Several other antimicrobial peptides have been investigated in the context of IBD. sPLA2 (secretory phospholipase A2) in PC granules is a constitutively expressed AMP with preferential activity against bacterial membranes of Gram-positive bacteria (Nevalainen et al. 2008). The C-type lectin Reg3α (also known as HIP/PAP and referred to as Reg3γ in mice) is expressed in PC and enterocytes (Cash et al. 2006). Its pro-peptide is processed by trypsin, and it is bactericidal to Gram-positive bacteria (Medveczky et al. 2009). Bactericidal/permeability increasing protein (BPI) is involved in lipid-mediated killing and the attenuation of proinflammatory signaling by bacteria. Its spectrum encompasses Gram-negative bacteria (Canny et al. 2002, 2006). An overview on important AMPs in intestinal mucosa is presented in Table 1.

4 The Intestinal Mucus Layer

Of course, the specific microenvironment in which AMPs function has to be taken into consideration. In the intestine, spatial segregation of microbiota and host is uniformly accepted as a prerequisite for a healthy mucosa. More so, the composition of the mucus layer secreted by ubiquitous goblet cells has been elucidated in murine models. To begin with, the apical surface of enterocytes is capped by a glycocalix composed of transmembrane mucins. In the small intestine, this glycocalix is covered by a single-layered mucus, which is not attached to the mucosa and permeable to bacteria (Johansson et al. 2011a). In the large intestine,

Table 1 Antimicrobial peptides of the intestinal mucosa

Antimicrobial peptide *Gene*	Chromosomal location	Mass (kDa)	Secretory stimuli	Distribution in gastrointestinal tract	Biological function	Changes in inflammatory bowel disease
BPI	20q11.23	50	LPS	Epithelial cells, neutrophils	Antimicrobial, binds LPS-compounds	No changes observed, regular induction in IBD
Cathelicidin antimicrobial peptide (also known as hCAP18 or LL-37) *CAMP*	3p21.3	18	Butyrate, vitamin D, bile acids, MDP	Epithelial cells, leukocytes	Antimicrobial, chemotactic	Attenuated induction in colonic CD Ileal CD and UC show regular induction
Elafin	20q13.12	9.8	IL-1, TNF-α	Epithelial cells, Leukocytes	Antiprotease with antimicrobial and chemotactic properties	Attenuated induction in colonic CD
hBD-1 *DEFB1*	8p23.1	3.5–4.5	Constitutive in epithelial cells, IFN-γ and LPS in monocytes	Ubiquitious in epithelial cells of small and large intestine, monocytes, monocyte-derived dendritic cells	Antimicrobial, chemotactic	Reduced mRNA expression in colonic IBD in inflamed and non-inflamed tissue
hBD-2,3,4 *DEFB4, DEFB103, DEFB104*	8p23.1	3.5–4.5	LPS, flagellin mediated by NF-κB and AP-1	Epithelial cells, monocytes	Antimicrobial, chemoattractant for macrophages and monocytes hBD-2: mast cells and neutrophils	Attenuated induction observed in colonic CD Reduced copy numbers for hBD-2 in colonic CD
HD-5 and HD-6 *DEFA5* and *DEFA6*	8p23.1	3.5–4.5	NOD2 activation (MDP, LPS) TLR	Granules of ileal Paneth cells (also metaplastic Paneth cells in other areas of intestinal tract)	Antimicrobial, induction of IL-8	Reduction in ileal CD, more pronounced in patients with NOD2 mutation

(continued)

Table 1 (continued)

Antimicrobial peptide *Gene*	Chromosomal location	Mass (kDa)	Secretory stimuli	Distribution in gastrointestinal tract	Biological function	Changes in inflammatory bowel disease
						HD-5 and HD-6 expression due to metaplastic Paneth cells in UC and CD colon
HIP/PAP (homolog of Reg3γ in mice)	2p12	16	TLR	Pancreas and small intestine	Antimicrobial, lectin	Unknown
Lysozyme	12q15	16.5	Unknown	Gastric, pyloric, and duodenal glands, small intestine, macrophages, and monocytes, not in colonic tissue	Antimicrobial against Gram-positive bacteria, chemotactic	Small intestine: no changes observed Increased colonic expression due to metaplastic Paneth cells
sPLA2	16p13.1-p12	14	LPS	Epithelial and inflammatory cells, Paneth cell granules	Acute phase protein Eicosanoide metabolism Small intestinal defense	Unknown

Adapted from Jager et al. (2010) and Stange (2009)

Abbreviations: *hBD* human β-defensin, *HD* human defensin, *HIP/PAP* hepatocarcinoma–intestine–pancreas/pancreatitis-associated protein, *BPI* bactericidal/permeability increasing protein, *LPS* lipopolysaccharide, *MDP* muramyldipeptide, *NF-κB* nuclear factor κB, *NOD2* nucleotide oligomerization domain 2, *sPLA2* secretory phospholipase A2, *TLR* Toll-like receptor

a densely packed inner layer and a loose outer layer can be distinguished. In mice, the thickness of the colonic mucus layer steadily increases from the proximal to the distal colon. The firmly attached inner layer of about 50-μm thickness forms a physical barrier to bacterial entry (Johansson et al. 2011b) and is devoid of bacterial colonization, whereas Lactobacilli and Bifidobacteria populate the outer layer (Subramani et al. 2010).

Using isolated mucus from murine small intestine, Meyer-Hoffert et al. demonstrated a high antimicrobial activity of the mucus extract. Subsequently, α-defensins, lysozyme, and phospholipase A2 were identified by MALDI-TOF in these fractions. These peptides were detected in crypt isolates as well but not in luminal contents (Meyer-Hoffert et al. 2008). Furthermore, it has been demonstrated using universal 16S ribosomal RNA gene probes (detecting bacterial nucleic acids) that microbiota are kept at a distance of ~50 μm from the epithelial cells in mice. Presence of Gram-positive bacteria within this distance was noted in the small bowel of mice with a knockout of the antimicrobial peptide Reg3γ (Vaishnava et al. 2011). As the vast majority of bacteria found in the intestinal lumen are Gram-negative, these must be controlled by other AMPs, as for example defensins, to which many Gram-negative bacteria are susceptible. The studies were done in murine ileal tissue, but as mentioned above, the inner layer of colonic mucus is essentially clear of bacteria (Johansson et al. 2011b), and antimicrobial peptides most certainly play a role in this situation as well. To our knowledge , direct experimental evidence for this is not yet available though.

5 IBD, Genome-Wide Association Studies and Defensins

Recent years have witnessed a great success in identifying genetic variants associated with CD, UC, or both. Currently, 71 loci are confirmed by a meta-analysis of six GWAS in CD (Franke et al. 2010) and 47 in UC (Anderson et al. 2011), while 28 risk loci are shared. Most prominently discussed are the bacterial sensor *NOD2* (nucleotide-binding oligomerization domain 2), the autophagy protein *ATG16L1*, *XBP-1*, and *IL-23R* polymorphisms, which in contrast to the three former seem to be protective.

In the meta-analyses cited above, CD or UC have not shown linkage to chromosome 8p23.1, the region in which defensin genes are clustered. The complex structure of the human β-defensin gene locus with variable copy numbers in *DEFB4, DEFB103*, and *DEFB104* (these encode hBD-2, hBD-3, and hBD-4, respectively) might explain the lack of association, as a gene with variable copy numbers will not be effectively interrogated by genotyping chips assaying tagSNPs flanking its region (Hollox et al. 2008). Yet, Kocsis et al. have reported a genetic association of hBD-1 variants in colonic CD in a Hungarian cohort (Kocsis et al. 2008).

An important caveat remains that the associations are based on predominantly Caucasian cohorts, and different ethnicities might harbor additional and different

variants. Thus, in a Japanese cohort, CD was not associated with the most common polymorphisms in *NOD2* (Inoue et al. 2002), and recently, a non-synonymous SNP in another AMP, BPI, was associated with both CD and UC in a Turkish cohort, while western European studies reported an association either only with CD or no association at all (Akin et al. 2011). These limitations apart, current data already show that some of the SNPs conferring the greatest relative risk can be linked to the pathogenesis of ileal CD, where they affect excretion, function, or transcription of human α-defensins by different avenues. In summary, the genetic data support the hypothesis that different location-phenotypes are mechanistically different diseases even though they share similar clinical features once the disease is present.

6 Localization Remains Stable Over time: Small Intestinal CD

As mentioned before, small and large intestinal CD present with a different clinical course, and growing evidence points to a distinct genetic background and a different disease pathogenesis. In small intestinal CD, Paneth cells moved to the center of research efforts. The most abundant product of these cells are the constitutively secreted α-defensins (especially HD-5), whose expression is a hundred times higher than that observed for lysozyme and sPLa2 (Wehkamp et al. 2005), which are other important PC products.

After activation of pattern recognition receptors (Toll-like receptors or NOD-like receptors) by pathogen-associated molecular patterns (PAMPs), derived from resident and pathogenic bacteria, PC secretions are released into the intestinal lumen (Vaishnava et al. 2008). Expression of intracellular receptors like NOD2 itself depends on the presence of commensal bacteria (Petnicki-Ocwieja et al. 2009). Vice versa, the composition of microbial species found in the small intestinal lumen can be regulated by the luminal antimicrobials (Salzman et al. 2010).

A link between *NOD2* and ileal CD has been demonstrated by Cuthbert et al. (Cuthbert et al. 2002) nearly ten years ago, when the mutations R702W, G908R, and 3020insC were identified as strong independent risk factors in patients of Caucasian decent. The odds ratio is 3.99 (Barrett et al. 2008). NOD2 protein and mRNA were found to be most prominently expressed in the terminal ileum, with localization to crypt base cells and mononuclear cells of the lamina propria (Lala et al. 2003). Subsequent work from our group reported decreased α-defensin mRNA levels in ileal biopsy specimens, which were even more pronounced in patients carrying *NOD2* mutations (Wehkamp et al. 2005). What is more, bactericidal activity of crypt secretions of the terminal ileum was severely compromised by *NOD2* deletion in a murine model (Petnicki-Ocwieja et al. 2009). Studies with different knockout animals, which were lacking functional cryptins (mouse-homologs to defensins) due to deficiency of the cryptdin-processing enzyme matrilysin, revealed that intestinal crypt secretions had decreased antimicrobial activity (Wilson et al. 1999). Additionally, these mice are more susceptible to orally administered bacterial pathogens or to DSS-induced colitis.

Nevertheless, others suggested that reduced α-defensin mRNA expression is not a primary effect. The authors report that this finding is most probably due to epithelial loss, as they did not observe reduced α-defensin levels in non-inflamed ileal mucosa in their CD patients (Simms et al. 2008). The association between NOD2 mutation (L1007fsinsC) and particularly low α-defensin levels could not be found in their cohort either. Yet, in an assessment of luminal HD-5 levels in ileostomy fluids, significantly lower defensin levels in CD patients than in controls were observed, and especially in those with homozygous/compound heterozygous NOD2 mutations (Elphick et al. 2008). In this publication, processing of pro-HD-5 to mature HD-5 by trypsin was found to be impaired, i.e., HD-5 in the ileostomy fluid of CD patients is predominantly present in inactive complexes with trypsin or chymotrypsin.

Unmethylated cytidine-phosphate-guanosine (CpG) is a ligand for another important PC pattern recognition receptor, TLR-9. Administration of oligonucleotides containing these CpG sequences leads to PC degranulation and was shown to salvage mice from subsequent disease after exposure to *S. typhimurium* (Rumio et al. 2004). Even more, TLR-9 might thus cooperate with NOD2 by promoting the degranulation of antimicrobial peptides from PC.

Mutations in pattern recognition receptors like *NOD2* are but one of the findings that underline the link between PC, defensins and ileal Crohn's disease. In 2001, a genome-wide association study identified *ATG16L1* as susceptibility locus for small intestinal CD (Hampe et al. 2001). The ATG16L1 protein mediates degradation of phagocytosed or invasive bacteria, although it is commonly known for its involvement in autophagy, a process responsible for the degradation of intracellular structures. Cadwell et al. provided evidence that in mice with conditional *Atg16l1* knockout, granule exocytosis is abnormal and that in patients with the variant T300A, morphologic distortions in PC can be observed. As PC granules contain huge amounts of α-defensins, impaired exocytosis of these antimicrobials presumably weakens the mucosal barrier. This could explain part of the increased disease susceptibility found with mutations in *ATG16L1*, though other mechanisms for the phenotypic development of ileal CD with this genetic background have to be considered as well. ATG16L1-deficient macrophages for example exhibit proinflammatory properties (Cadwell et al. 2008), which points to an additional involvement of myeloid cells in disease pathogenesis.

Employing transmission electron-microscopy, a recent study identified a significant decrease in the number of secretory granules within PC, which was specific for small intestinal CD and independent of mucosal inflammation. The authors report that the reduced number of granules results from an abnormal induction of autophagosomes which target the secretory granules (a process called crinophagy) (Thachil et al. 2012). Once more, parallels between reduced defensins (the major component of the granules) and intestinal CD can be drawn.

A conditional deletion of X-box binding protein (*Xbp-1*) shed light on the association of *Xbp-1* variants with inflammatory bowel disease (CD and UC). XBP-1 normally functions as transcription factor for the unfolded protein response under conditions of endoplasmic reticulum (ER) stress, maintaining proper folding,

export, and degradation of proteins by the ER. Knockout of *Xbp-1* in the intestinal epithelium led to spontaneous small bowel inflammation, altered PC morphology, and a decreased antimicrobial activity of crypt secretions (Kaser et al. 2008) .

In a murine model, inhibition of the K^+-pump/Ca^{2+} channel KCNN4, which regulates Ca^{2+}-fluxes important for the secretion mechanisms for AMPs from PC, led to a reduced bactericidal activity and AMP secretion (Ayabe et al. 2002). An association of the SNP r2306801 with CD in general, and with ileal CD the strongest, was recently observed in an combined cohort from Australia and New Zealand (Simms et al. 2010), though reproduction of the results in a larger cohort are not yet available. *KCNN4* mRNA expression in non-inflamed mucosal biopsies of individuals with *NOD2* mutations was reduced, leading to the assumption that functional NOD2 is important for the expression of *KCNN4*.

Yet another association between ileal CD and defensins becomes apparent when alterations in the Wnt pathway, which governs PC differentiation (van Es et al. 2005), are considered. More specifically, the transcription factor TCF-4 has been linked to α-defensin expression, as heterozygous *Tcf-4* knockout mice with decreased levels of *TCF-4* exhibit compromised cryptdin expression and weakened antimicrobial activity of crypt extracts (Wehkamp et al. 2007). Furthermore, a reduced mRNA expression of *TCF-4* was observed in patients with ileal CD. Investigations aimed at the regulatory regions of *TCF-4* revealed that in ileal CD, a SNP in the *TCF-4* promotor region (rs3814570) was significantly more frequent than in controls, exclusively colonic CD or UC (Koslowski et al. 2009). Taken together, the genetic variant in the promotor of *TCF-4* gives a further rationale for the α-defensin deficiency on the basis of PC differentiation pathways.

Additional alterations in the Wnt-pathway are evident from the analysis of LRP6 (low-density lipoprotein receptor-related protein 6), which is a Wnt co-receptor of intestinal cell surfaces. A functional mutation in this receptor was more frequent in early onset ileal CD and was also associated with fistulating disease. Importantly, these findings could be linked to an especially low HD-5 expression in ileal biopsies from patients who carried the mutation. Concurrently, cell culture experiments showed that overexpression of LRP6 leads to increased promotor activity in the HD-5 gene (Koslowski et al. 2012).

All the mentioned genetic variants and functional studies converge on the PC and its ability to provide the crypt lumen with antimicrobial peptides, which are predominantly α-defensins (Wehkamp and Stange 2010). Different genetic alterations thus lead to dysfunctional PCs, result in a weakened mucosal barrier and account for the increased disease susceptibility (Fig. 1).

7 Localization Remains Stable Over Time: Colonic CD

The first defensin identified in the large intestine was hBD-1, and in the healthy, non-inflamed colon, it is the major defensin. Its constitutive expression relies *inter alia* on signaling through peroxisome proliferator-activated receptor (PPAR-γ) (Peyrin-Biroulet et al. 2010), for which rosiglitazone is a well-known activator.

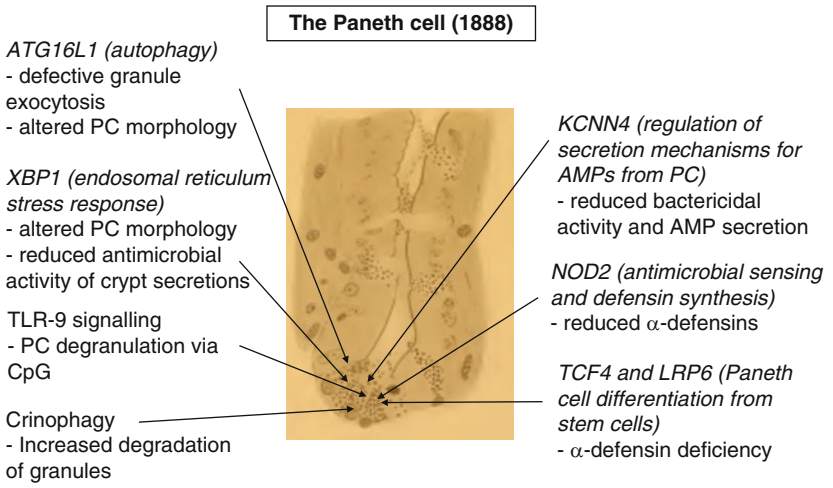

Fig. 1 Paneth cells and granules at the crypt base. The original photograpy is from Josef Paneth's paper in *Archiv für mikroskopische Anatomie, 1888*. The figure is adapted from Wehkamp J, Stange EF Journal of Crohn's and Colitis (2010)

Peyrin-Biroulet et al. also found a compromised defensin expression as a feature of colonic CD, where macroscopically and histologically non-inflamed mucosa already showed significantly decreased transcripts level of *DEFB1*. In addition, a further reduction of hBD-1 expression in inflamed mucosa of IBD patients compared to healthy controls has been noted (Wehkamp et al. 2003).

We recently reported that hBD-1 shows enhanced activity against enteric commensals after its three characteristic disulfide bonds have been reduced (Schroeder et al. 2011). Thus, antimicrobial activity of hBD-1 has to be evaluated in the light of specific conditions because it will exhibit a different antimicrobial spectrum in the human colon with its reducing environment versus the oxidizing environment on the skin. Reduced hBD-1 is active against Bifidobacteria, Lactobacilli, and several strains of the opportunistic fungus *Candida albicans* and could thus help to regulate the commensal microbiota in the colon. The oxidoreductase thioredoxin might be a physiological mediator catalyzing reduction of hBD-1 in human epithelia. While thioredoxin mRNA is upregulated in malignancy and inflammatory conditions like rheumatoid arthritis (Maurice et al. 1999), it was decreased in inflamed colonic CD (Schroeder et al. 2011). Yet, whether this relative lack of thioredoxin has a role in maintaining inflammation by compromising the intestinal antibiotic barrier function is still under investigation.

hBD-2 and hBD-3 are normally absent in healthy mucosa and only induced during inflammation or infection. Stimuli for hBD-2 expression include both bacteria and cytokines, like *Campylobacter jejuni* (Zilbauer et al. 2005) or the bacterial component flagellin from the *E. coli* strain Nissle 1917, which is used as probiotic in the maintenance treatment of ulcerative colitis (Mondel et al. 2008). On the cytokine level, the induction is mediated by proinflammatory cytokines such as

IL-1β (through NF-κB-dependent and AP-1-dependent pathways) (Wehkamp et al. 2004) and TNF-α or IL-17 (Gaffen 2009).

In the event of inflammation, CD and UC show remarkable differences in hBD-2 mRNA expression. In patients with active UC, hBD-2 and hBD-3 are strongly upregulated, while the induction is attenuated in Crohn's disease (Wehkamp et al. 2002, 2003; Aldhous et al. 2009). Furthermore, the killing capacity towards various commensal bacteria was low in colonic mucosa of CD patients (Nuding et al. 2007). The mechanism behind the reduced hBD-2 expression in inflamed colonic Crohn's has not been elucidated up to now. In a European and US cohort of IBD patients, gene copy numbers for hBD-2 (Fellermann et al. 2006) were reduced, but results from a New Zealand cohort were inconsistent with this finding (Bentley et al. 2010). Data from psoriasis studies is conflicting as well; there, susceptibility to disease was increased with increased copy numbers, and the authors could even show a linear relationship with gene dosage (Hollox et al. 2003). As mutations in the intracellular bacterial sensor NOD2 have been associated with reduced α-defensin expression levels in ileal CD, researchers also investigated the effects of *NOD2* mutations on the expression of β-defensins. Voss et al. demonstrated that the expression of hBD-2 is mediated by NOD2 activation (Voss et al. 2006), but a subanalysis stratified for *NOD2* mutation status could not identify differences in colonic hBD-2 expression (Wehkamp, unpublished observation).

Many lines of evidence thus point to a major role of β-defensins in inflammatory processes of the colon. Data for the *α-defensins* from a mouse model show that the PC cryptdins synthesized in the ileum are conserved in structure and functionality after passing into the colonic lumen (Mastroianni and Ouellette 2009), suggesting a role for α-defensins in the large bowel as well. PC metaplasia is a reactive change noted in different sites of inflammation along the gastrointestinal tract, including the colon (Cunliffe et al. 2001). This metaplastic response could represent a mechanism providing additional antimicrobial protection by α-defensins at sites of chronic inflammation. Furthermore, a significant elevation of hBD-2 peptide could be detected in fecal samples from patients with irritable bowel syndrome, a condition which demonstrates no macroscopic visible inflammation on colonoscopy and is generally thought to be of non-inflammatory nature (Langhorst et al. 2009). The antimicrobial peptide elafin has a similar expression pattern as the inducible β-defensins, LL-37, and secretory leukocyte protease inhibitor (SLPI). It has been suggested that its antiprotease activity balances the proteolytic effects of HNE (human neutrophilic elastase) from polymorphonuclear cells in healthy tissues (Hiemstra 2002). Elafin expression levels are reduced in colonic CD, which could result in a protease-antiprotease dysbalance. The penetrating, transmural type of inflammation often found in colonic CD might be explained in part by these findings (Schmid et al. 2007).

Cathelicidin-LL37 shows induction in inflamed tissues of UC, while in active CD the induction seems to be attenuated (Schauber et al. 2006). In mutant mice, cathelicidin restricts colonization of the epithelial by adherent bacterial pathogens like *Citrobacter rhodentium* (Iimura et al. 2005), confirming its vital role in the armamentarium of the innate immune system.

8 Pouchitis and Defensins

Restorative proctocolectomy is a valid, definite therapy for UC refractory to medical treatment. Nonetheless, acute or chronic inflammation of the reservoir (termed pouch) created from ileal loops is a common complication (50 % and 5 %, respectively, during the first 10 years). Kiehne et al. investigated expression of antimicrobial peptides in pouchitis and found a decreased defensin expression (Kiehne et al. 2005). Conversely, a recent study investigated chronic/relapsing pouchitis, and the authors concluded that high levels of HD-5 were an independent predictor of pouchitis (Scarpa et al. 2012). It was proposed that the increased number of CFU in mucosa-associated Clostridiaceae spp. might result from HD-5 mediated antimicrobial activity against Enterobacteriaceae, facilitating the growth of Clostridia species. In fact, from clinical experience, *C. difficile* infection has to be ruled out in cases of pouchitis, and antibiotics successfully used in treatment of pouchitis (e.g. metronidazole) have proven activity against Clostridiaceae (Dignass et al. 2011). In the setting of pouchitis, HD-5 can thus mediate shifts in the composition of the microbiota that favor inflammation.

9 Therapeutic Implications

Beyond currently used treatment options for patients with inflammatory bowel disease, more sophisticated strategies to interfere with the effector mechanisms of the immune system will become available in the near future. The therapeutic pipeline offers *inter alia* new immunomodulators, inhibitors of leukocyte migration to the gut (anti-MadCAM or anti-integrin, e.g., phase III vedolizumab trials in CD and UC), various interleukin-antagonists, chemokine receptors, Jak3 or protein kinase inhibitors, and alternative anti-TNF-α antibodies (Danese 2012). Nonetheless, the majority of these new agents is directed against an already established inflammatory process. Thus, enhancing or correcting innate immune functioning *before* an acute flare or even chronic inflammation occurs remains an elusive goal.

Strengthening of the mucosal barrier with phosphatidylcholin has shown promise in UC, and embryonated eggs from the wipe-worm *Trichuris suis* are currently tested in a phase II trial in moderate active CD. Pilot studies had demonstrated efficacy in CD an UC alike, but small patient numbers and lack of a double-blind study design were limitations (Summers et al. 2005a, b). Infection with these parasite ova, which do not survive in the human intestine for longer than 12 days, is thought to modulate the immune response. Evidence for these findings comes from the observation that children with helminthic infections have reduced atopy (Yazdanbakhsh et al. 2002) and peripheral blood mononuclear cell from these children show increased production of anti-inflammatory mediators IL-10 and TGF-ß (Doetze et al. 2000). Whether the therapeutic effect is mediated by a shift in adaptive immune function or whether stimulation of the production of antimicrobials is significantly involved can be addressed in the current trial.

However, recent animal studies have provided evidence that infection with other helminths, in this case the tapeworm *Hymenolepis diminuta*, can cause a significant disease exacerbation (Hunter et al. 2007). An already completed study using *Trichuris suis* ova in allergic rhinitis could not show benefits compared to placebo; moreover, the most frequent side effects were gastrointestinal disorders like diarrhea and abdominal pain which is certainly no encouraging news for patients with IBD (Bager et al. 2010).

Directly employing recombinant antimicrobial peptides like HD-5 in the treatment of CD of the ileum, though certainly attractive, will have to deal with many challenges as well. As shown above, defensins are mainly located in the crypt lumen and inner mucus layer. Orally administrated peptides presumably remain to a great extent in the lumen, and it is not clear if they would be more effective there than already available wide-spectrum antibiotics. Thus, testing strategies to induce expression of defensins in mucosal cells (e.g., in the Paneth cell) seems to be more promising to increase defensin levels at their proper site of action. Interestingly, mechanisms to induce antimicrobial peptides have already been demonstrated in vivo, e.g., calcipotriol ointment (a vitamin D analog used in the treatment of psoriasis) applied to healthy human skin can induce cathelicidin protein production (Weber et al. 2005). Other research along these lines demonstrated that 1,25-dihydroxyvitamin induces expression of cathelicidin (Wang et al. 2010), and phenylbutyrate induces cathelicidin in a variety of cell lines (Steinmann et al. 2009). The importance of antimicrobial peptides is underlined by the finding that pathogenic *Shigella flexneri* has developed mechanisms to downregulate intestinal cathelicidin production (Islam et al. 2001). Induction of hBD-2 has been demonstrated for *E. coli* Nissle 1917 (brand name Mutaflor®, approved for use in mild cases of ulcerative colitis) and for other therapeutic probiotic *E. coli* strains and *Lactobacilli* (Wehkamp et al. 2004; Mondel et al. 2008; Schlee et al. 2008). hBD-2 can also be induced by 1,25-dihydroxyvitamin D3 (Wang et al. 2010). These approaches show that induction of antimicrobials is already feasible and clinical trials assessing efficacy are warranted.

Given the unknown combined effects of genetic predisposition, diet, microbial colonization, and alterations in innate and adaptive immune function, the multifactorial pathogenesis of IBD will remain an exciting field for research. Given their important role towards a cause- and not just symptom-directed treatment, we predict that antimicrobial defensins and other barrier protective molecules will have an important role in coming acts on the IBD stage.

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Cystic Fibrosis and Defective Airway Innate Immunity

Jennifer A. Bartlett and Paul B. McCray Jr.

Abstract Cystic fibrosis is a common autosomal recessive disease caused by mutations in the *CFTR* gene that encodes an anion channel expressed in epithelia and other cell types. While the disease affects multiple organ systems, it is progressive pulmonary disease, characterized by airway infection and inflammation, that is life limiting. The origins of the lung disease associated with loss of CFTR function are complex and likely multifactorial. Current research is defining how loss of CFTR anion channel activity alters the volume and composition of respiratory secretions and thereby impacts host defenses. Here we review the current understanding of the defect in innate immunity that characterizes the airway disease in cystic fibrosis. Advances in cystic fibrosis basic science research and the development of new animal models of disease are shedding new light on the causes of lung disease and may lead to new, more targeted therapies.

1 Introduction

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (Rommens et al. 1989). *CFTR* encodes an anion channel regulated by nucleotides and phosphorylation. Over 1,500 disease-associated *CFTR* mutations have been reported. The most common mutation is a three-base deletion in exon 10 resulting in the loss of a phenylalanine residue at position 508 (ΔF508), present on ~70 % of mutant alleles (Kerem et al. 1989). In addition to primary or secondary CFTR-associated changes that lead to

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pulmonary disease manifestations, polymorphisms in other genetic loci may influence the CF phenotype (Garred et al. 1999; Henry et al. 2001; Salvatore et al. 2002). Several candidate modifier genes have been proposed for CF including mannose-binding protein (Garred et al. 1999), HBD-1 and HBD-2 (Salvatore et al. 2002), alpha 1-antitrypsin enhancer (Henry et al. 2001), and HLA class II (Aron et al. 1999). A recent genome-wide association study identified two new modifier loci for CF (Wright et al. 2011).

CF is a multiorgan system disease affecting the gastrointestinal tract (liver, gall bladder, small and large intestine, pancreas), sweat glands, reproductive system, sinuses, and respiratory tract. While the signs and symptoms of the disease have been recognized for centuries, it was only in the 1930s that Fanconi (Fanconi et al. 1936) and Andersen (1938) recognized and characterized the disorder as a distinct pathologic entity and noted its genetic basis. Although loss of CFTR function causes disease in many tissues and cell types, it is the effect on the respiratory tract that is most life limiting. More than 90 % of people with CF die of progressive lung disease associated with chronic bacterial infection and inflammation within the airways (Rowe et al. 2005). CF lung disease is associated with the eventual chronic colonization of the airways with large numbers of bacteria, notably *Haemophilus influenzae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Rosenfeld et al. 2001). It is increasingly recognized that CF-associated airway infections are polymicrobial and involve biofilm formation (Bjarnsholt et al. 2009; Sibley and Surette 2011; Singh et al. 2000a). Remarkably, these infections are confined to the respiratory tract and spread to other organs is extremely rare.

In this chapter, we will focus our attention on the host defense problem within the airways. In CF, bacteria grow in regions of the lung that are normally sterile. The clinical course of CF lung disease correlates with the acquisition of bacterial infection and its progression. Whatever the cause underlying this propensity for infection, it is lung specific. These features indicate that loss of CFTR activity impairs the innate defenses of the lung. The precise link between loss of CFTR function in the airways and the host defense defect remains an area of intense study and scientific debate. Here we review our current understanding of CF lung disease.

2 Overview of CF Lung Disease

The loss of CFTR anion channel activity has a profound impact on the function of many organs, most notably those lined by epithelia and involved in the elaboration of secretions at mucosal surfaces (Welsh et al. 2001). In addition, there is growing evidence that CFTR may be important to the function of non-epithelial cell types, including alveolar macrophages (Di et al. 2006; Zhang et al. 2010), neutrophils (Painter et al. 2006, 2008, 2010), lymphocytes (Bubien 2001; Bubien et al. 1990; Mueller et al. 2011), smooth muscle cells (Robert et al. 2004, 2005; Vandebrouck

et al. 2006), neurons (Rogan et al. 2010), and others. Here we will confine our focus to the impact of loss of CFTR function on the onset and progression of lung disease.

Lung disease in infants and preschool-aged children with CF can be remarkably asymptomatic at its earliest stages. A series of bronchoscopy and bronchoalveolar lavage (BAL) studies helped document that infants with CF may have significant inflammation and bacterial infection in the face of no respiratory symptoms or signs (Armstrong et al. 2005; Balough et al. 1995; Khan et al. 1995). Early laboratory evidence of lung disease includes the presence of neutrophils, proinflammatory cytokines, and culturable bacteria such as *H. influenzae* and *S. aureus* in BAL fluid (Khan et al. 1995; Muhlebach et al. 1999). High-resolution chest CT scans are among the most sensitive early radiologic measures of disease and may demonstrate inhomogeneity of aeration, subsegmental atelectasis, bronchial wall thickening, and airway obstruction in healthy-appearing young children. As the disease progresses, bronchial dilatation and bronchiectatic changes are observed in the airways. These findings indicated that the respiratory tract host defenses of children with CF are compromised early on in their ability to eradicate bacteria encountered by inhalation or microaspiration. While aggressive, early treatments have slowed the rate of progression of lung disease, CF remains a serious chronic disease. Lung transplant is currently the only option available for patients with advanced disease.

While studies of the host defense defect associated with CF usually focus on bacterial infections, there is evidence that people with CF may have problems in their ability to tolerate infections by respiratory viruses. One prospective study of infants and children with CF reported an increased morbidity associated with respiratory syncytial virus infections (Abman et al. 1988). Hiatt and coworkers found that compared to non-CF subjects, infants with CF were more likely to develop lower respiratory tract infections associated with hospitalization and reduction in lung function (Hiatt et al. 1999). In contrast, Ramsey and colleagues studied school-aged children with CF prospectively and did not identify any significant adverse effect of respiratory viral infections on pulmonary function compared with age-matched non-CF controls (Ramsey et al. 1989). In experiments using cultured primary CF and non-CF airway epithelia, Erzurum et al. noted an increase in parainfluenza type III replication in CF cells (Zheng et al. 2003). This increase in virus replication was associated with reduced nitric oxide synthase 2 (NOS2) and 2′,5′-oligoadenylate synthetase (OAS) 1 induction in response to virus or interferon gamma. The investigators linked these reductions in antiviral defenses to an impaired activation of signal transducer and activator of transcription (STAT)1. Recently, Sutanto and coworkers studied primary cells from CF and non-CF subjects and noted that human rhinovirus 1B replicated to higher levels in CF respiratory epithelia (Sutanto et al. 2011). This finding was also associated with a reduced apoptotic response and increased release of IL-8 in the CF epithelia. These studies are intriguing as some antimicrobial peptides and proteins exhibit antiviral properties (Daher et al. 1986), and signaling mediated by type I and type III interferons may also promote antibacterial defenses (Li et al. 2008). In addition, intracellular pathogen sensing mediated by NLRP1 and NLRP3

inflammasomes may influence both antiviral and antibacterial host defense responses (Cassel and Sutterwala 2010; Guarda et al. 2011; Poeck et al. 2010; Poeck and Ruland 2011; Strunk et al. 2011). Further studies are needed to determine if mutations in *CFTR* also cause a defect in antiviral innate immunity.

While our discussion focuses on how loss of CFTR function results in the primary manifestations of CF lung disease, it is important to understand that disease progression results in secondary complications that further compromise airway defenses. As airway bacterial infection in children with CF evolves from intermittent infection to chronic colonization, the host mounts an impressive response. Predominant findings include the release of proinflammatory cytokines by epithelia and immune effector cells that include IL-1, TNF-alpha, IL-6, 1L-17 (Decraene et al. 2010; Dubin and Kolls 2011; McAllister et al. 2005; Tan et al. 2011), and 1L-23 (Decraene et al. 2010; McAllister et al. 2005) and neutrophil chemoattractants such as IL-8, IL-17, and CCL20 (Armstrong et al. 2005; Balough et al. 1995; Khan et al. 1995; McAllister et al. 2005; Muhlebach et al. 1999) and the secretion of pathogen-specific antibodies (Doring et al. 1988). Intense neutrophilic infiltration of the airways ensues, and the associated inflammatory responses include the release of enzymes such as neutrophil elastase, myeloperoxidase, cathepsins, and others. In addition, airway pathogens can release proteases and other inflammatory stimuli that gradually lead to disease progression. These secondary inflammatory responses contribute to the destruction of airway tissue and stimulate proliferative responses in the airway epithelium (Leigh et al. 1995) with associated remodeling, such as goblet cell metaplasia (Bedrossian et al. 1976; Davis and Dickey 2008; Groneberg et al. 2002). This burden of proteases causes further secondary compromise in antimicrobial defenses by directly degrading host defense proteins such as lactoferrin (Britigan et al. 1993), defensins (Taggart et al. 2003), SLPI (Weldon et al. 2009), and elafin (Guyot et al. 2008). In addition, the protease-rich environment of the CF airways can cleave TLR-2 and TLR-4, which may further impair innate immune signaling (Greene et al. 2004). These persistent host responses to polymicrobial infection add to the difficulties in defining the host defense defects directly linked to loss of CFTR function and distinguishing them from those that arise as a consequence of the host inflammatory response.

3 Innate Immune Defenses in the Airways

The airways face a daily burden of inhaled or aspirated bacteria, viruses, and other potentially damaging particulates. To protect against these threats, the epithelium of the respiratory tract has evolved multiple mechanisms to prevent microbial infection and minimize tissue damage in response to infectious agents. At the most basic level, the epithelium protects the airways from such insults by serving as a physical barrier between the environment and the underlying tissue. However, the defensive capacity of this mucosal surface extends far beyond its simple barrier function. The conducting airways are lined by a pseudostratified columnar

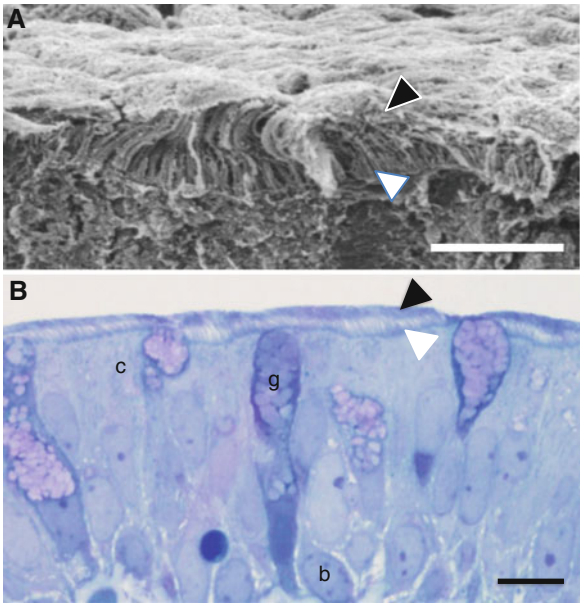

Fig. 1 The airway surface liquid (sol) and mucus (gel) layers provide an optimal environment for the function of secreted host defense factors. (**A**) Scanning electron microscope image of osmium and perfluorocarbon fixed cultured well-differentiated non-CF primary human airway epithelial cells. *Black arrowhead indicates gel layer; white arrowhead indicates sol layer*. This sentence refers to fig. 1B. (**B**) Note ciliated cells (c), goblet cells (g), and basal cells (b). Light microscopy image of osmium and perfluorocarbon fixed newborn non-CF pig tracheal epithelium stained with *toluidine blue*. *Black arrowhead* indicates gel layer; *white arrowhead* indicates sol layer. Scale bars in both images indicate 10 μm

epithelium consisting of ciliated and non-ciliated surface cells, mucin-producing goblet cells, and a progenitor cell type termed basal cells. The conducting airway epithelium also possesses submucosal glands, which supply bulk liquid secretion, additional mucins, and other molecules with antimicrobial, anti-inflammatory, or other host defense functions to the airway surface. Together, the surface airway epithelia and submucosal glands are responsible for generating the airway surface liquid (ASL)—a mixture of secreted proteins and peptides with innate immune functions, as well as lipids, surfactants, and electrolytes important for ASL volume homeostasis. The ASL is organized into two compartments: an aqueous phase near the cell surface that bathes the cilia (the *sol* or periciliary layer) and a layer of hydrated mucus (the *gel* layer) that rests atop the sol phase (Fig. 1). Inhaled microbes and other particles are trapped in this mucus layer, which, propelled by the coordinated beating of the cilia, slides along the top of the periciliary fluid layer in a process known as mucociliary clearance. In this way, particles are swept up and out of the airways to the nasopharynx, where they are eliminated by swallowing. Similarly, cough clearance also removes particles from the airway lumen (Bartlett et al. 2008a).

In addition to these physical and mechanical defense mechanisms, the airway epithelium actively interfaces with the environment and constitutively or inducibly secretes an array of innate immune effector molecules into the ASL that sense and respond to microbial threats (Table 1). This "chemical shield" includes cationic peptides with broad-spectrum antimicrobial activity, such as the beta-defensins (Pazgier et al. 2006; Schutte and McCray 2002; Singh et al. 1998), CCL20 (Starner et al. 2003), and the human cathelicidin LL-37 (Bals et al. 1998b), as well as the prototypic antimicrobial protein lysozyme (Fleming 1922; Fleming and Allison 1922), which kills bacteria by degrading peptidoglycan in the bacterial cell wall. There are also innate immune molecules that combat bacteria in ways that do not involve direct killing, such as iron sequestration by lactoferrin (Masson et al. 1966; Oram and Reiter 1968) or binding of bacterial siderophores (iron-chelating molecules) by the neutrophil gelatinase-associated lipocalin (NGAL, or lipocalin-2) (Goetz et al. 2002). Many of these molecules are multifunctional. For example, the collectins surfactant protein A (SP-A) and surfactant protein D (SP-D) bind microbes and act as opsonins (Kuan et al. 1992; Tenner et al. 1989; van Iwaarden et al. 1990, 1991, 1994) and also help to modulate inflammation through their interactions with phagocytes and inflammatory-signaling molecules (Murakami et al. 2002; Sano et al. 1999; Sato et al. 2003). The abundant secreted protein PLUNC (palate, lung, nasal epithelium clone) possesses potent surfactant activity and is proposed to contribute to airway epithelial defenses through antimicrobial as well as anti-biofilm effects (Chu et al. 2007; Gakhar et al. 2010; Lukinskiene et al. 2011; McGillivary and Bakaletz 2010; Zhou et al. 2008). Airway secretions also contain proteins with anti-inflammatory functions, such as the protease inhibitors elafin and secretory leukocyte protease inhibitor (SLPI) (Butler et al. 2006; Henriksen et al. 2004; Sallenave 2010). Porter and coworkers demonstrated that airway epithelia secrete lipids that exert direct antimicrobial activity and synergize with host defense proteins (Do et al. 2008).

In addition to polypeptide- and lipid-based defenses, the secreted enzyme lactoperoxidase (LPO) contributes to an oxidative host defense system. In this system, LPO uses H_2O_2 produced by the dual oxidases DUOX1 and DUOX2 (members of the NOX gene family) to catalyze the oxidation of the secreted anion thiocyanate (SCN^-) in the ASL. This reaction generates the antibacterial product hypothiocyanate ($OSCN^-$), which is toxic to several relevant airway pathogens (Conner et al. 2002; Forteza et al. 2005; Gerson et al. 2000; Moskwa et al. 2007; Wijkstrom-Frei et al. 2003). In all cases, these defensive molecules may be either constitutively expressed or induced in response to various inflammatory stimuli. This redundancy in the number of host defense factors that act by multiple mechanisms is further augmented by their ability to act together in synergistic or additive manners (Singh et al. 2000b).

A third arm of lung host defense involves the resident phagocytic cells, including alveolar macrophages and neutrophils that may be recruited to sites of airway infection and inflammation. In addition to engulfing and destroying bacteria, both macrophages and neutrophils release a variety of potent antimicrobial factors to augment the epithelial responses to invading microorganisms. Macrophages and

Table 1 ASL host defense factors

Product	Relative concentration in ASL	Cellular source	Function(s)	References
Lysozyme	μg-mg/mL	Epithelia, neutrophils	Antibacterial	Fleming (1922), Fleming and Allison (1922), Hiatt et al. (1952)
Lactoferrin	μg/mL	Epithelia, neutrophils	Antibacterial, antiviral, antifungal, inhibition of microbial growth through iron sequestration	Arnold et al. (1980), Masson et al. (1965, 1966), Oram and Reiter (1968), Sano et al. (2003), Xu et al. (1999)
Surfactant protein A (SP-A)	ng-μg/mL	Epithelia	Microbial aggregation, opsonization, modulation of inflammation	Hartshorn et al. (1998), Korfhagen et al. (1996), LeVine et al. (1999a, b, 2000), Murakami et al. (2002), Sano et al. (1999), Sato et al. (2003), Tenner et al. (1989), van Iwaarden et al. (1990, 1991)
Surfactant protein D (SP-D)	ng-μg/mL	Epithelia	Microbial aggregation, opsonization, modulation of inflammation	Hartshorn et al. (1998, 1994), Kuan et al. (1992), LeVine et al. (2000), Yoshida et al. (2001)
Secretory leukocyte protease inhibitor (SLPI)	μg/mL	Epithelia, neutrophils, macrophages	Protease inhibition, anti-inflammatory, antimicrobial	Henriksen et al. (2004), Hiemstra et al. (1996), Mihaila and Tremblay (2001), Saitoh et al. (2001), Sallenave et al. (1994, 1997)
Peptidase inhibitor 3 (elafin)	ng/mL	Epithelia, neutrophils, macrophages	Protease inhibition, anti-inflammatory, antimicrobial, opsonization	Henriksen et al. (2004), King et al. (2003), Mihaila and Tremblay (2001), Sallenave et al. (1994), Simpson et al. (1999), Wilkinson et al. (2009)
Cystatin S	*Unknown*	Epithelia	Cysteine protease inhibitor	Isemura et al. (1984), Lindahl et al. (1999)
Lipocalin-1 (tear lipocalin)	*Unknown*	Epithelia	Cysteine protease inhibitor, iron sequestration through siderophore binding	Fluckinger et al. (2004), Lindahl et al. (1999), Redl et al. (1998), van't Hof et al. (1997)

(continued)

Table 1 (continued)

Product	Relative concentration in ASL	Cellular source	Function(s)	References
Neutrophil gelatinase-associated lipocalin (NGAL, or lipocalin-2)	ng/mL	Epithelia, neutrophils	Iron sequestration through siderophore binding	Cowland et al. (2003), Goetz et al. (2002), Kjeldsen et al. (1993), Triebel et al. (1992)
Bactericidal/permeability-increasing protein (BPI)	*Unknown*	Neutrophils	Antibacterial, anti-inflammatory, endotoxin neutralization, opsonization	Iovine et al. (1997), Marra et al. (1990), Weiss et al. (1978)
PLUNC (or SPLUNC1, LUNX, NASG, SPURT, BPIFA1)	μg/mL	Epithelia, neutrophils	Antimicrobial, biofilm inhibition	Bartlett et al. (2008b), Bingle and Bingle (2000), Chu et al. (2007), Gakhar et al. (2010), Lindahl et al. (2001), Lukinskiene et al. (2011), McGillivary and Bakaletz (2010), Zhou et al. (2008)
Phospholipase A2	μg-mg/mL	Epithelia, neutrophils	Antibacterial, proinflammatory	Elsbach et al. (1979), Rosenthal et al. (1995), Weinrauch et al. (1996), Zallen et al. (1998)
IgA secretory component	μg/mL	Epithelia	Prevents bacterial adhesion to mucosal surfaces	Fiedler et al. (1991), Phalipon et al. (2002)
Complement factor C3	*Unknown*	Epithelia	Complement signaling, antibacterial, antifungal	Candiano et al. (2007), Nordahl et al. (2004), Sonesson et al. (2007)
S100A8 (calgranulin A) and S100A9 (calgranulin B) (together known as calprotectin)	*Unknown*	Epithelia, neutrophils, macrophages, monocytes	Antibacterial, antifungal effects through sequestration of divalent cations, neutrophil chemotaxis	Corbin et al. (2008), Cornish et al. (1996), Murthy et al. (1993), Nisapakultorn et al. (2001a, b), Ryckman et al. (2003), Sohnle et al. (1991, 1996), Steinbakk et al. (1990), Vandal et al. (2003), Xu et al. (1999)
S100A7 (psoriasin)	*Unknown*	Epithelia, macrophages	Antibacterial	Andresen et al. (2011), Glaser et al. (2005), Lee and Eckert (2007)
S100A12 (calgranulin C)	*Unknown*	Neutrophils, monocytes	Antibacterial, antifilarial	Cole et al. (2001), Gottsch et al. (1999), Guignard et al. (1995)
Defensins (alpha and beta)	ng-mg/mL	Epithelia, neutrophils, macrophages	Antibacterial, antifungal, antiviral, chemotactic for T cells, monocytes, dendritic cells	Bals et al. (1998a), Chertov et al. (1996), Daher et al. (1986), Ganz et al. (1985), Garcia et al. (2001a, b), Goldman et al.

				(1997), Harder et al. (2001), Jia et al. (2001), Lehrer et al. (1988), McCray and Bentley (1997), Singh et al. (1998), Territo et al. (1989), Yang et al. (1999)
CCL20	ng/mL	Epithelia, neutrophils, macrophages	Chemokine, antibacterial, antifungal	Starner et al. (2003), Yang et al. (2003)
LL-37	*Unknown*	Epithelia, neutrophils	Antibacterial, endotoxin neutralization, wound repair, angiogenesis, chemotactic for mast cells, neutrophils, monocytes, T cells	Agerberth et al. (2000), Bals et al. (1998b), Cowland et al. (1995), Heilborn et al. (2003), Koczulla et al. (2003), Larrick et al. (1995), Niyonsaba et al. (2002), Sorensen et al. (1997), Tjabringa et al. (2006), Turner et al. (1998), Yang et al. (2000)
Cholesteryl esters (cholesteryl linoleate, cholesteryl arachidonate)	μg/mL	Epithelia	Antibacterial	Do et al. (2008)
Lactoperoxidase (LPO)	μg/mL	Submucosal gland epithelia	Catalyzes oxidation of SCN^- to form $OSCN^-$	Conner et al. (2002), Gerson et al. (2000), Lorentzen et al. (2011), Moskwa et al. (2007), Wijkstrom-Frei et al. (2003)
H_2O_2 (via DUOX)	0.1 μM	Epithelia	Oxidizes SCN^- to form antibacterial $OSCN^-$	Conner et al. (2002), Forteza et al. (2005), Gerson et al. (2000), Lorentzen et al. (2011), Moskwa et al. (2007), Wijkstrom-Frei et al. (2003)
SCN^-	μM	Epithelia	Antibacterial in oxidized form ($OSCN^-$)	Conner et al. (2002), Gerson et al. (2000) Lorentzen et al. (2011), Moskwa et al. (2007), Wijkstrom-Frei et al. (2003)

neutrophils store and secrete many of the same innate immune factors released by airway epithelia, such as alpha- and beta-defensins (Ganz et al. 1985; Garcia et al. 2001a), LL-37 (Cowland et al. 1995), lysozyme (Hiatt et al. 1952), lactoferrin (Masson et al. 1969), NGAL (Kjeldsen et al. 1993), and PLUNC (Bartlett et al. 2008b), as well as phagocyte-specific products such as the anti-inflammatory bactericidal/permeability-increasing protein (BPI) (Weiss et al. 1978). Macrophages and neutrophils actively communicate with airway epithelia by releasing and responding to cytokines and other molecules involved in amplification and/or regulation of inflammatory responses.

In airway epithelia, as well as in macrophages and neutrophils, many innate immune responses are mediated by Toll-like receptors (TLRs), a family of pattern recognition receptors that evolved to recognize a variety of pathogen-associated molecular patterns (PAMPs). Engagement of a TLR typically initiates a signaling cascade that results in an immune response tailored to the invading organism. TLRs respond by activating the transcription of numerous cytokine genes, including TNF, IL-6, and IL-12 as well as a variety of chemokine genes (Akira and Takeda 2004). TLRs also induce proforms of IL-1β and IL-18, which are then processed and secreted by NOD-like receptor-mediated caspase-1 activity. Additional intracellular receptors also coordinate epithelial innate immune responses to pathogens, including the NOD-like receptors, RIG-I, and MDA5 (Andrejeva et al. 2004; Yoneyama et al. 2004). Reactive oxygen species (ROS) are among the most evolutionarily conserved pathway of responses to infection or injury and are triggered by all danger-associated molecular patterns (DAMPs) and PAMPs. A ROS-dependent pathway triggers the inflammasome complex formation in myeloid cells like macrophages (Cruz et al. 2007).

4 How Does Loss of CFTR Function Alter Airway Host Defenses?

It has been challenging to explain the complex pathogenesis of CF lung disease. While it is well established that CF is caused by absent or reduced CFTR anion channel activity, it has been difficult to draw a direct line from this molecular defect to the varied CF disease manifestations. In the case of CF lung disease, the critical question is: How does the loss of anion channel activity cause defective innate immunity in the airways? This question has given rise to a number of hypotheses, which touch on multiple components of mucosal innate immunity. We stress that these hypotheses are not necessarily mutually exclusive and that it is possible, even likely, that the primary pathogenesis of CF lung disease is multifactorial.

4.1 Altered Na^+ and Cl^- Transport in CF

CFTR is a Cl^- channel that also conducts HCO_3^-, SCN^-, I^-, and other anions (Anderson et al. 1991a, b). For this reason, considerable effort has focused on identifying links between altered electrolyte transport by airway epithelia and the functions of the innate immune system. As epithelia use Cl^- secretion and Na^+ absorption to coordinately regulate the volume of secretions, it is important to understand both Cl^- and Na^+ transport and osmotically coupled liquid movement across the airway epithelium in CF patients. Several groups have investigated how CF may alter the volume or composition of respiratory secretions.

One hypothesis posits that CFTR activity is essential for the regulation of ASL volume and mucus hydration and that absence of CFTR causes dehydration of the ASL and reduced mucociliary clearance (Boucher 2004, 2007). This model rests on the concept that the depth of the ASL periciliary layer is tightly regulated to ensure a height of ~7 μm (Matsui et al. 1998). This ~7 μm depth, the approximate length of a cilium, is thought to be optimal for efficient ciliary beating and movement of the mucus layer. Early studies assessing the nasal and airway transepithelial voltage (Vt) in CF subjects indicated that the CF epithelium had a lower (lumen-negative) Vt and also exhibited a greater reduction in nasal Vt after application of amiloride, an inhibitor of the epithelial Na^+ channel (ENaC), relative to non-CF epithelia (Knowles et al. 1981, 1983a, b). Based on this observation, it was suggested that Na^+ hyperabsorption and concomitant liquid absorption reduces ASL volume in the CF airways. This depletion of ASL would then lead to dehydration of the mucus layer, resulting in thick, sticky, adherent mucus that is difficult to move, ultimately impairing mucociliary clearance in the CF airways. In support of this hypothesis, evidence for altered ASL volume homeostasis, thickened mucus, and reduced mucociliary transport has been reported using cultured airway epithelia from human CF and non-CF patients (Matsui et al. 1998). In a study of bronchial tissue biopsies from human CF and non-CF subjects, CF samples showed a trend toward reduced ASL depth, although this did not reach statistical significance (Griesenbach et al. 2011). In the same study, a comparison of the periciliary liquid height of the nasal epithelium in wild-type and CFTR-null mice revealed a significant decrease in samples from the CFTR-null animals (Griesenbach et al. 2011).

Data from recent experiments in cultured CF and non-CF epithelia provide an alternative interpretation for the increased nasal Vt observed in people with CF. Itani and coworkers studied cultured primary well-differentiated human airway epithelia and failed to find evidence for increased Na^+ conductance in CF epithelia (Itani et al. 2011). Under basal or cAMP-stimulated conditions, the transepithelial conductance of CF epithelia was reduced compared with non-CF epithelia, and the reduction could be accounted for solely by the loss of the CFTR channel conductance. The addition of amiloride resulted in greater decreases in Vt and short-circuit current in CF epithelia than non-CF epithelia. However, amiloride caused similar reductions in conductance and Na^+ absorption in cells from both genotypes, indicating that the effects of amiloride were due to a loss of Cl^- conductance. These results suggest that loss of anion conductance is the critical defect in electrolyte transport in CF epithelia.

Inhibition of Na^+ transport in the respiratory tract as a therapy has been investigated in CF subjects given the ENaC inhibitor amiloride by inhalation. A multicenter randomized double-blind placebo-controlled clinical trial investigated the efficacy of thrice-daily inhaled amiloride on pulmonary function of CF patients over a 6-month period. The study failed to demonstrate significant benefit (Pons et al. 2000).

A second hypothesis proposes that changes in ASL ionic strength might adversely affect the activity of innate immune effector proteins. In this model, loss of CFTR-dependent Cl^- transport results in increased ASL NaCl concentrations due to an inability of the surface epithelium to modify ASL composition by absorption of Cl^- and Na^+. A predicted outcome of this scenario is a reduction in the activity of salt-sensitive antimicrobial factors. This hypothesis emphasizes the central importance of antimicrobials, particularly cationic peptides and proteins, in protecting the airways from microbes (Cole et al. 1999, 2002). It is known that the activity levels of numerous antimicrobial factors, including lysozyme, lactoferrin, alpha- and beta-defensins, and others, are diminished in solutions of increased ionic strength (Goldman et al. 1997; Porter et al. 1997; Singh et al. 1998, 2000b; Travis et al. 1999; Valore et al. 1998). In experiments using cultured well-differentiated human primary airway epithelia derived from CF and non-CF donors, Smith and colleagues found that CF epithelia exhibited a killing defect when a small bacterial inoculum was applied directly to the apical surface (Smith et al. 1996). However, when in vitro killing assays were performed using ASL that had been removed from the apical surface of the cultures using water as a diluent, there was no significant difference in killing between CF and non-CF-derived samples. This result implied that it was unlikely that CF ASL lacked a critical bactericidal factor, and suggested instead that the activity of a bactericidal factor or factor(s) was inhibited by some aspect of the ASL environment in the CF epithelia. To test the hypothesis that the NaCl concentration influences antimicrobial activity in CF ASL, the authors replaced the ASL of both CF and non-CF cultures with solutions containing either high (182 mM) or low (92 mM) Cl^- concentrations and repeated the bacterial killing assays. Bacterial killing was significantly reduced in non-CF cells that received the high salt solution, while killing activity was restored in CF cells under the low salt conditions (Smith et al. 1996).

While this hypothesis remains a subject of debate, the idea that loss of CFTR function alters the ASL milieu in a manner that impairs the activity of host defense factors remains attractive. A confounding factor has been the difficulty of accurately measuring ASL salt concentrations in vivo. Some investigators reported that Cl^- concentrations are indeed elevated in CF (Gilljam et al. 1989; Joris et al. 1993; Kozlova et al. 2006a, b; Vanthanouvong et al. 2006; Zabner et al. 1998), while others found no significant difference in NaCl concentrations between CF and non-CF (Caldwell et al. 2002; Grubb et al. 2002; Jayaraman et al. 2001a, b; Knowles et al. 1997). If a difference in the concentration of ASL NaCl is not validated in CF, the experimental findings of the study of Smith and colleagues (1996) continue to point to a compositional change in CF ASL that negatively impacts the function of host defense factors.

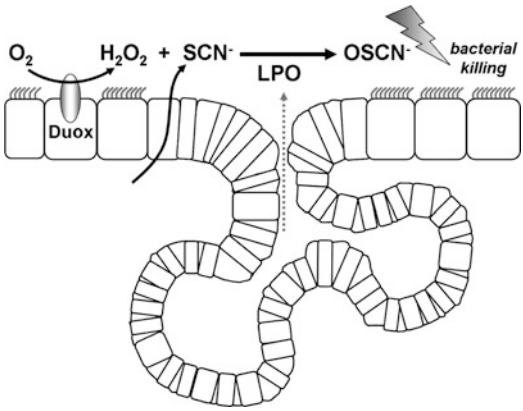

Fig. 2 Model of the oxidative host defense system at the apical side of airway epithelia. DUOX enzymes are the H_2O_2-generating cytochromes in the apical membrane of airway epithelia. LPO is secreted by submucosal glands. SCN^- is utilized by LPO for $OSCN^-$ generation

4.2 Altered SCN^- Transport in CF

This hypothesis builds from the observation that CFTR conducts thiocyanate (SCN^-) (Tabcharani et al. 1993). While many secreted host defense proteins and peptides are well characterized, the recognition of an airway epithelial oxidative microbicidal system is recent. This oxidative system consists of two H_2O_2-generating enzymes of airway epithelia, dual oxidases (DUOX)1 and 2, along with a pseudohalide anion (thiocyanate, SCN^-), and the enzyme lactoperoxidase (LPO) (Conner et al. 2002; Forteza et al. 2005; Gerson et al. 2000; Moskwa et al. 2007; Wijkstrom-Frei et al. 2003). The DUOX enzymes generate H_2O_2 into the apical extracellular space where H_2O_2 reacts with SCN^- in a LPO-catalyzed reaction to form the antibacterial molecule $OSCN^-$ ($H_2O_2 + SCN^- \rightarrow OSCN^-$) (Fig. 2). Both LPO and SCN^- are highly concentrated in the airway surface liquid. SCN^- is secreted apically by airway epithelia and accumulates in the ASL in concentrations of approximately 400–460 μM (Lorentzen et al. 2011; Wijkstrom-Frei et al. 2003). The DUOX/LPO/SCN^- system can generate sufficient $OSCN^-$ to eliminate bacteria in vitro and in vivo (Conner et al. 2007; Moskwa et al. 2007). SCN^- secretion is reduced in CF cells and tissues (Conner et al. 2007; Moskwa et al. 2007), leading to the hypothesis that diminished SCN^- (and therefore reduced $OSCN^-$) availability impairs defenses against airway pathogens. In support of this, Moskwa and coworkers reported that $OSCN^-$-mediated killing of *Staphylococcus aureus* was inhibited on the apical surface of cultured CF airway epithelia (Moskwa et al. 2007). The relative contribution of this system to normal airway defenses in vivo, and how relevant its inactivation may be to CF pathogenesis, is currently unresolved. In a recent study, Lorentzen and colleagues found that the concentrations of SCN^- were variable, and not significantly different, in the nasal secretions of humans with and without CF, suggesting that the bacterial killing activity of $OSCN^-$ was unlikely to be different between genotypes (Lorentzen et al. 2011). However, lung function correlated positively with SCN^- levels in the CF

subjects in this study. These findings suggest that while oxidative defenses are important for overall lung health, CFTR activity may not be the only determinant of ASL SCN^- levels; the authors cite SCN^- transport by alternative ion channels as potential compensating mechanisms in the CF airways (Lorentzen et al. 2011). One candidate for alternative SCN^- transport, the sodium-independent chloride/iodide transporter pendrin (also known as SLC26A4), is expressed by human airway epithelia and is upregulated in response to IL-4 (Pedemonte et al. 2007) and to viral infections (Nakagami et al. 2008). Therefore, it is possible that, in some individuals, increased pendrin expression might partially compensate for loss of CFTR activity in inflamed CF airways.

4.3 Altered HCO_3^- Transport in CF

It has been long recognized that loss of CFTR function results in the acidification of pancreatic secretions and that CF pancreatic secretions fail to properly alkalinize in response to secretagogues (Gaskin et al. 1982; Kopelman et al. 1988). CFTR expressed within the airways also transports bicarbonate (HCO_3^-) and thereby helps buffer ASL (Fischer and Widdicombe 2006; Smith and Welsh 1992). In the airways, loss of HCO_3^- secretion via CFTR is predicted to result in a diminished capacity to alkalinize respiratory secretions. A number of studies have assessed ASL pH using in vitro systems and in vivo models. There is evidence that secretions from submucosal glands derived from human CF nasal tissues are hyperacidified relative to non-CF secretions (Song et al. 2006) and that cultured CF human bronchial epithelia acidify their ASL more rapidly than do non-CF cells (Coakley et al. 2003). In studies comparing CFTR-null and wild-type mice, Jayaraman and coworkers noted no significant differences in the in vivo airway pH as measured using a tracheal window preparation (Jayaraman et al. 2001b). Reductions in ASL pH could negatively influence innate immunity by several mechanisms [reviewed in (Coakley and Boucher 2001; Poschet et al. 2002)] including inhibiting the activity of antimicrobials (Dorschner et al. 2006; Lehrer et al. 1983; Selsted et al. 1985), altering the viscosity of secretions (Bhaskar et al. 1991; Holma 1985), and decreasing ciliary beat frequency (Clary-Meinesz et al. 1998).

4.4 CF-Associated Changes in Airway Submucosal Gland Function and Secreted Mucins

Alterations in airway submucosal gland physiology are also implicated in the innate immune defects in CF. Submucosal glands are responsible for the secretion of liquid and mucins, such as the gel-forming mucin MUC5B (Groneberg et al. 2002), as well as numerous proteins and peptides with antimicrobial, anti-inflammatory,

and other host defense functions (Wine and Joo 2004). A submucosal gland is formed by invagination of the airway epithelial surface to form a single collecting duct, into which multiple mucous tubules empty (Wine and Joo 2004). The more proximal portions of these tubules are lined by mucous cells, responsible for mucin production, while the distal acini of the tubules contain serous cells that secrete liquid, electrolytes, and various protein and peptide components of ASL. CFTR is expressed primarily in the serous acini (Engelhardt et al. 1992), where its anion channel activity is thought to play a key role in liquid secretion. As major sites of mucus production, the submucosal glands contribute to the quantity and quality of mucus in the airways and are studied for their possible involvement in generating the abnormal mucus that is a hallmark of CF lung disease.

Several investigators hypothesize that liquid secretion by serous cells is reduced in CFTR-deficient submucosal glands, leading to altered hydration of secreted mucins and the ASL. The resulting mucus is predicted to be unusually viscous and would impede the efficient mechanical clearance of particles from the airways. Such changes in mucus rheologic properties could also lead to obstruction of submucosal gland ducts. In support of this hypothesis, there is abundant evidence that liquid secretion in response to secretory signals is reduced when CFTR function is impaired. A number of studies confirmed that CF glands fail to secrete in response to forskolin and vasoactive intestinal peptide (VIP), agonists that stimulate secretion by elevating intracellular cAMP levels (Choi et al. 2009; Joo et al. 2002, 2006, 2010; Lee and Foskett 2010). Similarly, secretion in CF glands is impaired in response to the neuropeptide substance P (Choi et al. 2009; Joo et al. 2010) and to carbachol when administered in combination with other agonists (Choi et al. 2007). In keeping with these data, studies of secretions collected from individual submucosal glands suggest that viscosity is significantly elevated in secretions from CF individuals (Jayaraman et al. 2001a; Salinas et al. 2005).

Defective HCO_3^- secretion is also hypothesized to impact mucin release and viscosity (Quinton 2008). One model posits that, early in the process of mucus gel formation and extrusion from the submucosal glands, polyanionic mucin molecules are packed together into condensed mucin granules. To maintain this structure, the mucin molecules are highly cross-linked and their charges neutralized by a "shield" of Ca^{2+} ions. HCO_3^-, which effectively chelates Ca^{2+}, can sequester these Ca^{2+} ions, thereby releasing the cross-links between mucins and allowing the mucins to rapidly expand as they are released from submucosal glands and/or goblet cells. In this way, HCO_3^- may be intimately involved in the swelling and hydration of the ASL mucus layer. This model is supported by the demonstration that adding HCO_3^- to mucus gels increases mucus dispersal in vitro (Chen et al. 2010a). Therefore, in CF glands and tissues, the absence of CFTR-mediated HCO_3^- secretion may result in mucus granules that de-condense inappropriately, giving rise to viscous, under-hydrated mucus that impairs mucociliary clearance (Chen et al. 2010a).

In addition to the impact of impaired secretory responses of CF glands on the hydration status of mucins, it is suggested that this phenomenon could affect the abundance of secreted antimicrobials and other host defense factors from gland serous cells. While there is currently little direct evidence for this, Wine and

colleagues reported that a number of known innate immune molecules, including lysozyme, NGAL (lipocalin-2), HSC-71, and the protease inhibitors alpha 1-antitrypsin and alpha 1-antichymotrypsin, are secreted by Calu-3 cells (a serous cell model) in response to forskolin stimulation (Joo et al. 2004). This observation leads to the prediction that secretion of antimicrobials may be reduced in the absence of CFTR. Additionally, when this observation is considered with the above-mentioned defects in liquid secretion and mucus hydration, it is tempting to speculate that in CF glands, secreted innate immune molecules may become "trapped" within viscous polyanionic mucus and are therefore less likely to be properly presented to the airway lumen.

Increased mucin production by the surface epithelium may also contribute to impaired mucociliary clearance in CF. Airway surface goblet cells secrete the gel-forming mucin MUC5AC and, to a lesser extent, MUC5B (Groneberg et al. 2002; Hovenberg et al. 1996). Goblet cell metaplasia is a response to chronic inflammation and is commonly seen in CF lung disease (Bedrossian et al. 1976; Davis and Dickey 2008; Groneberg et al. 2002). Therefore, the increased viscous secretions associated with CF are derived in part from the surface epithelium. In keeping with this, Derichs and colleagues reported that the viscosity was increased in both the mucus and the periciliary liquid layers of the ASL from cultured CF bronchial epithelia, which do not possess submucosal glands (Derichs et al. 2011). This raises the possibility that increased mucin production from goblet cells may also impact innate immunity by providing more binding sites for cationic antimicrobials and other molecules that normally associate with the polyanionic mucin polymers in the airways (Felgentreff et al. 2006), making those antimicrobial factors less available to interact with their target pathogens.

5 New Animal Models of CF Lung Disease

A limitation in advancing knowledge of the molecular basis of CF lung disease has been the lack of animal models that recapitulate key features of lung and other organ disease pathology. The mouse models with CFTR-null alleles and human CFTR mutations available since the early 1990s have contributed greatly to disease understanding but do not develop spontaneous lung disease similar to humans with CF. Recently, several groups used somatic cell targeting of the *CFTR* gene, followed by nuclear transfer and cloning to develop novel models in pigs (Klymiuk et al. 2011; Rogers et al. 2008a, b) and ferrets (Sun et al. 2008, 2010). These new animal models recapitulate key features of CF disease (Klymiuk et al. 2011; Rogers et al. 2008b; Sun et al. 2010). At birth, the airways of *CFTR* targeted pigs are free of inflammation but manifest a bacterial host defense defect without the secondary consequences of infection (Rogers et al. 2008b; Stoltz et al. 2010). Pigs with targeted *CFTR* genes spontaneously develop hallmark features of CF including airway inflammation, remodeling, mucus, and infection within months of birth (Fig. 3). Their lungs contain multiple bacterial species (Gram-negative and

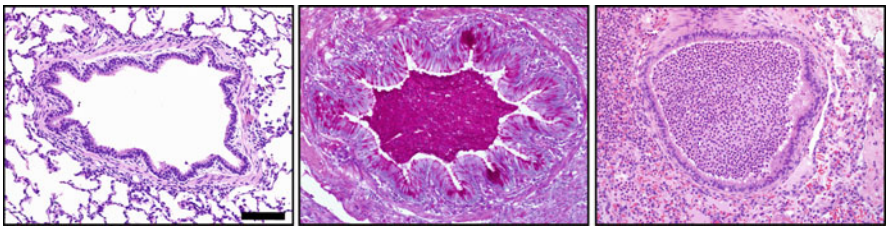

Fig. 3 Airway disease in a pig model of cystic fibrosis. Airways from a $CFTR^{+/+}$ (*left panel*, H&E stain) and a $CFTR^{-/-}$ pig (*middle* and *right*, PAS and H&E, respectively), bar = 90 μm. $CFTR^{-/-}$ pig is infected with the Gram-negative organism *Bordetella bronchiseptica*. Note the increased mucosal thickening and mucin expression in the epithelium with mucocellular lumen obstruction (*middle panel*). A CF pig airway is obstructed with neutrophils and mucopurulent debris (*right panel*)

Gram-positive), suggesting an equal opportunity host defense defect. While the lungs of newborn pigs show no inflammation, they are less often sterile than wild-type littermate controls. Moreover, after intrapulmonary bacterial challenge with *Staphylococcus aureus*, CF pigs fail to eradicate bacteria as effectively as wild-type pigs (Stoltz et al. 2010). These results suggest that impaired bacterial elimination is the pathogenic event initiating a cascade of inflammation and pathology.

Studies of CF pig submucosal glands have shown that the model exhibits responses to agonists qualitatively similar to glands from human patients with CF (Joo et al. 2010). CF pig glands produce almost no liquid in response to cAMP agonists and reduced volumes in response to all other stimuli except carbachol. Furthermore, glands from newborn CF pigs, like human CF glands, exhibit a reduced secretory response to substance P (Joo et al. 2010). Thus, CF glands have a reduced ability to respond to important secretagogues. These findings, combined with the hypoplastic submucosal glands of the newborn CF airways (Meyerholz et al. 2010), raise the possibility that the gland contributions to ASL are altered in their volume and composition in CF.

Recent studies in newborn CF pigs provide further support for the idea that an altered ASL environment is a primary cause of impaired innate immunity in the lung. In vitro and in vivo experiments showed that ASL pH was lower in CF pigs, and this reduction in pH reduced the antimicrobial activity of ASL (Pezzulo et al. 2012). Interestingly, a more acidic ASL pH also diminished bacterial killing in wild-type pigs, while increasing ASL pH increased antimicrobial activity in the ASL of pigs with CF. These results in newborn animals support the concept that reductions in ASL pH due to loss of CFTR-dependent HCO_3^- secretion impair the activity of endogenous antimicrobial proteins and peptides, allowing for the onset of airway infection.

Experiments in the CF pig model also yielded some unexpected findings. *CFTR* targeted pigs exhibit morphological abnormalities of the newborn trachea and large airways, including tracheal cartilage alterations, a decrease in submucosal gland mass, and more noticeable smooth muscle bundles in the posterior trachea

(Meyerholz et al. 2010). Interestingly, retrospective analysis of chest CT scans from children with CF revealed that the tracheas of CF subjects were less circular than the non-CF control individuals (Meyerholz et al. 2010), echoing the tracheal abnormalities observed in the CF piglets. These results are contrary to the dogma that the "lungs of children with CF are normal at birth," and raise the possibility that loss of CFTR function perturbs a developmental program in the lung resulting in structural abnormalities. Such changes could also contribute to the lung disease phenotype.

In a series of experiments using cultured airway epithelia and freshly excised nasal and tracheal tissues from $CFTR^{-/-}$ pigs, as well as in vivo electrophysiological measurements, Chen et al. reported that airway epithelia from newborn CF pigs displayed a reduced Cl^- conductance without an increase in Na^+ conductance (Chen et al. 2010b). The authors implicate the loss of the CFTR-dependent Cl^- conductance, rather than increased Na^+ conductance, as the explanation for the greater reduction in Vt in response to amiloride observed in CF epithelia relative to non-CF epithelia. Measurements of the periciliary liquid depth in fixed tracheal tissue from pigs indicated no significant difference in ASL depth between the CF and non-CF airways at birth. These data suggest that ASL/mucus dehydration due to Na^+ hyperabsorption in CF airways may not be a primary event in CF pathogenesis, although they do not rule out the possibility that perturbations in Na^+ transport may affect airway immunity as the disease progresses.

CFTR-null ferrets also develop multiorgan system disease, and neonatal animals manifest a pulmonary host defense defect in the airways associated with colonization by bacteria (Sun et al. 2010). Early results also indicate that adult CF ferrets develop a lung disease phenotype with similarities to human CF, including bacterial colonization (John Engelhardt, personal communication). Continued studies of animal models that reproduce key phenotypic features of human CF lung disease are likely to provide further insights into the links between loss of CFTR function and the initial pathologic events.

6 Future Directions

With recent advances in CF basic science research and the development of new animal models, the field is poised to make further breakthroughs in the understanding of disease pathogenesis, including a better grasp of the molecular basis of the airway host defense defect. Advancements in these areas are critical to the development of new pulmonary specific or systemic therapies to prevent the onset or slow the rate of disease progression.

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Antimicrobial Peptides in Chronic Obstructive Pulmonary Disease

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Abstract Chronic obstructive pulmonary disease (COPD) is a frequent, chronic lung disease associated with significant morbidity and mortality. Respiratory infections play a central role in the disease, not only during exacerbations but also in the stable phase of the disease. These infections contribute to the development and progression of the disease, and many patients are colonized by respiratory pathogens. The pathogens are present in the lung, despite the presence of large numbers of neutrophils, especially during acute states of inflammation. These neutrophils may release antimicrobial peptides (AMPs) that may not only serve to kill these pathogens but also contribute to tissue injury and inflammation. In addition, smoke affects many elements of the host immune system, including the expression of epithelial AMPs. Furthermore, the activity of AMPs may be decreased in the purulent airway secretions often present in COPD patients. Possibly vitamin D treatment may contribute to restoring local AMP deficiency and thereby to reducing exacerbations in COPD.

1 Introduction

Chronic obstructive pulmonary disease (COPD) is a frequent respiratory tract disorder characterized by persistent airflow limitation (Rabe et al. 2007). It is mainly caused by extensive exposure and an abnormal response toward harmful environmental substances and gases, most prominently, the exposure to cigarette smoke. Only ~20 % of the smoking population develops the disease, indicating the additional importance of genetics and other environmental predispositions.

The progressive decrease in lung function in COPD is caused by airflow obstruction resulting from a variety of structural changes in the lung, including

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destruction of the alveoli and alveolar attachments (emphysema), mucus hypersecretion, and subsequent airway plugging especially in small airways, and changes in the airway wall. These structural changes are accompanied by a chronic inflammatory process in which a variety of cell types play a role. Small airway pathology is characterized by increased accumulation of neutrophils, macrophages, and T-cells during disease progression and the occurrence of B-cell lymphoid follicles in severe disease stages (Hogg et al. 2004; Grashoff et al. 1997). Neutrophils are regarded as one of the driving forces of emphysema, causing excessive tissue damage by an imbalance between neutrophil-derived proteases and protease inhibitors. This imbalance is also observed in genetically predisposed patients with alpha-1 antitrypsin deficiency development (Ekeowa et al. 2011), a condition associated with liver disease and early onset emphysema.

2 Respiratory Infections in COPD

Chronic inflammation in stable COPD is frequently accompanied by bacterial and/or viral infections (Sethi 2010; Sethi et al. 2009). These infections contribute to persistence of airway inflammation and are thought to contribute to the etiology, pathogenesis, and clinical course of COPD. During acute exacerbations of COPD, a sudden decrease in airflow and an accompanying increase in airway inflammation is associated with the acquisition of new bacterial strains, most notable non-typeable *Haemophilus influenzae* (NTHi), *Moraxella catarrhalis*, and *Streptococcus pneumonia* (Sethi and Murphy 2001). Interactions between bacterial and viral pathogens may contribute to the intensity of exacerbations. This is illustrated by a study from Wilkinson et al., who showed that simultaneous detection of bacterial (mostly *H. influenzae*) and viral (mostly rhinovirus) pathogens during an exacerbation is associated with an increased bacterial load, inflammation, and symptoms and with decreased lung function (Wilkinson et al. 2006). It is now clear that infections not only contribute to increased inflammation during exacerbations but also in the stable phase of the disease. This is illustrated by a study by Bresser et al., showing that NTHi colonization may contribute to airway inflammation and airflow obstruction in COPD (Bresser et al. 2000).

Various mechanisms may explain the observed bacterial persistence in COPD, including antigenic variation, acquisition of new strains, tissue invasion, and biofilm formation. The observation that COPD patients are frequently unable to eradicate bacteria from their airways despite the presence of antibacterial antibodies and the abundant presence of neutrophils suggests that tissue invasion and biofilm formation are important processes. Adherence to airway epithelial cells is an important process in bacterial persistence. NTHi may penetrate between epithelial cells (van Schilfgaarde et al. 1999) and even into epithelial cells (Bandi et al. 2001), which may protect them from host defense systems in the lung as well as from antibiotics. This protection from host immunity and antibiotics is also achieved by formation of biofilms, in which bacterial adhesion is the first essential

step (Costerton et al. 1999). Biofilms are microbial communities that adhere to a surface and in which the microorganisms are embedded in a self-produced matrix. Importantly, microorganisms present in biofilms are not always readily detected by conventional culture techniques. Therefore, molecular techniques including microbiome sequencing are important to gain insight into the lung microbiome in COPD. Several recent studies have used such techniques and provided important insight in the microbial population composition in COPD. Hilty et al. demonstrated in bronchial brushings and bronchoalveolar lavage (BAL) specimens of COPD patients an increase in Proteobacteria, mainly *Haemophilus*, *Moraxella*, and *Neisseria* species, and a decrease in the phylum Bacteroidetes compared to healthy controls (Hilty et al. 2010). Erb-Downward et al. furthermore showed in severe COPD that the diversity of the microbiome is reduced (Erb-Downward et al. 2011). These initial findings show some differences with other studies which may have been caused by differences in study design, sampling techniques, and small sample sizes (Sze et al. 2012). Nevertheless, the overall conclusions are the same, showing that the lung microbiome in COPD differs from that in healthy subjects and smokers with normal lung function. The COPD patient lung microbiome also differs from the microbiome in patients with cystic fibrosis. Further microbiome studies in COPD are needed to explore a wide range of topics, including changes in the composition of the microbiome over time, its relation to disease severity and response to therapy, the influence of antibiotic therapy on the microbiome compositions, its role in development and progression of the disease, as well as regional heterogeneity.

3 Antimicrobial Peptides in COPD

The prominent role of respiratory infections despite intense inflammation in the COPD lung suggests that the pulmonary immune system does not function optimally in COPD. Antimicrobial peptides (AMPs) play a central role in host defense against infection in the lung (Bals and Hiemstra 2004), which is also supported by, e.g., in vivo mouse studies showing that gene deletion or overexpression of AMP genes affects pulmonary host defense against *Haemophilus influenzae* (Moser et al. 2002) and *Pseudomonas aeruginosa* (Bals et al. 1999). Based on this role of AMPs, several studies have investigated expression and activity of AMPs in COPD in search for an explanation for the increased susceptibility to infection and increased inflammation in COPD.

3.1 Neutrophil-Derived Antimicrobial Peptides

The excessive number of neutrophils in the lung consequently leads to high detectable quantities of neutrophil-derived AMPs. α-Defensins (human neutrophil peptides; HNP) are abundantly present in sputum samples of COPD patients and

found elevated in more severe disease stages compared to mild-to-moderate COPD (Paone et al. 2011). Analysis of sputum and bronchoalveolar lavage fluid (BAL) from COPD patients using a proteomic approach specifically demonstrated an increase of HNP1 and HNP2 levels, while HNP3 levels were similar to those of healthy controls (Terracciano et al. 2011; Merkel et al. 2005). In addition, also in alpha-1 antitrypsin-deficient patients, higher levels of HNPs were observed (Spencer et al. 2004; Wencker and Brantly 2005). Similar to HNPs, concentrations of the cathelicidin antimicrobial peptide LL-37 are elevated in induced sputum samples of mild-to-very severe COPD patients (Xiao et al. 2005; Golec et al. 2009). Compared to nonsmokers, LL-37 was already increased to some extent in smokers with a normal lung function, an observation not noticed for HNPs (Merkel et al. 2005). This suggests that cigarette smoking plays an important role in increasing LL-37 levels in the airway. Moreover, a study examining the relation of LL-37 with bacterial colonization during acute exacerbations revealed that increased levels of LL-37 in sputum samples correlated with the acquisition of NTHi and *M. catarrhalis* (Parameswaran et al. 2011).

The usually protective role of neutrophils in host defense against pathogens may be dysfunctional in COPD. High levels of neutrophil-derived HNPs and LL-37 are suggested to contribute to the chronic inflammatory state (Fig. 1) (Bals and Hiemstra 2006), and HNPs were found to increase bacterial adherence to epithelial cells (Gorter et al. 1998). The combined release of AMPs with reactive oxygen species and other neutrophil granule–derived proteins, such as cathepsin G, elastase, and S100 proteins, causes extensive tissue damage that contributes to airway remodeling and the maintenance of inflammation (Quint and Wedzicha 2007). The release of neutrophil extracellular traps (NETs), consisting of complexes of DNA including high levels of LL-37 and HNPs, has been shown to contribute to autoimmune diseases via the development of autoantibodies (Lande et al. 2011). However, although various studies have provided evidence for an autoimmune response in COPD [e.g., Nunez et al. (2011)], it is unclear whether autoimmunity contributes to COPD development and progression or may be a response to tissue injury that in itself is not pathogenic.

In vitro experimental data support the increased cytotoxic activity and immunomodulatory properties of HNPs and LL-37 at high concentrations. Exposure of airway epithelial cells to HNPs may result in cell death, whereas lower concentrations increase the secretion of pro-inflammatory cytokines, including the neutrophil chemoattractant IL-8 (Van Wetering et al. 1997a, b, 2002). Furthermore, HNPs also increase the transcription and secretion of the mucin MUC5AC by an airway epithelial cell line (Aarbiou et al. 2004b; Ishimoto et al. 2009). This finding indicates a potential role in promoting mucus hypersecretion. A further role in decreased mucociliary clearance was suggested by the observation of an association between increased HNP levels and squamous metaplastic epithelium (Aarbiou et al. 2004a). This finding combined with the mitogenic activity of neutrophil defensins toward airway epithelial cells (Aarbiou et al. 2002, 2004b) suggests a possible role in airway epithelial remodeling. HNPs can also contribute to the increased bacterial colonization in acute exacerbations as it has been demonstrated

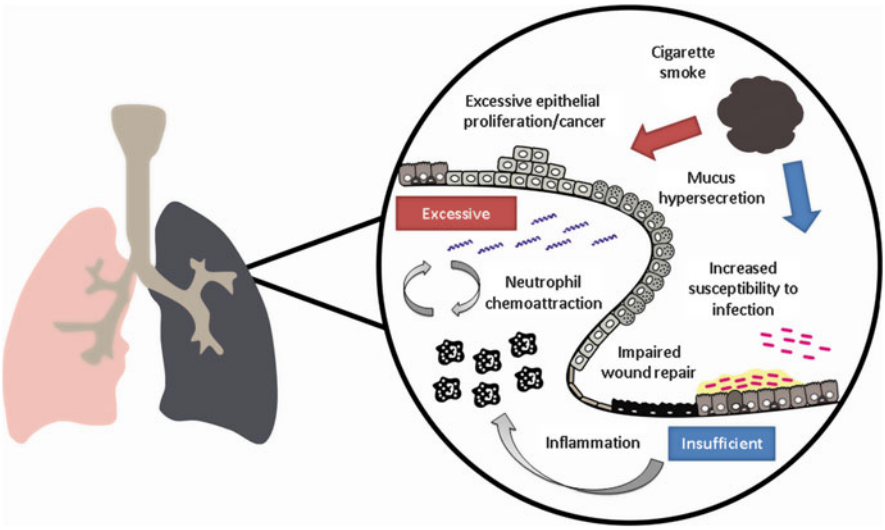

Fig. 1 Dysregulation of antimicrobial peptide expression and activity by cigarette smoke. Cigarette smoke-induced chemoattraction of neutrophils causes excessive levels of neutrophil-derived AMPs in the lung, which may contribute to airway epithelial remodeling (including proliferation and mucus hypersecretion) and possibly carcinoma development, and further enhancement of neutrophilic inflammation. This mechanism may be increased by posttranslational modification of AMPs such as HNPs and LL-37, which may be increased in smokers and alter the activity of these peptides. Cigarette smoke furthermore not only impairs expression of AMPs by airway epithelial cells exposed to microbial stimuli but also decreases ciliary activity and increases mucus hypersecretion. These mechanisms contribute to an increased susceptibility to respiratory tract infections and may impair proper wound healing. Moreover, these respiratory infections lead to a further increase in airway inflammation

that the peptides increase the adhesion of NTHi, *M. catarrhalis*, and *S. pneumonia* toward the airway epithelial surface (Gorter et al. 1998, 2000). Adhesion may be the first step in biofilm formation, or facilitate intra- or intercellular localization, mechanisms that may provide protection against the host immune system.

Similar to HNPs, LL-37 can contribute to the increased neutrophilic infiltration in the lung by inducing the expression of IL-8 by airway epithelial cells and airway smooth muscle cells (Tjabringa et al. 2003; Zuyderduyn et al. 2006) or via a direct chemotactic activity (Yang et al. 2000). Furthermore, it has been shown that LL-37 can drive macrophage differentiation toward a pro-inflammatory phenotype, thereby potentially increasing the inflammatory state in COPD (van der Does et al. 2010). Similar to HNPs, it has been shown that LL-37 also increases airway epithelial cell proliferation (Shaykhiev et al. 2005), which may contribute to epithelial remodeling. The switch in function from antimicrobial effectors toward harmful mediators is not a unique property of HNPs and LL-37 at high concentrations. Several studies report that cigarette smoke-related posttranslational modifications of both AMPs may affect their function. The converting enzyme responsible for ADP-ribosylation of HNP1, arginine-specific ADP-ribosyltransferase 1, is increased in smoking individuals, and

levels of ADP-ribosylated HNP1 are increased in BAL fluid from smokers (Paone et al. 2006). This may have important functional consequences, since ADP-ribosylation of HNP1 decreases the antimicrobial effect of the peptide, while increasing the cytotoxic and IL-8 inducing properties (Paone et al. 2002, 2006). Recently, citrullination was described as a novel mechanism for posttranslational modification of LL-37 that may also be increased in smokers, and it was demonstrated that citrullination of LL-37 decreases antimicrobial activity and increases chemotactic activity (Kilsgard et al. 2012). Citrullination of proteins is mediated by members of the peptidylarginine deiminase (PADI) family and expression of PADI2, and presence of citrullinated proteins was found to be increased in the lungs of smokers (Makrygiannakis et al. 2008; Kilsgard et al. 2012). This points toward a potential mechanism by which modification of HNPs and LL-37 alters the activity of these peptides, which may contribute to the development and progression of COPD.

3.2 Epithelial Expression of Antimicrobial Peptides

In contrast to HNPs and LL-37, levels of human β-defensin-2 (hBD-2) are decreased in induced sputum samples and BAL of COPD patients (Tsoumakidou et al. 2010). Furthermore, Herr et al. found an association between decreased hBD-2 levels and cigarette smoking in patients hospitalized with an acute pneumonia (Herr et al. 2009). The cigarette smoke–mediated suppression of hBD-2 expression seems to be persistent, as 1 year smoking cessation did not result in an increase in hBD-2 sputum levels in asymptomatic smokers (Bouloukaki et al. 2011). In COPD patients, hBD-2 expression is predominantly decreased in the central airways, while in the distal airways the expression was increased compared to controls (Pace et al. 2012). A previous report showed that hBD-2 depletion in BAL supernatants increased the number of apoptotic neutrophils, suggesting a role of hBD-2 in protection of cells from apoptosis (Pace et al. 2011). In contrast to HNPs, hBD-2 does not induce IL-8- expression in airway epithelial cells (Sakamoto et al. 2005), so it remains to be further investigated if hBD-2 contributes to inflammation in COPD.

In vitro antibacterial assays demonstrate efficient killing activity of hBD-2- toward acute exacerbation-associated bacteria (Lee et al. 2004). This suggests that the observed suppression of hBD-2 expression in COPD in central airways contributes to the increased bacterial colonization during acute exacerbations. As the airway epithelium is regarded as the main cellular source of hBD-2 in the airways (Singh et al. 1998), it is hypothesized that airway epithelial cells display an impaired host defense activity (Fig. 1). In vitro experiments demonstrate that prior cigarette smoke exposure of air–liquid interface-cultured airway epithelial cells inhibits *P. aeruginosa*- and *M. catarrhalis*-induced expression of hBD-2 (Herr et al. 2009; Zhang et al. 2011). Furthermore, it was shown that apical surface fluid derived from these cells displayed decreased antimicrobial activity. Stimulation of epithelial cells with the cigarette smoke–derived compound acrolein similarly

showed an inhibition of hBD-2 expression (Lee et al. 2007). In contrast to in vitro experiments, cigarette smoke exposure in murine models showed increased hBD-2 expression in the lung (Shibata et al. 2008), which is not in line with the findings in these in vitro studies, and observations in central airways and airway secretions in human studies (Pace et al. 2012; Herr et al. 2009; Tsoumakidou et al. 2010).

Recent studies have highlighted a role for vitamin D in the regulation of expression of antimicrobial peptides in epithelial cells and macrophages (Zasloff 2006; Hughes 2009). Furthermore, vitamin D deficiency is frequent in COPD and correlates with disease activity (Janssens et al. 2010a). This would suggest that vitamin D supplementation may be beneficial in COPD and may be a good strategy to prevent exacerbations that are so frequently associated with infections (Decramer et al. 2012). A recent intervention study showed that high-dose vitamin D supplementation may indeed reduce exacerbations in those COPD patients with severe vitamin D deficiency (Lehouck et al. 2012).

3.3 Genetic and Epigenetic Mechanisms

Several studies have investigated the association between genetic and epigenetic differences of AMPs with COPD development. Both α- and β-defensin-coding genes are localized at highly polymorphic regions (Hollox 2008). Genetic association studies assessing the role of single nucleotide polymorphisms (SNPs) in the gene encoding human β-defensin-1 (hBD-1) with COPD development revealed population-dependent outcomes. The hBD-1 gene contains polymorphisms in both the promoter region and the two coding exons (Dork and Stuhrmann 1998). In a Japanese population study, the nucleotide polymorphism in exon 2, resulting in a change of valine to isoleucine at position 38, was more frequent in COPD patients compared to controls (Matsushita et al. 2002). A study in a Chinese Han population describes an association between a polymorphism localized at exon 1 (44 C/G) with COPD susceptibility (Hu et al. 2004). In Caucasians, an association between SNPs in the hBD-1 gene and COPD could not be found (Wallace et al. 2006; Hersh et al. 2006). In addition, also polymorphisms in HNP1/3 were not associated with COPD development in such populations (Wallace et al. 2006). This indicates that the relation between polymorphisms and disease may differ between populations and that further studies on the functional consequences of these polymorphisms are needed.

Janssens et al. examined the association of copy-number variations of the hBD-2 gene with COPD development (Janssens et al. 2010b). Using in vitro cultured epithelial cells, it was shown in this study that five and higher diploid copy numbers of the hBD-2 gene was significantly more often present in COPD patients compared to controls. Moreover, it was shown that epithelial cells with high diploid copy number displayed a higher expression of hBD-2 induced by TNF-α and furthermore have a higher bacterial killing activity. These results are in contrast with earlier mentioned observations of a decrease in hBD-2 levels in BAL and induced sputum

and inhibition of hBD-2 expression by airway epithelial cells after cigarette smoke exposure. Therefore, the additional effects of environmental factors on the induced expression of hBD-2 in COPD patients with high copy numbers of the hBD-2 gene should be taken into consideration. Andresen et al. investigated the role of epigenetics in hBD-1 expression in COPD (Andresen et al. 2011). Using airway epithelium and cells derived from BAL, it was shown that mRNA levels of hBD-1 were higher in cells of COPD patients with mild to very severe disease compared to cells of healthy controls. The difference in mRNA levels was not due to a difference in DNA methylation of the hBD-1 gene promoter, but rather correlated with histone H3 lysine 4 methylation. These studies highlight that copy-number variations and epigenetic mechanisms may contribute to the control of expression levels of antimicrobial peptides in COPD.

3.4 Activity of Antimicrobial Peptides in COPD

During airway inflammation and infection, the local environment in which AMPs are active undergoes dramatic changes. Increased production of mucus as a result of smoking, inflammation, and infection may impact on local host defense against infection. Whereas the mucus layer that is positioned on top of the periciliary layer (Fig. 2) normally acts to trap and remove inhaled particles and pathogens, decreased mucociliary clearance in COPD prevents this process. Mucus itself does display antimicrobial activity because of its barrier and biochemical properties, but microorganisms have developed escape mechanisms such as flagella-mediated motility and enzymatic degradation of mucus (McGuckin et al. 2011). Mucins are large, heavily glycosylated glycoproteins that are essential components of mucus. Mucins have been shown to restrict the antimicrobial activity of LL-37 (Felgentreff et al. 2006; Bucki et al. 2008), and therefore, these peptides may not contribute optimally to antimicrobial activity of mucus. In healthy airways, this is not a problem, since mucus is removed. Furthermore, the bacteria that escape from the mucus layer are likely to be killed by antimicrobial peptides present in the periciliary layer (Fig. 2). However, because of excess mucus production and impaired ciliary activity, in COPD more bacteria may penetrate the mucus layer and reach the epithelial surfaces.

However, also other factors in the inflamed airways of COPD patients may have a negative impact on the antimicrobial activity of antimicrobial peptides. It has been shown that a wide range of microbial and host proteases are able to degrade and inactivate antimicrobial peptides (Taggart et al. 2003; Weldon et al. 2009; Schmidtchen et al. 2002). In addition, products such as F-actin and DNA (Weiner et al. 2003) that are released by dead cells, glycosaminoglycans (Baranska-Rybak et al. 2006; Bergsson et al. 2009), and bacterial polysaccharides (Herasimenka et al. 2005) may inhibit antimicrobial activity of AMPs. Finally, bacteria have evolved mechanisms to escape the antimicrobial activity of peptides. One such mechanism is the development of biofilms that provide protection against the action of the host

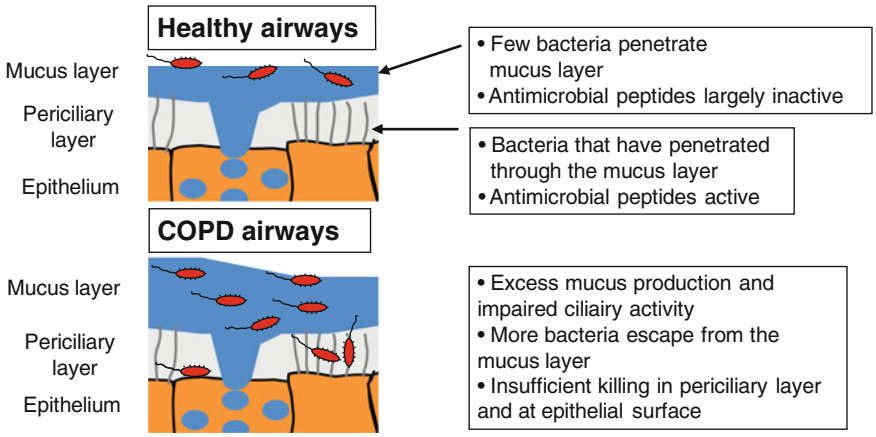

Fig. 2 Mucus, antimicrobial peptides, and microbial pathogens. In COPD, mucus hypersecretion and decreased mucociliary clearance may allow bacteria to escape from the mucus layer in numbers that are too large for efficient killing in the periciliary layer and epithelial surface

immune system. Interestingly, bacteria in biofilms may not be fully protected against the action of antimicrobial peptides, since, e.g., LL-37 (Overhage et al. 2008) and lactoferrin (Singh et al. 2002) have been shown to prevent biofilm formation and to act on bacteria present in biofilms.

4 Concluding Remarks

Recent studies point to a clear role of respiratory infection and AMPs in COPD. Microbiome analysis using unbiased molecular biological methods is still in its infancy but is pointing toward an altered microbiome also in stable COPD. Local excessive release of AMPs by, e.g., neutrophils may contribute to inflammation and possibly autoimmunity, whereas a local deficiency as a result of epithelial smoke exposure or inactivation of AMPs may contribute to respiratory infections. Biofilm formation impairs host defense against infection, but some AMPs may contribute to the fight against biofilm formation. Whether enhancement of local AMP production by, e.g., vitamin D treatment or administration of novel drugs based on the structure of endogenous AMPs holds a future in COPD treatment requires additional studies.

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Host Defense Peptides: Immune Modulation and Antimicrobial Activity In Vivo

Nicole J. Afacan, Laure M. Janot, and Robert E. W. Hancock

Abstract Cationic host defense peptides (HDPs), a vital component of the innate immune system, are amphipathic molecules of 12–50 amino acids in length and are produced by numerous cell types, either constitutively or in response to inflammatory stimuli. In addition to their antimicrobial and immunomodulatory properties, novel roles have been attributed to HDPs including promoting chemotaxis of immune cells, limiting inflammation/sepsis, promoting wound healing, regulating metabolism, and enhancing vaccine responses. These properties make HDPs a novel class of anti-infectives that can be exploited to treat immune and inflammatory disorders as well as infectious diseases. The emergence of multi-resistant bacteria is a major challenge facing modern healthcare since very few novel antibiotic agents are available. HDPs and their synthetic derivatives provide extremely valuable leads in the development of new treatment strategies for multi-resistant bacterial infections. This chapter reviews our basic knowledge on HDPs and synthetic cationic peptides and focuses on their current clinical application as anti-infectives, immunomodulators, and anticancer treatments. Challenges to their development as new therapeutics are also discussed.

Keywords Host defense peptides • Antimicrobial • Immunomodulatory • Cathelicidins • Defensins • Innate defense regulators

Abbreviations

ATP	Adenosine triphosphate
BPI	Bactericidal/permeability-increasing protein

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BMDC	Bone marrow-derived DCs
BRSV	Bovine respiratory syncytial virus
CAMP	Cathelicidin antimicrobial peptide
CDC	Centers for Disease Control and Prevention
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
EGFR	Epidermal growth factor receptor
FDA	Food and Drug Administration
FMOC	Fluorenylmethoxycarbonyl
GAS	Group A *Streptococcus*
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophages colony-stimulating factor
hst-5	Histatin 5
HDP	Host defense peptide
hBD	Human beta defensin
HIF-1α	Hypoxia-inducible factor-1α
HLA	Human leukocyte antigen
hLF	Human lactoferrin
HNP	Human neutrophil protein
Ig	Immunoglobulin
IDSA	Infectious Disease Society of America
IDR	Innate defense regulator
IOM	Institute of Medicine
IP-10	Interferon gamma-induced protein 10 kDa
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MIP	Macrophage inflammatory protein
MC-1R	Melanocortin-1 receptor
MSH	Melanocyte-stimulating hormone
MRSA	Methicillin-resistant *S. aureus* strains
MCP	Monocyte chemotactic protein
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
pDC	Plasmacytoid dendritic cell
PolyIC	Polyinosinic-polycytidylic acid
PP	Polyphosphazene
QSAR	Quantitative structure-activity relationship
RI	Retro-inverse
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

1 Introduction to Host Defense Peptides

Multicellular organisms are continually exposed to infectious agents and yet rarely develop infections. The innate immune system is the first line of defense against infectious agents. It is a rapid and relatively non-specific response made up of various cells and molecular components that include host defense peptides (HDPs) (Hancock and Diamond 2000). HDPs can influence the host response to infection in many ways including direct antimicrobial killing, modulation of chemokine and cytokine production, angiogenesis, and wound healing (Bowdish et al. 2005b). Due to the diverse antimicrobial and immunomodulatory properties of HDPs and their synthetic derivatives, innate defense regulators (IDRs), they are attractive candidates for use as novel anti-infectives.

1.1 Physical Properties of Host Defense Peptides

HDPs are between 12 and 50 amino acids in length, of which 2–9 residues are positively charged and approximately 50 % are hydrophobic. This unique make-up allows HDPs to fold into amphipathic secondary structures with both hydrophilic and hydrophobic patches (Hancock and Rozek 2002). Classification of HDPs is difficult owing to their enormous sequence diversity; however, folded peptides fall into one of four structural groups: β-sheets (e.g., human α- and β-defensins), α-helical (e.g., the human cathelicidin LL-37), extended structures (e.g., indolecidin) and looped peptides (e.g., bactenecin) (Guani-Guerra et al. 2010; Hancock and Sahl 2006). Although humans produce numerous HDPs, this chapter will focus primarily on cathelicidins and defensins, the best studied of the peptides.

Cathelicidins are defined by their highly conserved N-terminal cathelin domain and signal sequence. The biologically active C-terminal domains exhibit significant sequence variability resulting in peptides with a variety of secondary structures including α-helical, β-hairpin, β-turn, and extended conformations (Zanetti et al. 2000). Defensins are a structurally distinct family, subdivided into three classes: α-, β-, and Θ-defensins. The α- and β-defensin classes adopt similar β-sheet secondary structures, sometimes with an additional small α-helix, while Θ-defensins, only found in Old World monkeys, are cyclic peptides (Selsted and Ouellette 2005).

1.2 Expression of HDPs

Expression of HDPs is coordinately regulated at both the transcriptional and translational level, allowing multiple HDPs to be produced at a single body site. Gene expression and protein secretion of HDPs depend on the species, tissue, and cell type and stage of differentiation. In mammals, numerous cell types including

neutrophils, monocytes, T and B lymphocytes, and epithelial cells produce both cathelicidins and defensins. Expression and secretion of an HDP can be constitutive or induced by specific stimuli such as pro-inflammatory cytokines or microbial agents (Brown and Hancock 2006). For example, human keratinocytes constitutively express the β-defensin hBD-1, but hBD-2 and hBD-4 are induced by stimulation of Toll-like receptors (TLR) or pro-inflammatory cytokines (Selsted and Ouellette 2005). The expression of the human cathelicidin, LL-37, in leukocytes and epithelial cells is induced by inflammatory stimuli, and histone modification and vitamin D both play an important role in this process (Gombart et al. 2005; Kida et al. 2006; Schauber et al. 2004, 2008; Wang et al. 2004a). In addition to vitamin D, recent evidence suggests that cathelicidin expression is regulated by the transcription factor hypoxia-inducible factor-1α (HIF-1α) (Cramer et al. 2003; Fang et al. 2009; Peyssonnaux et al. 2005, 2008).

Once translated, HDPs exist in an inactive pro-form that can be stored at high concentrations in intracellular granules. Human neutrophils store β-defensins and LL-37 within their granules, releasing them into phagosomes or secreting them extracellularly upon activation, e.g., at the site of infections. HDPs must be proteolytically cleaved to release the mature, biologically active C-terminal domain (Valore and Ganz 1992; Wilson et al. 1999).

1.3 Functions of HDPs

A variety of functions have been described for HDPs. These functions fall within two broad categories: direct microbicidal activity and immunomodulation.

1.3.1 Direct Antimicrobial Activity

The role classically ascribed to HDPs is that of direct antimicrobial activity. In vitro, virtually all purified HDPs with a net positive charge and possessing a reasonable proportion (>40 %) of hydrophobic residues exhibit antibacterial, antifungal, and antiviral activity (Brown and Hancock 2006). The mechanism behind their antimicrobial activity is an area of active research, with several models having been proposed. All models involve the cationic HDPs interacting with the negatively charged phospholipid head groups and inserting into the membrane. In some cases, this is proposed to result in formation of aggregated peptide in the membrane leading to loss of cell membrane integrity, leakage of cytoplasmic contents, and cell death (Pazgier et al. 2006). Some HDPs cross the cell membrane and interact with intracellular anionic targets resulting in perturbation of normal cell function and inhibition of functions such as nucleic acid or protein synthesis (Hong et al. 2003; Kragol et al. 2001; Patrzykat et al. 2002; Zhang et al. 2000). However, under in vivo conditions such as high salt concentrations, 1–2 mM divalent cations and the presence of negatively charged glycosaminoglycans and serum proteins such as

apolipoprotein A-I and B, there is a very substantial attenuation of the direct antimicrobial activity of HDPs (Maisetta et al. 2008; Sorensen et al. 1999). Therefore, HDPs must be present at extremely high concentrations (mg ml^{-1}) such as with the α-defensins found in phagocytic granules and intestinal crypts in order to overcome the inhibitory effects of salt and serum components. Alternatively, HDPs may display direct microbicidal activity at anatomical sites with low salt concentrations, while at other sites, the immunomodulatory properties of HDPs will likely predominate (Mayer et al. 2010).

1.3.2 Immunomodulation by HDPs

In addition to the inhibitory effects of high salt concentrations and serum proteins, the minimum inhibitory concentrations of HDPs such as LL-37 and β-defensins in vitro are often much higher than the physiological concentrations of these peptides, e.g., at mucosal sites. Under such circumstances, their many other functions are likely to predominate. Defensins and cathelicidins modulate the innate and the adaptive immune responses, displaying both pro- and anti-inflammatory activity and can do so at low concentrations, in the presence of in vivo salt concentrations and serum components (Hancock et al. 2012; Mookherjee et al. 2006; Soehnlein et al. 2008).

HDPs recruit immune effector cells to sites of inflammation. Some human α- and β- defensins and LL-37 act as chemotactic agents for monocytes, macrophages, neutrophils, and T cells. This is mediated in part by their ability to stimulate production of chemokines and cytokines such as interferon gamma-induced protein 10 kDa (IP-10), monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-3α, interleukin (IL)-6, and IL-10 by a variety of cells including keratinocytes and monocytes (Chertov et al. 1996; De et al. 2000; Garcia et al. 2001; Niyonsaba et al. 2007; Territo et al. 1989).

HDPs also suppress pro-inflammatory responses induced by TLR activation of neutrophils and monocytes (Bowdish et al. 2004, 2005a; Yu et al. 2007). Neutrophils stimulated with lipopolysaccharide (LPS) or Gram-negative bacteria in the presence of LL-37 exhibit reduced secretion of tumor necrosis factor (TNF)-α, IL-1β, IL-6, and IL-8 (Alalwani et al. 2010). The reduction in TNF-α is partly due to modulation of downstream signaling pathways and partly due to binding of LL-37 to both LPS and CD14 blocking activation through TLR4 (Cirioni et al. 2006; Mookherjee et al. 2006; Nagaoka et al. 2001; Scott et al. 2002). Interestingly, while LL-37 and the α-defensin, HNP-1, inhibit *N. meningititis* lipooligosaccharide-stimulated reactive nitrogen species (RNS) production by macrophages, they enhance endotoxin-induced reactive oxygen species (ROS) production (Alalwani et al. 2010; Larrick et al. 1995; Zheng et al. 2007; Zughaier et al. 2005), consistent with pathway modulation.

Defensins and cathelicidins significantly enhance wound repair and angiogenesis, in part through regulation of macrophage differentiation (discussed further in Sect. 3.1). Finally, HDPs modulate apoptosis of a variety of cells, and this HDP-mediated suppression or activation of apoptosis is dependent upon cell type

and stimulation conditions (e.g., HDP concentration). Human α-defensins, β-defensins, and LL-37 are capable of suppressing neutrophil apoptosis (Barlow et al. 2006; Nagaoka et al. 2006, 2008, 2010). More recently, LL-37 was also shown to suppress endotoxin-induced apoptosis of endothelial cells and hepatocytes (Suzuki et al. 2011). Suppression of apoptosis by HDPs prolongs the life of cells, which may be advantageous to host defense against infectious agents. LL-37 also activates apoptosis in certain cells. Bacterially infected airway epithelial cells stimulated with physiological levels of LL-37 exhibit enhanced apoptosis. This may constitute a novel mechanism by which LL-37 can promote innate immune clearance of respiratory pathogens (Barlow et al. 2006, 2010). LL-37-induced apoptosis of cytotoxic and regulatory T cells is initiated by granzyme leakage and may influence adaptive immune responses to tumors (Mader et al. 2011a, b).

The mixed pro- and anti-inflammatory properties of HDPs may allow tailoring of the immune response in inflammatory settings such as infection, autoimmune disorders, and cancer. They promote a rapid and effective innate immune response while simultaneously restricting potentially excessive and damaging inflammation. This makes them attractive candidates for novel therapeutics that modulate the immune system.

2 In Vivo Role of HDPs in Infection and Inflammation

Studies correlating HDP expression levels with resistance or susceptibility to bacterial infections in humans and mice provide clear evidence for the importance of HDPs in the innate immune response. Individuals suffering from specific granule deficiency syndrome experience frequent, severe bacterial infections due to a lack of α-defensin production (Ganz et al. 1988). Others with a condition known as morbus Kostmann exhibit severe neutropenia. Although neutrophil levels can be restored through recombinant human granulocyte colony-stimulating factor (G-CSF) treatment, these individuals continue to present with frequent oral bacterial infections and periodontal disease (Carlsson et al. 2006; Joazlina et al. 2005; Putsep et al. 2002). This has been linked to a deficiency in both α-defensins (HNP-1–3) and LL-37 production by neutrophils (Putsep et al. 2002). Although these studies establish a role for HDPs in the innate immune system and in prevention and clearance of infections, they do not delineate their mode of action.

2.1 Antimicrobial Activities

2.1.1 Antibacterial Activities

Clinical studies and experimental animal models have begun to elucidate the role of cathelicidins and β-defensins in prevention and clearance of bacterial infections. In a rabbit model of *Shigella* infection, cathelicidin induction correlated with

reduced bacterial burden and improved clinical markers. Similarly, patients suffering from severe *Shigella* infections exhibited diminished levels of LL-37 in intestinal biopsies (Islam et al. 2001). Furthermore, cathelicidin antimicrobial peptide (CAMP) knockout mice exhibit increased susceptibility to infection by group A *Streptococcus* (GAS) and *Escherichia coli* (Chromek et al. 2006; Lee et al. 2005; Nizet et al. 2001). Protection against GAS can be attributed in part to mast cell-mediated cathelicidin production, as mast cell-deficient mice exhibit severe GAS skin infections, which could be alleviated after reconstitution with wild type but not $CAMP^{-/-}$ mast cells (Di Nardo et al. 2008). While these studies were intended to demonstrate the direct antimicrobial activity of these peptides, they do not exclude the possibility that enhancement of innate immunity is a contributing factor or even the dominant mechanism in the modest protective efficacy of these peptides. Indeed, it is extremely difficult to discriminate these two possibilities as the outcome of more efficient innate immunity would be bacterial killing by the various effector mechanisms of this system (e.g., phagocytic killing, various proteinaceous and non-peptide antimicrobial molecules and complement-mediated killing). For example, Welling et al. demonstrated that as little as 4 ng of human α-defensin 1 (HNP-1; several orders of magnitude below the bactericidal concentration) was able to reduce infection by *Klebsiella* in the lungs of infected mice, presumably by increasing neutrophil recruitment, while 400 ng could assist in clearance of intramuscular *Klebsiella* and *Staphylococcus aureus* infections (Welling et al. 1998).

2.1.2 Antiviral Activities

In vivo antiviral activity has been described for both cathelicidins and defensins. CAMP-deficient mice exhibit increased susceptibility to both herpes simplex virus and vaccinia virus (Howell et al. 2004, 2006b). Defensin expression is induced by viral infections, including influenza virus and human rhinovirus (Chong et al. 2008; Ding et al. 2009). HDPs exert their antiviral activity through a variety of mechanisms including disruption of viral envelope membranes, inhibiting binding to viral glycoproteins, inducing aggregation of virions for subsequent phagocytic uptake, inhibition of virion uptake into cells, and a variety of intracellular mechanisms including inhibition of macromolecular synthesis and stimulation of host cell antiviral immune mechanisms (Jenssen et al. 2006). For example, defensins can act on target cells, preventing viral entry and replication (Daher et al. 1986; Guo et al. 2004; Hartshorn et al. 2006; Tecle et al. 2007; Wang et al. 2004b).

2.1.3 Antifungal Activities

In vitro antifungal activity has been described for HDPs; however, there are limited studies on their in vivo role. There is some evidence to suggest that alteration of

HDP structure or production is associated with *Candida* carriage levels. For example, a single-nucleotide polymorphism at position −44 in the DEFB1 gene encoding hBD-1 is associated with enhanced protection from oral *Candida* colonization (Jurevic et al. 2003). Histatin 5 (hst-5) is a 24 amino acid histidine-rich HDP found in saliva with potent antifungal activity (Aerts et al. 2008). HIV^+ individuals are heavily colonized with *C. albicans*, which correlates with low levels of hst-5 in their saliva (Torres et al. 2009). Furthermore, hst-5 proved efficacious in protecting oral tissue in an ex vivo murine model of *C. albicans* infection, suggesting that hst-5 exerts a protective effect against oral colonization by *C. albicans* (Peters et al. 2010). The mechanism behind the candidicidal activity of HDPs is unclear, although in vitro studies suggest it is an energy-dependent process that results in adenosine triphosphate (ATP) release (Aerts et al. 2008; Koshlukova et al. 1999). Mounting evidence suggests that some HDPs may act through membrane disruption/permeabilization, cell lysis, and interaction with fungal mitochondria (Jenssen et al. 2006).

2.1.4 Antiparasitic Activities

Like HDP antifungal activity, most research into their antiparasitic activities has been carried out in vitro. While a number of mammalian HDPs exhibit in vitro antiparasitic activity against several *Leishmania* and *Trypanosoma* species, (McGwire and Kulkarni 2010) the physiological role of these peptides during infection is poorly understood. In a murine *T. brucei*-infection model, mice given exogenous cathelicidins late in the infection exhibited reduced parasitemia and delayed mortality (McGwire et al. 2003). Mice deficient in CRAMP experience more severe cutaneous leishmaniasis, as a result of uncontrolled parasite growth within lesions (McGwire and Kulkarni 2010). Several models for the mechanism of HDP action have been proposed, including disruption of parasite surface membranes increasing permeability and leakage of intracellular contents, interaction with cell surface receptors inducing signaling pathways and collapse of mitochondrial membrane potential and function.

2.2 Immunomodulatory Activities of HDPs in Infections and Inflammatory Diseases

Although the studies presented above have been interpreted as suggesting that HDPs act via direct antimicrobial activity, several studies that have come out in recent years provide compelling evidence for an alternative (or additional) mode of action. In vitro studies show that immunomodulatory effects can occur at very low physiological concentrations and at physiological salt concentrations (Filewod et al. 2009; Mookherjee et al. 2006). This is consistent with the landmark Welling study

mentioned above (Welling et al. 1998). Induction of rat β-defensin 2 during chronic *Pseudomonas aeruginosa* lung infection has also been shown to increase recruitment of neutrophils to the lungs and elevated cytokine/chemokine production. This is associated with improved survival rate, reduced bacterial load, and milder lung pathology (Hu et al. 2010). A recent study examined the ability of human α-defensins to modulate the immune response of rainbow trout under resting conditions. Trout injected with recombinant HNP-1 exhibited enhanced pro-inflammatory cytokine-, chemokine-, and interferon-related gene expression in different tissues. HNP-1 was also able to chemoattract isolated trout leukocytes. These results shed light on the in vivo immunomodulatory properties of HDPs and demonstrate that this property is active across species (Falco et al. 2008).

Excessive HDP production can lead to exaggerated inflammatory responses as a result of their immunomodulatory properties. Excessive production of LL-37 is observed in psoriasis and rosacea, two inflammatory skin disorders. In psoriasis, LL-37 facilitates the uptake of extracellular self-DNA fragments by plasmacytoid dendritic cells (pDCs), inducing a type I interferon response and maturation of pDCs (Lande et al. 2007). The mature DCs activate autoreactive T cells leading to the production of interferon (IFN)-γ, IL-17, and IL-22, which further induce LL-37 driving inflammation through a positive feedback mechanism (Ma et al. 2008; Wilson et al. 2007). Excess LL-37 produced in rosacea is proposed to be processed by serine proteases into a pro-inflammatory form that stimulates inflammation and irregular angiogenesis. In vitro studies and a mouse model of contact skin irritation indicate that the inflammation is a result of LL-37 inducing cytokine and chemokine production, neutrophil recruitment, thrombosis, and hemorrhage (Yamasaki et al. 2007).

In contrast, some inflammatory skin disorders are associated with reduced HDP expression; patients with atopic dermatitis have diminished expression of LL-37, hBD-2, and hBD-3 in skin lesions and dermcidin in sweat compared to patients suffering from other inflammatory conditions (Howell et al. 2006; Kisich et al. 2008; Ong et al. 2002; Rieg et al. 2005). Th2 cytokines produced in atopic lesions are thought to inhibit HDP expression at these sites (Howell et al. 2005; Nomura et al. 2003). These studies serve to underscore many in vitro studies that demonstrate that the effects of HDPs are modulated by other molecules in the environment. For example, in vitro studies have demonstrated that LL-37 antagonizes LPS and lipoteichoic acid (LTA) pro-inflammatory response but shows synergy with polyinosinic-polycytidylic acid (PolyIC) or mixed effects (CpG) with other TLR agonists and synergy or antagonistic interactions with host molecules such as granulocyte–macrophage–CSF (GM–CSF), IL-1β, and IFN-γ (Bowdish et al. 2004; Filewod et al. 2009; Mookherjee et al. 2006; Yu et al. 2007).

In addition, variation in defensin copy number has been associated with the strength of inflammatory responses. HIV-positive children exhibit lower DEFB104 (hBD-4) copy number than uninfected children exposed to HIV, suggesting that hBD-4 expression may confer a level of protection against HIV infection (Linzmeier and Ganz 2005; Milanese et al. 2009). High defensin copy numbers have also been associated with excessive inflammatory responses. A high copy

number for DEFA1/DEFA3 (α-defensins) is associated with increased risk of developing severe sepsis, a systemic inflammatory reaction (Chen et al. 2010). Similarly, individuals with psoriasis exhibit increased β-defensin copy number compared to healthy individuals, with a linear increase in the risk of developing psoriasis as copy number increases. The higher copy numbers are thought to result in increased/excessive defensin expression and production and overstimulation of immune effector cells responding to these peptides (Hollox and Armour 2008; Linzmeier and Ganz 2005; Yang et al. 2002).

Variation in defensin copy number and expression levels is also associated with several inflammatory bowel disorders, including ulcerative colitis and Crohn's (Guani-Guerra et al. 2010). In ulcerative colitis, expression of HD5, HD6, and hBD-2-hBD-4 by epithelial cells is dramatically upregulated. In pediatric ulcerative colitis patients, the expression levels of hBD-2 and hBD-3 are strongly correlated to the expression of the pro-inflammatory cytokines, IL-8 and TNF-α (Zilbauer et al. 2010). The high levels of these defensins could result in excessive inflammation similar to that seen in psoriasis and rosacea (Fahlgren et al. 2003; Lai and Gallo 2009). Furthermore, lower β-defensin copy number and impaired expression of hBD-2 and hBD-3 and HD-5 and HD-6 have been linked to the self-perpetuating inflammation observed in patients with Crohn's colitis (Fellermann et al. 2006; Guani-Guerra et al. 2010). Many of the recent studies into the role of defensins in Crohn's colitis have suggested that it is their antimicrobial activity that is essential to maintaining normal flora homeostasis. However, these are inflammatory diseases and there is evidence to suggest that the immunomodulatory (see below) activities of some HDPs such as LL-37 may also be quite influential (Marks et al. 2006; Schauber et al. 2006).

3 Novel Immunomodulatory Roles of Endogenous HDPs

3.1 Wound Healing and Angiogenesis

In addition to the activities described above, several novel immunomodulatory functions have been attributed to HDPs (Fig. 1). Cathelicidins and defensins significantly enhance wound repair and angiogenesis (Hao et al. 2009; Hirsch et al. 2009). Many of the studies on wound repair and angiogenesis have focused on the cathelicidins LL-37 and PR-39, a porcine cathelicidin, which are highly expressed in wound beds and are involved in re-epithelialization and neovascularization through distinct mechanisms (Heilborn et al. 2003). LL-37 induces keratinocyte proliferation and migration by inducing the release of active epidermal growth factor receptor (EGFR) ligands (Tokumaru et al. 2005; Yin et al. 2007). PR-39 induces expression of syndecans, cell surface heparan sulfate proteoglycans that regulate cellular proliferation and migration in response to heparin-binding growth factors (Gallo et al. 1994). LL-37 directly activates formyl peptide receptor-

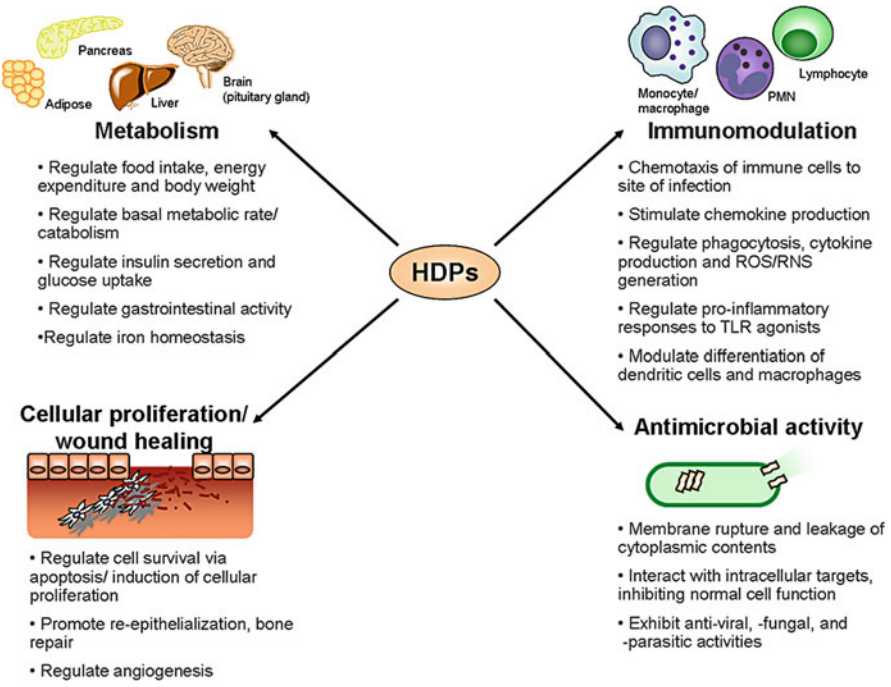

Fig. 1 Biological activities of HDPs. The two major biological functions ascribed to HDPs are direct antimicrobial activity and potent immunomodulatory activity, which includes regulation of wound healing and angiogenesis. In addition, many peptides have been identified that are major regulators of both metabolism and the immune response. *PMN* polymorphonuclear cell, *ROS* reactive oxygen species, *RNS* reactive nitrogen species, *TLR* Toll-like receptor

like 1 on endothelial cells, while PR-39 induces endothelial cells to express vascular endothelial growth factor (VEGF) and its receptor (FLT1) via stabilization and activation of the transcription factor HIF-1α. The actions of LL-37 and PR-39 both result in cellular proliferation and formation of vascular structures (Koczulla et al. 2003; Li et al. 2000).

A recent study has linked LL-37 to bone repair. Transplantation of LL-37-differentiated monocytes into nonobese diabetic/severe combined immune deficiency mice results in formation of bone-like structures. The LL-37-differentiated monocytes, named monoosteophils, appear to be distinct from monocyte-derived DCs and macrophages, instead possessing characteristics of osteoblasts and osteoclasts (Zhang and Shively 2010). The induction by HDPs of these bone-forming cells could constitute a novel therapeutic approach for healing fractures and diseases such as osteoporosis.

The role of defensins in wound healing and angiogenesis in vivo is being increasingly appreciated. In animal models of wound healing, upregulation of hBD-2 and hBD-3 expression in the wound bed is associated with accelerated wound closure (Hao et al. 2009; Hirsch et al. 2009). In vitro studies on intestinal wound healing suggest that hBD-2 induces migration of intestinal epithelial cells by

signaling through the chemokine receptor CCR6, preventing apoptosis and upregulating expression of mucins 2 and 3, which are essential to the protective barrier between epithelial cells and the intestinal lumen (Otte et al. 2008; Vongsa et al. 2009). hBD-2, hBD-3, and hBD-4 have also been shown to act in a similar manner to LL-37, inducing keratinocyte migration and proliferation, through activation of EGFR and production of cytokines/chemokines (Niyonsaba et al. 2007). In vitro, hBD-2 is also capable of inducing endothelial cell proliferation, migration, and vascular structure formation, three processes essential to angiogenesis, although the underlying mechanisms and its relevance in vivo remains unclear (Baroni et al. 2009). α-Defensins have the opposite effect on neo-vascularization, preventing in vivo neo-vascularization in the chicken chorioallantoic membrane assay. In vitro they were found to inhibit VEGF-induced migration and adhesion of endothelial cells to fibronectin, as well as their proliferation and survival (Chavakis et al. 2004). In the murine model of hypoxia-induced retinal angiogenesis, local and systemic administration of α-defensins was able to reduce neo-vascularization by over 40 % (Economopoulou et al. 2005). This property of α-defensins is therefore being studied as a possible treatment for pathologic retinal neo-vascularization, such as that observed in diabetes.

Histatins cell proliferation: Veerman publications, starting in 2008.

3.2 Modulation of Immune Responses and Metabolism

Many major physiological processes impact the development of an immune response. For example, metabolism controls the synthesis and degradation of biomolecules as well as the energy availability within an organism (Matarese and La Cava 2004), and the immune response requires substantial amounts of energy and metabolic intermediates for biosynthesis of macromolecules. It is predicted that the basal metabolic rate of leukocytes activated during an acute inflammatory response increases by 9–30 %. During a major inflammatory response such as that seen in sepsis, the metabolic rate can increase by 50 %. Thus, regulation of energy-rich fuel availability and utilization will have wide-reaching effects on host immune responses and homeostasis (Straub et al. 2010).

Research into the crosstalk between these two processes has focused primarily on identifying and characterizing the activity of small molecule regulators of both systems, with the majority of regulators identified being adipose-derived molecules known as adipokines. Leptin, a 16-kDa non-glycosylated peptide hormone, is an indirect gauge of total body fat, acting to decrease food intake while increasing basal metabolism and catabolism under nutrient-rich conditions (Friedman and Halaas 1998). It is also important to the immune response as mice deficient in leptin signaling are immunocompromised (Howard et al. 1999). Leptin is involved in activation of macrophages, promoting phagocytosis and leukotriene B4, nitric oxide, and pro-inflammatory cytokine production. Leptin also increases chemotaxis of neutrophils and lymphocytes (Lago et al. 2007). Mice lacking leptin have low levels of Th1-associated cytokines such as IL-2, IFN-γ, TNF, and IL-18 and high

levels of Th2-associated cytokines, such as IL-4 and IL-10, suggesting that the presence of leptin favors the development of Th1 responses (Busso et al. 2002; De Rosa et al. 2006; Faggioni et al. 2000; Frisullo et al. 2007). Because immunological roles are being identified for leptin and it contains a cationic amphipathic α-helix at its N terminus, this peptide could also be classed as an HDP. Immunomodulatory activity has also been described for several other cationic peptide hormones and neuropeptides classically associated with metabolic regulation (Table 1).

The neuropeptide α-melanocyte-stimulating hormone (α-MSH) has been shown to regulate both metabolism and immune responses. α-MSH reduces food intake while increasing basal metabolic rate. It also plays a substantial role in resolving inflammation by enhancing anti-inflammatory activities in leukocytes and lymphocytes. The immunomodulatory properties of α-MSH have been reviewed extensively elsewhere (Brzoska et al. 2008). Briefly, α-MSH mediates its immunomodulatory activities by interacting with melanocortin-1 receptor (MC-1R), found on monocytes, neutrophils, and lymphocytes (Andersen et al. 2005; Bhardwaj et al. 1997; Cooper et al. 2005). Stimulation of MC-1R in vitro results in suppression of pro-inflammatory cytokines, lipid mediators (i.e., prostaglandins), ROS/RNS, and chemotaxis with an upregulation of anti-inflammatory cytokines and activation of T regulatory lymphocytes. Much like the classical HDPs described above, α-MSH is also capable of modulating apoptosis in neuronal and non-neuronal cells, exhibiting cytoprotective activity as well as promoting postlesional repair of nerves. The in vivo immunomodulatory activity of α-MSH has been characterized using several animal models, and in all instances, α-MSH acts as a potent anti-inflammatory agent (Brzoska et al. 2008).

There has been very little work done on the role classically antimicrobial or immunomodulatory HDPs play in integrating the innate immune response and metabolism, although the area is beginning to spark some interest. A link between HDPs and the endocrine system, the major regulator of allostatic systems such as metabolism, was recently established in the paper of Radek et al. (2010) which demonstrated that CRAMP tissue levels are regulated, in part, by the cholinergic component of the endocrine system (Radek et al. 2010). Additionally, CRAMP (and other HDPs) modulates the production of a number of molecules associated with the immune response, including TNF-α and IL-6 that are also potent regulators of metabolism (Ouchi et al. 2011). This provides a potential mechanism for HDP-mediated modulation of the immune response and metabolism, although more research is required to determine whether HDPs do regulate metabolism within an organism or a single cell and whether this could occur directly or indirectly.

One human HDP α-defensin HNP-1 has been shown to reduce blood glucose levels in mice primarily by inhibiting hepatic glycogenolysis and gluconeogenesis, using an insulin-independent mechanism (Liu et al. 2008). This novel area of research is very much in its infancy but in the future could lead to a better understanding of the interconnection of metabolism and immunity as well as identify novel therapeutic targets in infectious disease and immunological disorders (Fig. 1).

Table 1 Cationic peptide hormones and neuropeptides that regulate metabolism and exhibit immunomodulatory activity

Cationic peptide hormones/ neuropeptides	Metabolic regulation	Immunomodulatory activity	References
Leptin	Decreases food intake Increases basal metabolism/ catabolism under nutrient-rich conditions	Activates macrophages and promotes phagocytosis and leukotriene B4, nitric oxide, and pro-inflammatory cytokine production. Increases chemotaxis of neutrophils and lymphocytes Promotes Th1 responses	Friedman and Halaas (1998), Lago et al. (2007) Ouchi et al. (2011)
Ghrelin	Energy homeostasis and weight regulation Stimulates appetite/ food intake Decreases insulin secretion Promotes gastrointestinal motility	Anti-inflammatory Inhibits pro-inflammatory cytokine release Protective in many inflammatory disease models	Himmerich and Sheldrick (2010)
Vasoactive intestinal peptide Pituitary adenylate cyclase-activating polypeptide-27 and -38	Stimulates insulin and glucagon secretion Regulates energy and lipid metabolism as well as body weight Regulate gastrointestinal function	Cytoprotective Extensive modulation of innate and adaptive immune effector cell functions Promotes mast cell degranulation and pro-inflammatory cytokine production Reduces cell recruitment Inhibits production of pro-inflammatory cytokines/ chemokines and ROS/ RNS by macrophages Promotes anti-inflammatory cytokine production Promotes Th2 responses	Smalley et al. (2009), (Moody et al. (2011), (Gomariz et al. (2006)
Hepcidin	Iron homeostasis Inhibits intestinal iron absorption Promotes iron sequesterization in macrophages/ liver	Expression is induced by inflammatory stimuli Diminishes iron availability for bacteria and tumors Inflammation-induced hypoferremia leads to anemia Type II acute-phase protein	Malyszko and Mysliwiec (2007)

(continued)

Table 1 (continued)

Cationic peptide hormones/ neuropeptides	Metabolic regulation	Immunomodulatory activity	References
Adrenomedullin (natriuretic peptide)	Natriuretic/diuretic Decreases salt appetite Inhibits insulin secretion Increases circulating glucose levels	Vasodilator Inhibits production of pro-inflammatory cytokines/ chemokines Promotes anti-inflammatory cytokine production Induces regulatory T lymphocytes	Martinez et al. (1996), Schell et al. (1996), Delgado and Ganea (2008)
Cortistatin-17/ somatostatin	Inhibits insulin and ghrelin secretion	Inhibits production of pro-inflammatory cytokines/ chemokines Promotes anti-inflammatory cytokine production Induces regulatory T lymphocytes	Broglio et al. (2008), Delgado and Ganea (2008)
MSH (α- and γ-MSH)	Reduces food intake Increases basal metabolic rate	Cytoprotective Inhibits production of pro-inflammatory cytokines, lipid mediators, and ROS/RNS Suppresses chemotaxis Enhances anti-inflammatory activity of leukocytes Activates T regulatory lymphocytes	Brzoska et al. (2008)
Substance P	Regulates adipose tissue responses Inhibits insulin-mediated glucose uptake by adipocytes	Modulates inflammatory responses in monocytes/ macrophages via differential activation of neurokinin 1 receptor isoforms Proliferative effect on cells of myeloid lineage Modulation of chemokine-induced responses in monocytes	Chernova et al. (2009), Tuluc et al. (2009), Endocrinology. 2011 152: (Karagiannides, I.)

4 Therapeutic Potential and Applications

4.1 Reasons for Developing New Therapeutics

The discovery of penicillin, a compound able to selectively kill bacteria, heralded the golden age of antibiotic discovery (Fleming 2001). The widespread use of antibiotics has resulted in bacteria resistant to every known class of antibiotic

(Gulay et al. 2001). A study by Katz et al. estimated that more than 70 % of pathogenic bacteria were resistant to at least one antibiotic (Katz et al. 2006). In response to plasmid-mediated penicillin resistance in 80 % of *S. aureus* isolates by 1960, a penicillin derivative, methicillin, was introduced to treat infections caused by *S. aureus* and other penicillin-resistant bacteria. Only 2 years later, methicillin-resistant *S. aureus* strains (MRSA) were detected and are now widespread in healthcare units throughout the world. Vancomycin is used as therapy for MRSA infection, but in 2002, strains of MRSA also resistant to this antibiotic began to emerge in hospitals. Although originally confined to hospitals and nursing homes, resistant strains are now prevalent in the community (David and Daum 2010). Different organizations like the Infectious Diseases Society of America (IDSA), the Institute of Medicine (IOM), the Food and Drug Administration (FDA), and Centers for Disease Control and Prevention (CDC) have identified the emergence of these "superbugs" as a major challenge facing modern healthcare. The problem of widespread multidrug resistance is compounded by the fact that there are very few new antibiotics in development as drug innovation is expensive, success rates are low, there are substantial regulatory hurdles for antibiotic approval and antibiotics bring in relatively low profits to pharmaceutical companies, compared to drugs that treat chronic conditions (FDA 2004; Nathan 2004; Sellers 2003).

Infections caused by multidrug-resistant bacteria cause severe morbidity and mortality, and treatment and recovery costs are a significant burden on the healthcare system (Spellberg 2010). Research into and development of novel anti-infective therapeutics is important to mitigate this healthcare challenge (Levy and Marshall 2004). HDPs are a promising new therapeutic strategy, not only for bacterial infections but also other infectious agents, autoimmune disorders, and cancer.

4.2 The Assets of Peptides Compared to Conventional Antibiotics

Peptides have several assets that make them worthy of consideration as stand-alone therapies or adjunctive treatments to expand the efficacy of classical antibiotics. Owing to their amphipathicity and cationic charge, antimicrobial peptides can attach to bacterial membrane and cause channel formation (Brogden 2005) or translocate across the membrane to inhibit cell wall synthesis and DNA, RNA, and protein synthesis (Boman 1995; Futaki et al. 2001; Ramanathan et al. 2002; Scheller et al. 1999).Their multifaceted antimicrobial mechanisms and physical mechanism of interaction with essential targets make the development of resistance to them much less likely. Furthermore, they demonstrate extremely broad-spectrum activities that can encompass antibacterial, antiviral, antifungal, and antiparasitic activity (Benincasa et al. 2006; Gallo et al. 2006; Kollef et al. 2006; Scott et al. 2007; Tanaka et al. 2010). However, to date results in clinical trials have been disappointing as they have failed to meet their exciting promise as a next generation of antibiotics. In addition to their antimicrobial activities, HDPs are also able to

favorably modulate the immune response to enhance clearance of infectious agents, due in part to their chemotaxis and anti-inflammatory properties (Sawa et al. 1998; Scott et al. 2002; Zhang et al. 2000), and this has formed the basis of an exciting new (adjunctive) approach to antimicrobial therapy (Scott et al. 2007).

4.3 Rational Design of Synthetic Peptides

Exploiting the biological properties of HDPs requires the development of novel rational design techniques to develop peptides with enhanced therapeutic activity. Recent peptide array approaches have dramatically increased the rate at which the influence of sequence variation on antimicrobial activity can be surveyed (Hilpert et al. 2005). Natural or modified peptides can be used as templates to generate thousands of synthetic peptides by random amino acid substitution, scrambling, and truncation. These peptide libraries are then screened in vitro to determine their direct antimicrobial activities and, together with machine learning quantitative structure-activity relationship (QSAR) approaches, lead to optimally designed HDPs (Cherkasov et al. 2009; Fjell et al. 2009). This is an invaluable aid in rational design of novel peptides with stronger activities and reduces the cost of investment (Jenssen et al. 2008). Based on this strategy, 8–12 amino acid variants of the bovine bactenecin were generated that were more than tenfold more effective against Gram-positive and Gram-negative bacteria as well as *Candida* and for the first time were protective in systemic animal models (Cherkasov et al. 2009; Hilpert et al. 2005). Recently, this method was modified to screen short peptides exhibiting antimicrobial properties while tethered to a solid surface (Hilpert et al. 2009). This could be particularly important in preventing implant-associated infections, such as on catheters or prostheses. In this regard, the recent demonstration that LL-37 is able to suppress biofilm formation (which occurs in ~60 % of human infections) in *P. aeruginosa* (Overhage et al. 2008) and *Staphylococcus epidermidis* (Hell et al. 2010) provides a new avenue for peptide design.

Rational design of immunomodulatory peptides is more difficult as structure–activity relationships have not been extensively investigated, perhaps due to the very recent evolution of this field and the pleiotropic effects and targets of such peptides. Thus, research has focused on specific markers of immunomodulatory activity including the ability to suppress TNF-α production in response to TLR agonists (Mookherjee et al. 2006) and the capacity to enhance production of chemokines by human peripheral blood mononuclear cells (Scott et al. 2007). One of the first synthetic peptides produced, IDR-1, was selected based on these markers (Scott et al. 2007) and was protective in animal models despite a (designed) lack of direct antimicrobial activity. Through iterative design, synthetic peptides with immunomodulatory activity can be improved upon. Using this method, a peptide, IDR-1002, with greater anti-infective activity was generated (Nijnik et al. 2010).

Structure-function analysis is an important strategy in the optimization of natural peptides. For example, anti-endotoxic activity of LL-37 was improved by appropriate amino acid substitutions (Nagaoka et al. 2002). Antibacterial and immunomodulatory activities of LL-37 were also dissociated using this method (Braff et al. 2005) indicating that certain structural characteristics are linked to specific functions. While the design of synthetic peptide variants has greatly enhanced their ability to treat disease, further research is necessary to develop peptides with functions tailored to treat specific conditions.

4.4 Recent Clinical Development of HDPs

Several synthetic peptides developed using rational design are currently undergoing clinical or preclinical trials to determine their therapeutic potential (Table 2). The broad range of clinically applicable HDP functions is reflected in the investigations into their use as adjuvant compounds or wound-healing enhancers as well as novel anti-infectives and anti-inflammatories.

4.4.1 Clinical Applications of Antimicrobial and Immunomodulatory Peptides

Many peptides have been investigated as topical anti-infectives, including MSI-78 (pexiganan), IB-363 (iseganan), XOMA-629, HB-1345, and MX-226 (Table 2). Results to date have been quite disappointing as none of these agents have received new drug approval despite encouraging efficacy results The indolicidin derivative MX-226 was efficacious when provided topically as an anti-infective in two phase IIIa and b clinical studies into prevention of central venous catheter colonization (http://www.migenix.com, 2009 annual report). However, it twice failed to reach its primary endpoint of physician-determined infections (although it did demonstrate statistically significant efficacy in suppressing microbiologically confirmed infections and reducing colonization of catheters).

The use of synthetic peptides as modulators of the innate immune response provides the best prospects as these peptides simultaneously reduce the potential risk of harmful inflammation and septic shock and the development of resistance by the pathogen to the treatment. It should be stated that the goal for such peptides would be as adjunctive treatments to enhance the outcomes of antimicrobial therapy, especially in the face of antibiotic resistance, rather than serving as stand-alone treatments. MX-226, originally developed as an antimicrobial peptide, was also shown in phase II clinical trials to be efficacious as an anti-inflammatory topical treatment for severe acne, an inflammatory condition triggered by infection, and rosacea, a chronic inflammatory skin disease, for which it will likely enter phase III trials. Human lactoferrin, hLF1-11, an antimicrobial peptide, has demonstrated the ability to enhance the differentiation of macrophages into cells

Table 2 Cationic immunomodulatory peptides in clinical development

Drug	Description	Intended use	Progress	References*
Immunomodulatory anti-infective peptides with antimicrobial activities				
Omiganan [MX-226] (Migenix)	Indolicin derivative	Prevention of central venous catheter infections	Phase IIIb	NCT 00231153 (completed)
		Topical skin antisepsis, rosacea	Phase II	NCT 00608959 (completed)
hLF1-11 (AM-Pharma)	Human lactoferrin derivative	Prevention of bacteremia and fungal infections in immunocompromised patients	Phase I/II	NCT 00509834 NCT 00509938 (completed) Velden et al. (2009)
Immunomodulatory anti-infective peptides lacking antimicrobial activities				
IMX942 (Inimex)	IDR derivate form indolicin	Nosocomial infections, neutropenia	Phase Ia	http://www.inimexpharma.com
Immunomodulatory peptides				
IC31 (Intercell)	Peptide KLKL5KLK combined to deoxyinosine/deoxycytosine ODN	Subunit of tuberculosis vaccine	Phase I	NCT 01003093 (completed) NCT 00929396 (completed)
			Phase I	NCT 01049282 (completed)
			Phase I	Ottenhoff et al. (2010), van Dissel et al. (2010)
AP214 (Action Pharma)	α-MSH derivative	Prevention of organ failure after surgery	Phase II	NCT 00903604 (completed)
			Phase II	NCT 01256372 (completed)
Heptapeptide-7 (Helix Biomedix)	Derivative of HB-107, itself derived from cecropin	Wound healing, skin regeneration	Phase II	http://www.helixbiomedix.com Falla and Zhang (2010)
Anti-infective peptides with unknown immunomodulatory activities				
HB-1345 (Helix Biomedix)	Lipohexapeptide	Acne	Pre-phase I	http://www.helixbiomedix.com
PAC-113 (Pacgen Biopharmaceutical)	Histatin derivative	Antifungal	Phase IIb	NCT 00659971 (completed)

(continued)

Table 2 (continued)

Drug	Description	Intended use	Progress	References*
Pexiganan acetate [MSI-78] (MacroChem)	22 amino acid magainin derivative	Topical antibiotic	Phase III	NCT 00563394 (completed) NCT 00563433 Lipsky et al. (2008)
Opebacan [rBPI21] (Xoma)	A 21-amino acid peptide derived from BPI	Prevention of endotoxemia in patients undergoing stem cell transplant	Phase I	NCT 00454155 (terminated)
		Prevention of infection in burn injuries	Phase II	NCT 00462904 (completed)
Xoma-629 (Xoma)	A 90-amino acid peptide derived from BPI	Impetigo	Phase IIa	http://www.xoma.com
Iseganan [IB-367] (Ardea Biosciences)	Protegrin-1 derivative	Prevention of ventilator-associated pneumonia	Phase II/III	NCT 00118781 (terminated)
		Prevention of oral mucositis	Phase III	NCT 00022373 Elad et al. (2012)
OP-145 (OctoPlus)	LL-37 derivative optimized for LPS and LTA binding	Chronic ear infection	Phase II	ISRCTN 84220089 Press release from OctoPlus, 28 July 2008

*International clinical trial registration number as indexed on http://www.clinicaltrial.gov

BPI bactericidal/permeability-increasing protein, *LTA* lipoteichoic acid, *LPS* lipopolysaccharide, *IDR* immune defence regulator 1, *HLA* human leukocyte antigen, *MSH* melanocyte-stimulating hormone

that are highly efficient against *C. albicans* and *S. aureus* (van der Does et al. 2009). hLF1-11 is a unique immunomodulatory peptide delivered systemically to immunocompromised patients to prevent candidemia (Table 2). A phase I study on the tolerability and early efficacy of hLF1-11 in immunocompromised patients reported that repeated daily intravenous injections of this peptide were well tolerated despite the slight elevation of transaminases (Velden et al. 2009).

IDR-1, a synthetic derivative of bovine Bac2A that lacks direct antimicrobial activity, provides broad-spectrum protection against multidrug-resistant bacteria such as MRSA, vancomycin-resistant *Enterococcus* and *Salmonella enterica* (Scott et al. 2007). Both local and systemic administration of IDR-1 was effective in recruiting monocytes and macrophages. In addition, this synthetic peptide exerts anti-inflammatory effects by decreasing both TNF-α and IL-6 production, thus avoiding a hyperinflammatory state. This demonstrates that IDRs without any direct antimicrobial effects can still be effective therapeutics. Based on this data and the observation that IDR peptides work even in neutropenic animals (Scott et al. 2007), a five amino acid subsequence peptide, IMX942, recently underwent phase I clinical trials for prevention of unexplained fevers and infections associated with chemotherapy or induced neutropenia (Incorporated 2009).

Another cationic peptide currently undergoing clinical trials, RDP58, is derived from sequences of human class I MHC molecule, (De Vry et al. 2005). RDP58 inhibits the production of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-2, and IL-12 and appears to be a strong candidate as a treatment for inflammatory diseases such as ulcerative colitis (Travis et al. 2005). This peptide is currently being investigated in two phase II clinical trials for the treatment of moderate ulcerative colitis and Crohn's disease (http://www.genzyme.com).

4.4.2 Adjuvant Activity

In addition to their effects on the innate immune system, HDPs can be used as adjuvants in vaccine formulations to elicit appropriately balanced Th1/Th2 responses. Intranasal co-administration of human neutrophil α-defensins with ovalbumin (OVA) results in greater systemic production of OVA-specific immunoglobulin (Ig)G (but not IgA) and OVA-specific CD4+ T cells producing both Th1 and Th2 cytokines including IFN-γ, IL-5, IL-6, and IL-10 (Lillard et al. 1999). Others studies on mice immunized intranasally with the human β-defensins hBD-2 or with the human neutrophil peptide HNP and OVA demonstrated a higher level of OVA-specific serum IgG compared to the control as well as a reduction of IFN-γ production by the OVA-stimulated splenocytes from mice immunized with OVA and HNP or hBD-2 (Brogden et al. 2003). These studies demonstrate the ability of α- and β-defensins to act as adjuvants, promoting a balanced Th1/Th2 response.

Fritz et al. were the first to report an in vivo adjuvant effect for a synthetic antimicrobial peptide, $KLKL_5KLK$, when coadministrated with OVA or with commercial influenza vaccine (Fritz et al. 2004). In addition, mice immunized with $KLKL_5KLK$ and a DNA vaccine showed an enhanced Th1 response after

challenge with a virulent *Mycobacterium tuberculosis* strain compared to the mice immunized with the Bacille Calmette–Guerin vaccine (Li et al. 2008). The safety and immunogenicity of different formulations of the synthetic adjuvant IC31 containing $KLKL_5KLK$, oligodeoxynucleotide (ODN) 1 and microbial antigens, which comprise the novel TB vaccine, are being tested in two phase I trials (Table 2).

Similarly, the combination of the synthetic peptide IDR-HH2 with CpG ODN has exhibited excellent adjuvanticity. Intranasal immunization of mice with IDR-HH2/CpG together with pertussis toxoid as an antigen induced considerably higher production of toxoid-specific IgG1 and IgG2a antibodies than did the same antigen with either adjuvant component alone, demonstrating a mixed Th1/Th2 response (Kindrachuk et al. 2009). Furthermore, the double adjuvant proved protective. Ex vivo studies demonstrated that the IDR-HH2/CpG combination induced a strong chemokine release without any significant production of pro-inflammatory cytokines such as TNF-α (Kindrachuk et al. 2009). In addition, using the model antigen OVA, combination of CpG ODN with polyphosphazene (PP) and the indolocidin peptide enhanced antigen-specific antibody and cell-mediated immune responses (Kovacs-Nolan et al. 2009a). This three-component adjuvant complex seems to improve the delivery of both antigen and adjuvants to antigen-presenting cells, such as DCs, resulting in their maturation and in production of cytokines promoting a type 1 response. Moreover, substitution of proline residues in indolicidin with arginine (peptide IN50) greatly enhanced OVA uptake by bone marrow-derived DCs (BMDCs) (Kovacs-Nolan et al. 2009a). The CpG/Indol/PP formulation was also studied in co-formulations with the recombinant truncated bovine respiratory syncytial virus (BRSV) fusion protein (ΔF) (Kovacs-Nolan et al. 2009b). Mice immunized with the vaccine ΔF/CpG/Indol/PP and BRSV challenged exhibited a significant reduction of Th2 cytokines in the lung, while IFN-γ, which plays an important role in establishing a protective Th1 response in BRSV infection, was increased (Kovacs-Nolan et al. 2009b). The neonatal immune system is functionally skewed toward a Th2-type response (Siegrist 2001, 2007), and the combination of CpG/PP with peptide may prove useful in the development of vaccines that induce a balanced Th1 response in infants. Co-formulation of pertussis toxoid with CpG/PP/IDR-HH18 induced a strong and long-lasting IgG2a response in neonatal mice, confirming the ability of the formulation to increase the Th1 response (Gracia et al. 2011). IgG2a titers detected after a single immunization were greater than those induced after immunization with the commercial vaccine, suggesting that the number of immunization can be substantially reduced. These promising results could aid in the design of new vaccines that are safer and more effective.

4.4.3 Wound-Healing Activities

It has been well established that HDPs play an important role in wound healing. As a result, HDPs and synthetic peptides are attractive candidates as therapeutics intended to improve would repair. Originally derived from the antimicrobial

cecropin B, HB-107 is a synthetic peptide lacking antimicrobial activity. In a murine model, it promotes keratinocyte proliferation and leukocyte infiltration resulting in accelerated wound repair (Lee et al. 2004). The synthetic peptide IDR-1018, selected from a library of bactenecin derivates, was shown to promote wound healing in mice (in submission). Although more research into the wound-healing properties of HDPs is required for them to move into the clinic, the results thus far provide a promising new avenue of study.

5 Challenges in the Development of Synthetic HDPs as Therapeutics

Drug development starts with the identification of optimized peptides. To exploit synthetic peptides as therapeutics for clinical use, there are certain issues to consider regarding their cost of manufacturing, toxicity, and stability while avoiding bacterial resistance for direct antimicrobials.

5.1 Cost of Manufacturing

To enable the commercial development of HDPs and their testing in a clinical setting, a cost-effective method to produce and purify large amounts of peptides is required. Current methods of solid phase using fluorenylmethoxycarbonyl (FMOC) chemistry synthesis need costly precursor components, making the price per dose quite high. Development of a commercial-scale peptide production platform is non-trivial and is difficult to implement; however, there have been some recent successes. Novozymes Inc. has developed a fungal-based system that is able to produce the peptide Plectasin in large scale and with high purity (Mygind et al. 2005). Bommarius et al. have described a method allowing the high-yield production of both antimicrobial and immunomodulatory peptides using a fusion protein partner (Bommarius et al. 2010). This procedure was efficient for the production and purification of peptides with ranging in length from 9 to 37 amino acids. The enzyme sumoase is employed to cleave the peptide from its fusion partner. Sumoase is cheap, easily produced, and purified, reducing the cost of production. Cleavage occurs at a precise site, leaving no unwanted amino acids at the N terminus of the peptide. In addition, this method is easily scalable for industrial application since it requires a simple two-step purification protocol (Bommarius et al. 2010). While these kinds of procedures greatly contribute to reducing the cost of manufacturing, strategies enhancing stability, and reducing the size and the number of doses necessary to obtain therapeutic benefit must be studied.

5.2 Biological Instability and Toxicity

The sensitivity of peptides to proteolytic degradation is also a concern, as it decreases their biological half-life. Several methods have been proposed to enhance in vivo stability of HDPs such as the synthesis of peptides in tetrabranched form, four peptides linked by a lysine core, making them highly resistant to proteolytic degradation (Pini et al. 2010). Construction of peptide isomers is another approach to improve peptide resistance to proteolytic degradation. Fischer et al. exploited the inefficiency of proteolytic machinery to metabolize D-amino acid polypeptides by creating a D-amino acid isomer of RDP58, which enhanced its stability (Fischer 2003). Given that the spatial conformation of the HDPs is important to their interaction with receptors, the reverse sequence of this D-amino acid peptide was generated to maintain its initial spatial positioning. This retro-inverse (RI) strategy successfully recapitulated the function of the parent peptide and has now been used to improve the stability of several peptides such as Bac2A. Comparison of L-, D-, and RI-Bac2A showed that RI-Bac2A is more resistant to proteolytic degradation (Scruten et al. 2010). This retro-inverse method offers a mechanism to stabilize HDPs, improving their antimicrobial and/or immunomodulatory capacities. Moreover, this approach seems to reduce toxicity effects of HDPs allowing full exploitation of their therapeutic potential.

Due to their amphipathic fold, peptides interact with the lipid bilayers of pathogens, disrupting pathogen membranes or inhibiting cytosolic targets (Powers and Hancock 2003). These non-specific interactions confer broad-spectrum antimicrobial activity to HDPs but are partly responsible for cytotoxicity observed when they interact with mammalian cell membranes (Johansson et al. 1998). Appropriate formulations and routes of administration must be investigated to overcome this toxicity. Although some peptides such as IDRs are protective in animal models of infections with low in vivo toxicity (Scott et al. 2007), most of HDPs in clinical trials are topically applied, while the systemic route is almost never used. Several studies demonstrate that the hemolytic activity of peptides, which is a main cause of peptide toxicity, is dependent upon high hydrophobicity, high amphipathicity, and high helicity of the peptide (Chen et al. 2005; Oren and Shai 1997). Substitution of L-amino acids by D-amino leads to reduced hemolytic and cytotoxic activities (Kondejewski et al. 1999; Oren et al. 1997; Oren and Shai 1997). Similarly, retro-inversion of the bovine cathelicidin BMAP28 enhances its stability and reduces its toxicity while maintaining both its antimicrobial and immunomodulatory properties (Kindrachuk et al. 2010).

The transition from animal models to clinical use should be considered a potential point at which unexpected effects of HDPs may be observed. At the heart of this issue is our incomplete understanding of not only the human immune system but also those of the animal models used in preclinical research. Rodents are primarily used to provide information about in vivo activity, formulation, and dosages, but none of these models fully reproduce the immune response occurring in human diseases. Genetically modified mouse strains are useful tools to study

human diseases such as cystic fibrosis (CF); however, the disease is often not fully recapitulated (Carvalho-Oliveira et al. 2007). Cystic fibrosis transmembrane conductance regulator (CFTR)-deficient mice do not reproduce all the abnormalities of the pancreas, lung, intestine, and liver seen in CF, and questions regarding the pathogenesis of the disease remain. Therefore, a CFTR-deficient pig model has been developed which closely matches the human disease (Rogers et al. 2008). Recent publication reported that CFTR-deficient pigs spontaneously developed features of CF lung disease with airway inflammation, mucus accumulation, and infection (Stoltz et al. 2010). This model will contribute significantly to the evaluation of new drugs to treat this disease. Toxicity studies in relevant animal models and in ex vivo studies on human material assist in predicting risks and dose ranges when crossing the species barrier and have already contributed to the successful development of some immunomodulatory drugs currently on the market.

5.3 Resistance

Direct antimicrobial activity raises the concern for the development of peptide resistance in pathogens if peptides are developed for widespread use as therapeutics. Indeed, a few bacterial species already exhibit resistance to peptides (Kraus and Peschel 2008; Nizet 2006; Peschel and Sahl 2006). However, unlike classical antibiotics, antimicrobial peptides possess diverse targets and mechanisms of action, constituting an opportunity to decrease the direct selective pressure on pathogens (Peschel and Sahl 2006). In addition, utilization of immunomodulatory HDPs also limits potential for development of resistance as they act on the immune system rather than via direct antimicrobial activity (Nijnik et al. 2010; Scott et al. 2007). Administration of peptides as therapeutics might promote resistance to endogenous HDPs (Bell and Gouyon 2003). However, use of the bacterial peptide nisin as a food preservative has not impacted on the capacity of our immune system to fight bacterial infections (Bell and Gouyon 2003; Hancock 2003). Only a small number of bacteria are naturally peptide resistant, and there is no evidence that resistance mechanisms are easily acquired (Hancock 2003).

6 Conclusions

One of the challenges facing modern medicine is the emergence of antibiotic-resistant bacteria and the limited number of new drugs. The powerful potential of natural and synthetic HDPs opens new avenues to treat multi-resistant bacterial diseases as well as viral, fungal, and parasitic infections. During the last few years, several attempts have been made to introduce immunomodulatory and antimicrobial peptides into clinical use with a moderate success. Some impediments to commercial and clinical development must be overcome for their widespread use as therapeutics.

Recently, issues concerning toxicity and instability of HDPs have begun to be resolved with research into retro-inverse peptides that exhibit reduced cytotoxicity and increased stability while maintaining their desired activities. Cost of manufacturing is another limitation to the commercial development of natural peptides and their derivates. Improving their biological activity and stability will reduce dose frequency and concentration, thus reducing manufacturing costs. Although more research is required, there have already been considerable advances in the resolution of these manufacturing issues. HDPs and their synthetic derivatives offer a novel alternative to the treatment of infectious diseases and inflammatory and autoimmune disorders. In addition, their immunomodulatory properties are being exploited through their use as novel vaccine adjuvants and stimulators of wound repair.

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Helping the Host: Induction of Antimicrobial Peptides as a Novel Therapeutic Strategy Against Infections

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Abstract Endogenous antimicrobial peptides (AMPs) are gene encoded and can be considered as our own antibiotics. These peptides represent an ancient system since they are widespread in nature and have been identified in invertebrates, vertebrates, mammals and also in plants. Defensins and cathelicidins are the main families of AMPs in mammals including humans. From an evolutionary point of view, AMPs have coevolved with microbes in specific niches and constitute an important parameter in host–microbe interactions. The development of bacterial resistance against classical antibiotics is a growing problem, and novel antimicrobial strategies are urgently needed. Here, we present a concept based on the idea of inducing endogenous AMP expression by small compounds, such as vitamin D and butyrate. The induction of multiple AMPs with different mechanisms of action would minimize the risk of bacterial resistance. Thus, such inducing compounds may open new avenues for pharmaceutical intervention in the treatment or prevention of infections. Additional novel targets for medical treatment may be identified by dissecting signaling pathways and regulatory circuits for induced expression of AMPs.

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1 Introduction

1.1 Antimicrobial Peptides Are Included in a Conserved Defense Concept

Gene-encoded antimicrobial peptides (AMP) are found among diverse life forms of eukaryotes from simple metazoan such as Hydra to complex mammals including humans (Bosch et al. 2009). There are even fungal species that are able to synthesize gene-encoded peptides, although fungi are better known for making antibiotics that are chemically distinct from peptides in order to defend themselves against bacteria. The first fungal peptide identified was plectasin, isolated from the saprophytic ascomycete *Pseudoplectania nigrella* (Mygind et al. 2005). More recently additional related fungal peptides were identified and found to be related to the animal defensins, indicating an ancient evolutionary origin for the mammalian defensins (Zhu 2008b). Furthermore, AMPs have also been identified in several plants (Sels et al. 2008). Characterized AMPs exhibit diverse structures but participate in a conserved defense concept. The expression at epithelial surfaces and phagocytes is the consensus among the diverse group of metazoans that are best studied. Epithelial cells are the first cells confronted by microbes, while phagocytes are the first cells recruited to the site of microbial challenge. Phagocytes contribute to various antimicrobial components including AMPs and amplify the first line of defense in metazoans. Thus, AMPs are included at the front of the host defense barrier. The fact that these peptides are widespread in nature emphasizes their importance and success in eukaryotic evolution. AMPs are grouped in families based on their primary structures, where defensins and cathelicidins constitute the main families of mammalian AMPs (Zasloff 2002). Defensins exhibit an extended evolutionary history and are present from fungi to humans, while cathelicidins appeared later in evolution and have only been found in vertebrates. The different peptide families show independent lineage-specific patterns, being expanded in some species but deleted in other. The comparison of different AMPs shows rapid divergence with alterations of primary structures, resulting in multiple forms that might reflect the evolutionary race with fast evolving microbes. In vertebrates AMPs are an important part of innate immunity that have influenced the coevolution with microbes and are still determinants of the niche for the natural microbial community. Thus, AMPs can be viewed as parameters of the intertwined evolution of host–microbe interactions. Importantly, this interaction is at many levels and influences not only defenses but also access to dietary energy for the host. The evolutionary time of AMPs assumes their inclusion in the platform that the advanced adaptive immunity was based on. The importance of AMP function in vertebrate defenses and immunity is established, and consequences of deficiency are known to result in susceptibility to infections (Nizet et al. 2001). Furthermore, an altered expression profile of AMPs, exemplified in transgenic mice, affects the composition of the microbial flora (Salzman et al. 2010). Importantly, this ancient

system has maintained the antimicrobial function, fending off microbes through millions of years of evolution. Thus, they are an example of a surviving defense system including different effectors that have been selected for in evolution. In contrast, the human use of classical antibiotics has resulted in widespread resistance that has evolved among bacteria only in decades, as a result of strong selection against a single antimicrobial target. Most likely the key to the antimicrobial success of AMPs is the sequence divergence and coexpression of multiple peptide forms together with defense proteins such as lysozyme and lactoferrin, lipocalin, and RegIII-gamma working together in synergy. This network builds a complex defense system, utilizing various signaling pathways (Cederlund et al. 2010; Gudmundsson et al. 2010). All these different defense effectors are directed toward vital functions or structure of the microbes, i.e., the cell wall, the cell membrane, or depletion of essential nutrients such as iron (Fe). Thus, there is no single simple solution for the microbe to escape the frontier of innate defenses. AMPs have affinity toward the bacterial cell membrane leading to membrane destabilization and loss of vital functions such as elimination of membrane potential with subsequent lysis of the microbe. A more specific target than the whole cell membrane was recently added for several defensins, which bind to lipid II and thereby disturb the synthesis of the microbial cell wall (Schneider et al. 2010). If this activity applies to other amphipathic antimicrobial peptides remains to be elucidated. The majority of AMPs are cationic with an amphipathic character, a quality that allows flexibility with respect to their primary structures but still maintaining the activity. This is one criterion allowing a rapid evolutionary divergence among AMPs. The outcome is that every species has its own repertoire of AMPs exemplified by the mammalian cathelicidins with one cathelicidin gene in humans, mice, and rats; three in horses; seven in sheep (Zanetti 2004); and 11 genes in pigs (Zhu 2008).

1.2 AMPs and the Normal Flora

The rapid divergence of AMPs has been an important host parameter, influencing initial defenses and making up defined barriers for the coevolving microbes. The composition of AMPs also determines the complexity of the microbial community (Salzman et al. 2010). The microbe interaction with the host is multifarious, including the span from beneficial symbionts to harmful pathogens. These different host–microbe interactions follow different routes in selection, where the mammalian gut can serve as a prime example. Using genomic and metabolomic approaches, considerable research effort has resulted in characterization of up to 1,000 different microbial species in the human gut with an estimation of up to 100 trillion (10^{14}) microbial cells on individual basis. This research also highlights the importance of the relationship between the microbial community and human diseases such as obesity, diabetes, and inflammatory bowel diseases (Hooper and Macpherson 2010).

A substantial part of the microbes in the gut are symbionts, contributing to vital processes for the whole organism. For the host, the symbionts are an important metabolic adduct by degrading dietary carbohydrates, and hence, the host gains access to energy as well as the microbe. In addition, symbionts synthesize essential components for the host such as vitamin K. Symbiotic microbes exhibit metabolic adaptation and thrive in the luminal space of the gut. They are dependent on a number of hydrolyzing enzymes encoded in the bacterial genome that degrade complex carbohydrates (Hooper 2009). Thus, the symbionts do not need host tissues for energy and are kept outside the internal milieu of the body by the epithelia.

Recently, an interesting opinion has been presented with respect to metabolic adaptation of characterized symbionts in comparison to virulent pathogens. Pathogens do not show the advanced metabolic adaptation; instead, they need access to host tissues for their survival (Hooper and Macpherson 2010). In order to break through the epithelial barrier, pathogens have evolved virulence systems and/or factors. In this process, the pathogens meet obstacles of the epithelial barrier, where AMPs are included as one part. Upon challenge the peptides are mobilized by protease cleavage of an inactive precursor or released from storage in the mucus or vacuoles. Thus, the activation is rapid and immediate. AMPs limit the access to tissues for the pathogens and define a protective zone. During evolution the pathogens have adopted approaches in order to escape initial defenses of the host such as degrading the peptides by secreted proteases, i.e., *Streptococcus* (Frick et al. 2003), modifying phospholipids rendering resistance through reduced AMP affinity, i.e., *Salmonella* (Prost et al. 2007), downregulating peptide expression, i.e., *Shigella* (Islam et al. 2001; Sperandio et al. 2008), and utilizing advanced pump systems, i.e., *Neisseria* (Shafer et al. 1998). By these strategies, pathogens are more resistant and can break through the complex defense barrier of the host. Additional factors of importance include the nutritional status of the host, where components of the diet influence the level of AMP expression directly or indirectly such as Zn, vitamin D_3, and butyrate (Steinmann et al. 2009; Talukder et al. 2011). Thus, the prediction is that the barrier may be strengthened by increased production of AMPs. The diverse virulence mechanisms for different pathogens underline the importance of complete gene characterization for every pathogen. Detailed knowledge of host–microbe interaction is also essential to be defined in addition to environmental signals at different epithelial surfaces.

A key question in this connection is if a strategy can be designed and utilized in therapy based on neutralizing pathogenic offense such as counteracting the downregulation of AMPs or inhibiting microbial proteases that degrade these defense effectors. If the escape routes are blocked, the pathogens will be exposed to the full arsenal of the innate defense barrier and can be eliminated in an efficient way. In this chapter, we will discuss the regulation of AMPs associated with innate defenses. Furthermore, inducers that are able to enhance the production of AMPs and can be drug candidates will also be discussed. Maybe a combination of compounds, where one inhibits virulence and the other induces AMP production, is an optimal solution? A strategy, where we can induce and control the production

of AMPs at tissue sites of pathogen entry, has the potential to be a therapeutic complement to classical antibiotic treatment. A system that has survived during evolution will most likely not have the problem with microbial resistance that is an increasing problem in modern healthcare.

2 Regulatory and Signaling Pathways of Endogenous Antimicrobial Peptides

The expression of defensins and cathelicidin in human tissue is constitutive or induced by external and/or internal signals. The induced AMP expression is included in the enhanced defense programs initiated upon exposure to danger signals, sensing the presence of bacteria, or intrinsic signals from pro-inflammatory cytokines.

The constitutive expression of AMPs is a part of cellular differentiation and thus included in the normal developmental program of cells and organisms. In insects, the link between development and immunity is recognized, where the transcription factor dorsal is important not only in the development of the body axis but also a key factor in induced immunity (Hoffmann and Reichhart 2002). This transcription factor in *Drosophila melanogaster* is indeed related to the mammalian transcription factor NF-κB, a key regulator of inflammation in mammals.

In bone marrow, AMP genes are constitutively expressed with transcription in progenitor cells of mainly neutrophils and eosinophils. Indeed, neutrophils are the major innate defense cells in blood with a high turnover rate. The human α-defensins HNP1–4 are estimated to constitute 20 % of total vacuolar proteins and are thus major components of neutrophils (Ganz 2003). The proforms of defensins are stored in granules of mature neutrophils cells and are immediately available for processing in order to eliminate microbial intruders. The human cathelicidin is also present in neutrophils but in lower amount compared to α-defensins. The cathelicidin proform hCAP18 has been suggested to be secreted from the neutrophilic progenitors into the bloodstream and to be the main source of hCAP18 found in plasma (Borregaard 2010).

The constitutive expression of AMPs in bone marrow leading to storage in or secretion from neutrophils is included in the differentiation program of these cells. This applies also to AMPs of the Paneth cells in the crypt of Liberkühn in the small intestine. Paneth cells are derived from the crypt stem cells and are unique with respect to the expression of α-defensins, since human defensins 5 and 6 (HD5 and 6) are only present in this cell type, representing a lineage-restricted expression. This cell-specific transcription resides mainly in the proximal promoter that is incorporated in the humanized HD5 mouse, resulting in Paneth cell-specific expression of HD5 in the *DEFA5*-transgenic mice (Salzman et al. 2003). Paneth cells express many other defense proteins in addition to the HD5 and 6 including lysozyme, secretory phospholipase A2, RegIII-gamma, and matrix

metalloproteinase 7 (MMP7), in addition to pro-inflammatory mediators such as interleukin-17A (IL-17A), tumor necrosis factor-alpha (TNF-α), and IL-1β (Ouellette 2010). The defense function of Paneth cells protects the niche of the stem cells in the crypt, and secreted factors of Paneth cells affect the composition of the microbial community in the gut (Salzman et al. 2010). Indeed, the Paneth cells represent the defense concept with multiple effectors working in synergy. During development, the progenitors of Paneth cells enter the base of the crypt in a process governed by the Wnt signaling pathway, influencing HD5 and 6 expression through the transcription factors β-catenin and T-cell factor-4 (TCF-4) (Ouellette 2010). These transcription factors are included in the differentiation program and contribute to the constitutive expression of innate effectors independent of bacterial components, since this expression takes place also in germ-free mice (Ouellette 2010).

In contrast to the α-defensins, the β-defensins are mainly expressed in epithelial cells. Transcription factors of importance for the expression of β-defensins have not yet been characterized in details except for NF-κB that is essential for the induced expression of β-defensin 2 (O'Neil et al. 1999; Duits et al. 2002). In cell lines, the expression of β-defensin-1 (hBD-1) has been linked to c-Myc regulation and certain binding sites in the promoter connected to regulators of circadian rhythms (Sherman and Froy 2008). This regulation might reflect the *in vivo* situation of the constitutive epithelial expression of hBD-1.

The expression pattern of the human cathelicidin LL-37 seems unique, since it is expressed both in pro-granulocytic cells (undifferentiated neutrophils and eosinophils) and epithelial cells. Essential transcription factors, activating the *CAMP* gene encoding LL-37, have only been partially defined. The initial study indicated several binding sites in the promoter of the *CAMP* gene, and an enhancer element was identified in intron 2 (Gudmundsson et al. 1996; Termen et al. 2008).

PU.1 is one transcription factor that is related to AMP gene expression and was first indicated to be important for the expression of α-defensins of neutrophils (Ma et al. 1998). Notably, PU.1 is essential for myeloid lineage commitment, and together with C/EBP, it is a determinant for the monocytic or granulocytic decision (Borregaard 2010). In relation to cathelicidins, PU.1 was first found to regulate the prophenin gene in pigs (Ramanathan et al. 2005). PU.1 is also crucial for epithelial expression of LL-37, where it is involved in both constitutively and induced expression by butyrate (Termen et al. 2008). Thus, both PU.1 and C/EBP are examples of transcription factors, essential for development and immunity.

Functional importance of transcriptional regulation of AMP encoding genes was first reported, when a conserved binding site for a C/EBP family member in mouse and human promoter of the cathelicidin genes was studied. Subsequently, it was found that the C/EBPε knockout mice did not express cathelicidin in bone marrow and suggested to contribute to the susceptibility of these mice to gram-negative bacteria (Verbeek et al. 1999). Later, binding of C/EBPε to the *CAMP* promoter was confirmed in expression studies in the monocytic cell line U937 (Gombart et al. 2005). In this study, binding of the vitamin D receptor (VDR) to the *CAMP* gene promoter was also established. The effects of vitamin D in relation to the induction of AMPs will be discussed below. VDR is a nuclear receptor binding 1,25

dihydroxyvitamin D_3 and lithocholic acid (LCA). Recently additional ligands of VDR have been identified such as ω3- and ω6-essential polyunsaturated fatty acids (PUFAs), i.e., docosahexaenoic acid (DHA) and arachidonic acid, respectively, and the vitamin E derivative γ-tocotrienol (Haussler et al. 2008). Additional nuclear receptors directly linked to AMP expression are farnesoid X receptor (FXR) (D'Aldebert et al. 2009) and peroxisome proliferator-activated receptor gamma (PPAR-γ) (Dai et al. 2010). FXR regulates *CAMP* gene expression and has been claimed important for biliary tract sterility and partially explaining the beneficiary effects of ursodeoxycholic acid (UDCA) against inflammatory biliary disease. The induced expression of hBD-3 and cathelicidin is also regulated by PPAR-γ signaling via AP-1 and p38 activity (Dai et al. 2010).

The involvement of nuclear receptors in innate immunity emphasizes the recent linkage to the diet, where ligands to these receptors are derived from the diet, mediating effects on innate defenses. Interestingly, nuclear receptors are direct targets of several drugs that are utilized in therapy today, such as the PPAR-γ agonist pioglitazone, which is FDA-approved for use in diabetes type 2 patients (Gillies and Dunn 2000).

Intracellular mediators of signals linked to AMP expression upstream of the transcription factors have also been identified. By using specific inhibitors, it has been shown in several studies that signaling pathways, involving MAP kinases, are central for *CAMP* gene expression (Schauber et al. 2003; Termen et al. 2008). It is clear that the current knowledge of signaling pathways associated to AMPs is fragmentary. However, the emerging picture indicates signaling modules including nuclear receptors and MAP kinases that may represent adaptation to bacterial challenge. This might be a result of positive selection in host cells, avoiding bacterial strategies to escape innate effectors by turning off signaling pathways. Despite variation in the structure of AMPs, where divergence is the hallmark, the regulation is linked to vital conserved processes, and hence, one would expect similar regulatory circuits in various species but linked to different effectors.

2.1 Epigenetic Control of AMP Expression

In gene regulation, the structure of chromatin is an additional regulatory level, determining the accessibility of transcription factors to specific binding sites, and controlled by histone modifications. Recently, this has been studied in relation to effectors of innate immunity upon LPS response and tolerance in mouse macrophages (Foster et al. 2007). Genes of innate effectors can be divided into two categories with respect to secondary responses, i.e., silenced or amplified. The mouse cathelicidin gene was demonstrated to belong to the latter category with enhanced secondary response, indicating transcriptional memory. This epigenetic control may be viewed as an adaptation to repeated infections and is relevant associated to immune regulation (Foster and Medzhitov 2009) (Fig. 1).

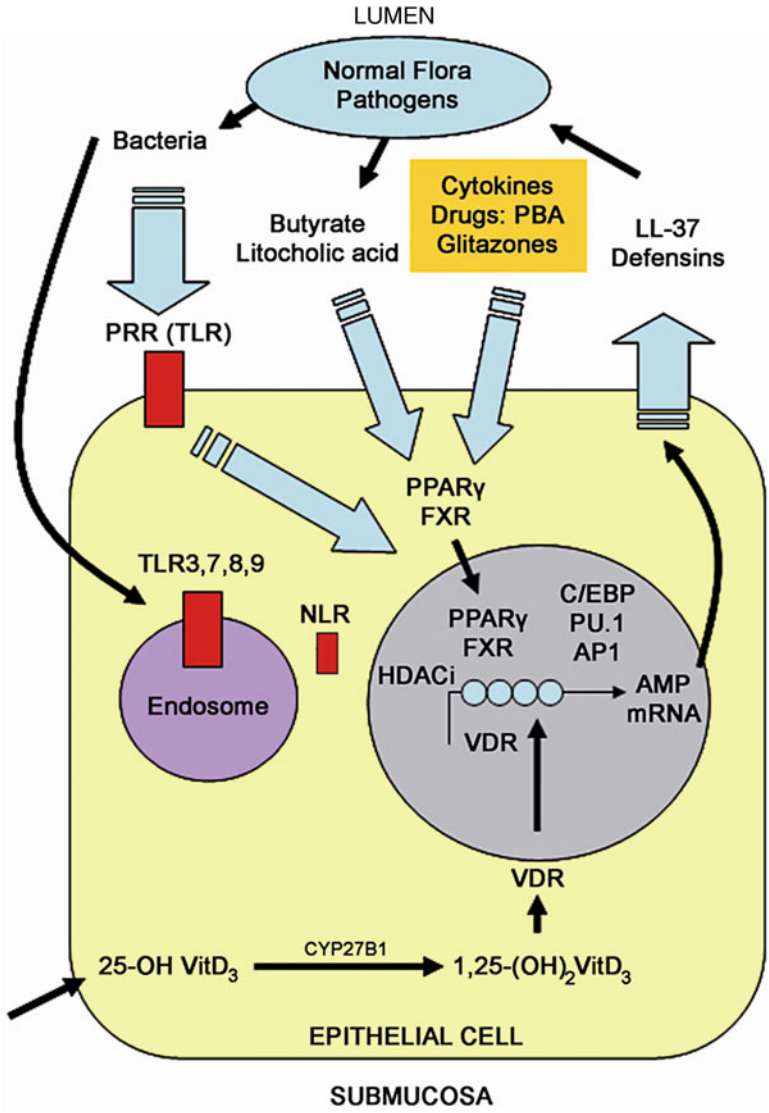

Fig. 1 *Inducers, receptors, and effectors in host-bacterial cross talk*. This schematic figure describes the main components discussed in this chapter. Bacteria carry chemical signatures, which are recognized by pattern recognition receptors (PRRs) on the cell surface or on the endosome, whereas NLRs are located in the cytoplasm. Commensal bacteria produce butyrate and lithocholic acid; the latter binds FXR in the cytosol. In addition, cytokines and exogenous drugs can affect AMP expression, a response that is mediated via specific receptors. Additional transcription factors, such as C/EBP, PU.1, and AP-1, have also been shown to be involved in AMP expression. The storage form, 25-OH vitamin D_3, is converted by 1-alpha-hydroxylase (CYP27B1) to the active form 1,25-$(OH)_2$-vitamin D_3, which binds to the vitamin D receptor (VDR). *TLRs* Toll-like receptors, *NLRs* nucleotide oligomerization domain-like receptors, *HDACi* histone deacetylase inhibitor and *PBA* phenylbutyrate

3 Inducers of Antimicrobial Peptide Expression

The production of AMPs can be stimulated by bacterial products and cytokines, leading to signals that induce clearance of microbes and hence prevent infection. Initially it was reported that IL-1alpha stimulation or an infection with enteroinvasive *Salmonella* upregulated the expression of human β-defensin-2 (hBD-2) in colon epithelial cells (O'Neil et al. 1999). The human β-defensin-1 (hBD-1) was found to be constitutively expressed and not affected by cytokine stimulation or infection. A similar pattern was demonstrated in gastric epithelial cells, where hBD-2 is induced by IL-1alpha or infection with *Helicobacter pylori* (O'Neil et al. 2000). Also in these gastric cells, hBD-1 exhibited a constitutively expression. Later in another study, LL-37 was demonstrated to be induced by *H. pylori* in gastric epithelial cells (Hase et al. 2003). In human tracheobronchial epithelial cells, the induction of hBD-2 was shown dependent on TLR-2 by bacterial lipopeptides (Hertz et al. 2003). Also the bacterial products lipopolysaccharide (LPS) and lipoteichoic acid increased the production of LL-37 in sinus epithelial air-liquid tissue culture (Nell et al. 2004). A role of hBD-2 in CNS pathogenesis was indicated when astrocytes in culture were treated with LPS, IL-1beta, or TNF-α with subsequent production of hBD-2 (Hao et al. 2001). We found the mouse cathelicidin upregulated in the blood–brain barrier by *Neisseria meningitidis* infection (Bergman et al. 2006). However, the role of the mouse cathelicidin was not linked to CNS protection; although, the number of bacteria in blood was significantly increased in the cathelicidin-deficient mice (Bergman et al. 2006). Recently, it was shown that flagellin from *Pseudomonas aeruginosa* stimulated the secretion of both LL-37 and hBD-2 in corneal epithelial cells (Gao et al. 2010). Flagellin binds to TLR-5 and trigger inflammatory responses via NF-κB in different epithelial cells (Ramos et al. 2004). Notably, flagellin has been demonstrated to induce a protective response against *Pseudomonas aeruginosa* that is dependent on the release of cathelicidin from lung epithelial cells (Yu et al. 2010). Furthermore, the peptide LL-37 in lung epithelia activates a metalloprotease that in turn liberates a ligand to the epidermal growth factor receptor (EGFR). Subsequently the EGFR dimerizes, resulting in intracellular MEK activation (Tjabringa et al. 2003). This links antimicrobial activity and epithelial repair in one response. Importantly, AMPs have evolved additional functions that are receptor mediated and relevant to defenses such as chemotaxis, angiogenesis, and cell proliferation. These properties are also fundamental in wound healing and tissue repair.

Interestingly, some pathogens escape elimination by the antimicrobial defense system at epithelial surfaces by downregulating the expression of AMPs (Islam et al. 2001). The downregulation represents an initial step in breaking through the mucus for the pathogen to gain access to the epithelial cells. By this strategy, pathogens come into proximity to the epithelial cell membrane, utilizing their virulence tools such as the type-three-secretion system. In this connection, inducers of AMP expression are of interest as they counteract the pathogenic downregulation of AMPs, and the pathogen cannot interact with the cell. Instead, the pathogen is killed by the AMPs present. The short-chain fatty acid butyrate was one of the first

molecules that were found to exhibit these inducing properties (Hase et al. 2002; Schauber et al. 2003). Interestingly, butyrate was earlier shown to affect clinical symptoms and decrease inflammation and bacterial load in experimental shigellosis in a rabbit model (Rabbani et al. 1999). Later we demonstrated a causative link between butyrate and AMP expression in shigellosis (Raqib et al. 2006). The counteraction by butyrate resulted in pathogen elimination from epithelial surfaces and recovery of the rabbits from the infection (Raqib et al. 2006). In normal colon function, butyrate is a byproduct of dietary fiber digestion and contributes to the important communication between the host and the microbial community, regulating AMP expression. Detailed knowledge of the mechanism is unknown but butyrate is known as an inhibitor of histone deacetylase (HDACi). In relation to the *CAMP* gene regulation, the HDACi activity has been suggested to be direct (Kida et al. 2006). However, also a secondary effect has been indicated, where the initial effect is the induction of a protein needed for the expression of the *CAMP* gene in lung epithelial cells (Steinmann et al. 2009). In general HDACi affects the packing of chromatin, keeping it open, and hence giving transcription factors access to their binding sites on target genes. However, for butyrate an alternative or complimentary mechanism involves the G-protein-coupled receptors GPR43 and GPR41 that have been shown to be important in mediating butyrate responses (Brown et al. 2003). The GPR43 receptor has recently been confirmed important for inflammatory regulation in mouse models and was suggested as a molecular link between diet, gastrointestinal bacterial metabolism, and immunological inflammatory responses (Maslowski et al. 2009).

In a search for additional inducers, phenylbutyrate (PBA) was found to be an interesting candidate with pronounced activity. Butyrate is a foul-smelling compound, and in that respect, PBA is a more suitable drug candidate. In addition PBA is a registered drug for the treatment of urea cycle disorders, working as a scavenger for ammonia (Brusilow 1991; Burlina et al. 2001). This offers an opportunity to use PBA in clinical trials, since safety measures have already been carried out.

Further research showed PBA to improve recovery from shigellosis in the rabbit model (Sarker et al. 2011). In this study, we also observed a systemic effect of butyrate, indicating that the inducers are not only working locally but also enter the bloodstream, since the rabbit cathelicidin CAP-18 also was induced in lung epithelia (Sarker et al. 2011).

Interestingly 1,25-dihydroxyvitamin D_3, the hormonal form of vitamin D_3, has been promoted as a therapeutic approach against infections due to its ability to induce AMP expression. This bioactive form of vitamin D_3 also stimulated the *defB2* gene encoding the antimicrobial peptide hBD-2 in monocytes and neutrophils (Wang et al. 2004). It has also been reported that vitamin D induces the expression of LL-37 in acute myeloid leukemia bone marrow cells, keratinocytes, colon cancer cell lines, and bone marrow-derived macrophages (Gombart et al. 2005). Interestingly, activation of Toll-like receptors (TLRs) in human macrophages upregulates the vitamin D receptor (VDR) and the vitamin D_3 hydroxylase genes, leading to the induction of LL-37 with subsequent killing of intracellular *Mycobacterium*

tuberculosis (Liu et al. 2006). The 1-alpha-hydroxylase is responsible for generating the biological active form of vitamin D_3 by transforming the proform 25-hydroxyvitamin D_3 into 1,25-dihydroxyvitamin D_3. In the study of Liu et al., it was also shown that low levels of 25-hydroxyvitamin D_3 in sera of African-American individuals, who are more susceptible to tuberculosis, correspond to lower induction of LL-37 in macrophages (Liu et al. 2006). This indicates a link to low LL-37 levels and susceptibility to tuberculosis and that vitamin D plays a key role in the production of LL-37, which can kill mycobacteria. In a recent randomized multicenter trial of vitamin D_3 as adjunctive therapy for active tuberculosis, it was found that vitamin D_3 did not significantly affect time to sputum culture conversion in the whole study population, but it did significantly hasten sputum culture conversion in participants with a specific genotype of the vitamin D receptor (Martineau et al. 2011).

We have recently shown that PBA and the active form of vitamin D act synergistically in inducing the expression of LL-37 (Steinmann et al. 2009), which might be utilized in therapy. Most recently, it was demonstrated that 1,25-dihydroxyvitamin D_3 significantly enhances hBD-3 and LL-37 expression in keratinocytes. This upregulation was shown to be mediated via the nuclear receptor PPAR-γ where AP-1 and p38 activity was shown to be involved (Dai et al. 2010).

More recently, we have found that lithocholic acid (LCA) also induces antimicrobial peptide expression (Termen et al. 2008). LCA has some characteristics of both butyrate and vitamin D_3. It is a bacterial product like butyrate made by bacteria from bile acid in the colon, and it is a known ligand to VDR like vitamin D (Adachi et al. 2005). A bacterial-derived bile acid with affinity to VDR is possibly involved in the physiology of the gut, affecting the microbial flora.

4 Role of Endogenous Antimicrobial Peptides in Human Diseases

Already in 1997, we demonstrated that the human cathelicidin LL-37 is upregulated in keratinocytes of psoriatic lesions (Frohm et al. 1997). This observation was confirmed by Ong et al. (2002), also showing that hBD-2 was upregulated in the lesions of psoriatic patients. In contrast, a downregulation of both LL-37 and hBD-2 was shown in the lesions of atopic dermatitis. The authors claimed that the low expression of these defense peptides in atopic dermatitis was the reason why these patients very often are infected in their lesions. This is not the case for psoriatic patients, who rarely get infections in their lesions. Psoriasis is a multifactorial autoimmune disease with unknown etiology. The trigger for the induced expression of LL-37 and hBD-2 has not been resolved. Interestingly, it was recently demonstrated that LL-37 can drive autoimmunity in psoriasis by binding to self-DNA. The complex of LL-37 and self-DNA is sensed by plasmacytoid dendritic cells (DCs) as a danger signal, and hence, this complex is translocated to endocytic compartments, where it is recognized by Toll-like receptor 9 (TLR-9), and a production of α-interferon is initiated (Lande et al.

2007). Normally, TLR-9 recognizes bacterial and/or viral DNA present in the endosomes. If LL-37 is a transporter of self-DNA into the endosomes in other autoimmune diseases needs to be investigated.

LL-37 is expressed also in atherosclerotic lesions more specifically in macrophages and endothelial cells. Its involvement in this disease is unclear. However, LL-37 induces the expression of the adhesion molecule intercellular adhesion molecule-1 (ICAM-1) and the chemokine monocyte chemotactic protein-1 (MCP-1) in endothelial cells (Edfeldt et al. 2006). Since ICAM-1 and MCP-1 promote atherosclerotic lesion development, LL-37 may accelerate the disease progress under conditions of chronic inflammation. Furthermore, it is well established that leukotriene B_4 (LTB_4) a powerful pro-inflammatory lipid mediator is involved in the development of atherosclerosis (Funk 2005). Recently, we reported that LL-37 can activate human neutrophils to promote synthesis and release of LTB_4, and one mechanism behind this response is the ability of LL-37 to elicit translocation of 5-lipoxygenase to the nuclear membrane (Wan et al. 2007). On the other hand, LTB_4 can trigger the release of LL-37 in neutrophils, an effect that is mediated via the leukotriene B_4 receptor type 1 (BLT1) receptor (Wan et al. 2007). This cross talk between LTB_4 and LL-37 may constitute an important parameter in inflammatory diseases.

During certain conditions, the delicate balance between bacteria and host can be disturbed, which may result in clinical symptoms and disease such as Crohn's disease (CD). This clinical entity belongs to the inflammatory bowel disorders, where also ulcerative colitis (UC) belongs. However, these two diseases are distinctly different with the most obvious difference being that UC is strictly located to the colon, whereas CD can involve the entire gastrointestinal system from esophagus to rectum. A general hypothesis in CD pathogenesis is that gut bacteria are physically located too close and even adhere to epithelial cells, which drive a chronic inflammatory process (Sartor 2001). Several lines of research support the hypothesis that the normal flora is not recognized properly and a large genetic screen has identified mutations in NOD2 among a subgroup of patients with ileal CD (Hugot et al. 2001; Ogura et al. 2001). NOD2 is the receptor for muramyl dipeptide (MDP), which is a cell wall fragment shared by many bacterial species, including the normal flora. The downstream effect of a NOD2 mutation has been shown to result in a lack of Paneth cell defensin expression (Wehkamp et al. 2004). Indeed, Wehkamp et al. reported in 2004 that patients with ileal CD exhibited low expression of α-defensins in Paneth cells of the ileum (Wehkamp et al. 2004). Notably, the expression levels of eight other Paneth cell components remained unchanged, and the decrease of α-defensins was not observed in the colon of Crohn's patients, in UC, or in pouchitis, indicating a very specific effect on Paneth cell alpha-defensins in ileal CD (Wehkamp et al. 2005). However, later studies showed that NOD2 mutations only could explain a fraction of all CD cases, which suggested the involvement of other genes (Hugot 2006). Indeed, a decrease of the HD5 and HD6 regulatory gene TCF7L2 (TCF-4) has been demonstrated to be associated with ileal CD (Wehkamp et al. 2007). Another gene important for Paneth cell biology is the autophagy-related gene ATG16L1, which has been linked to ileal

CD (Hampe et al. 2007; Cadwell et al. 2008). Recently, a connection between NOD2 and the autophagic process has been established (Cooney et al. 2010; Travassos et al. 2010), suggesting that aberrant bacterial sensing and intracellular processing are tightly linked and that errors in these processes may result in CD. Finally, novel data has shown that vitamin D can induce NOD2 expression in monocytic cells and that vitamin D together with MDP—the ligand for NOD2 – synergistically increased expression levels of hBD-2 (Wang et al. 2010; Verway et al. 2010). Taken together, mutations in a gene encoding a bacterial sensing system (NOD2), in a Paneth cell–associated gene (TCF4) and in an autophagic machinery gene (ATG16L1) have been implicated in CD. The final effects of these mutations appear to involve a low expression of the antimicrobial peptides (HD5 and HD6), which result in a compromised barrier function. Thus, there is a solid rationale for therapeutic attempts to restore the antimicrobial capacity of the mucosal barrier with exogenous compounds such as vitamin D, PBA, and/or LCA.

5 Final Comments

In summary, several active molecular inducers have been identified, which can enhance the production of AMP, working together to strengthen epithelial repair and defenses. In animal models, we have demonstrated that several inducers affect elimination of enteroinvasive pathogens such as *Shigella*. Thus, these inducers are possible drug candidates against infections and might also be useful in preventive medicine. Due to induction of multiple factors with different mechanisms of action, the risk of developing bacterial resistance can be considered low. This is an important advantage of utilizing induced endogenous defense molecules to fight infections. Elucidating the components of constitutively and induced expression of AMPs may open new avenues for pharmaceutical intervention in the treatment of infections. Important motive for future research is to understand basic defenses and host pathogen interaction together with the interaction between the host and the natural bacterial flora. Further, a detailed knowledge about transcriptional pathways dictating AMP expression in different tissues and during specific infectious or inflammatory conditions is necessary.

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