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Antimicrobial Peptides and Innate Immunity

Springer

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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)

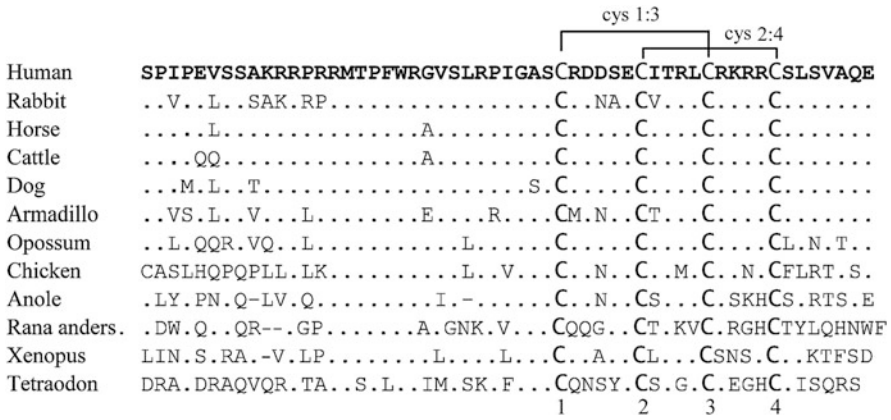

Fig. 1 Liver-expressed antimicrobial peptide-2 (LEAP-2). The sequence of human LEAP-2 is shown in its entirety. Residues that are identical to those in human LEAP-2 in the other peptides are represented by *dots*. Cysteine residues are numbered at the *bottom*, and their connectivity is shown at the *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

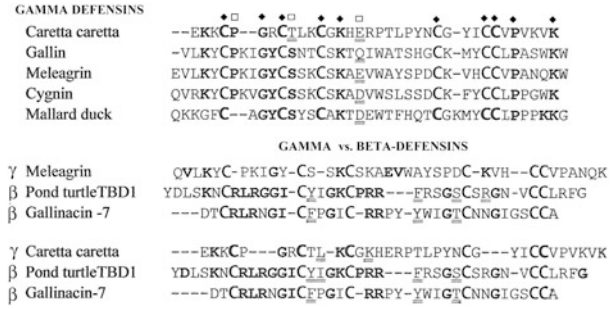

Fig. 3 Gamma defensins. Series I shows sequences of five γ-defensins, isolated from the white of various eggs. *Caretta caretta* is a turtle. Gallin, meleagrin, and cygnin came respectively from the eggs of chickens (*Gallus gallus*), turkeys (Meleagris gallopavo), and black swans (*Cygnus atratus*).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

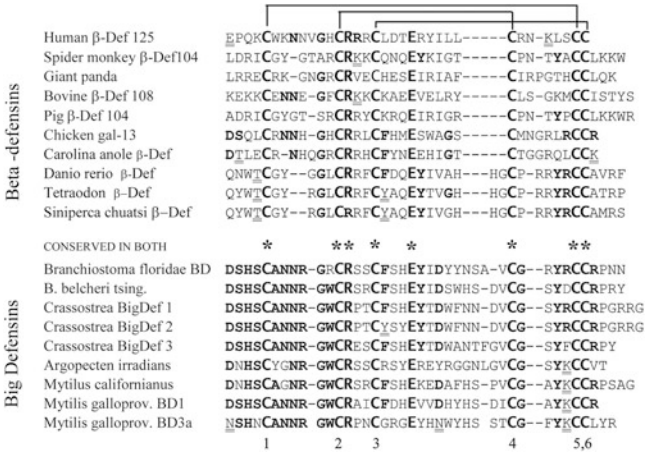

Fig. 4 Big defensins and beta-defensins. The upper ten sequences are of β-defensins from three fish (*Siniperca chuatsi*, *Tetraodon nigriviridis*, *Danio rerio*), a reptile (*Anolis californianus*), the chicken, (*Gallus gallus*), and five mammals. Residues (6 cys, 1 arg, 1 glu) shown in a larger font 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,