1
Antimicrobial Peptides: Their History, Evolution, and Functional Promiscuity

Summary

Eukaryotic antimicrobial peptides (AMPs) first became a research focus in the middle decades of the twentieth century with the description of cecropins from moths and magainins from frogs. Since then, the number of reported AMPs has burgeoned to over 2000 with representatives in virtually all eukaryotic organisms. The availability of databases of AMPs has facilitated phylogenetic analyses, which have shown that the origins of β-defensins are traceable to an ancestral gene over half a billion years old, indicating the evolutionarily ancient nature of AMPs. An emerging theme from research on AMPs is their multifunctional nature, which we review here for β-defensins, and show that these peptides play roles in wound healing and modulation of both the innate and adaptive immune systems via the dual ability to promote and suppress the proinflammatory response to microbial infection.

1.1
Introduction: The History of Antimicrobial Peptides

Antimicrobial peptides (AMPs) have been recognized in prokaryotic cells since 1939 when antimicrobial substances, named gramicidins, were isolated from *Bacillus brevis*, and were found to exhibit activity both *in vitro* and *in vivo* against a wide range of Gram-positive bacteria [1, 2]. Gramicidins were later shown to successfully treat infected wounds on guinea-pig skin, indicating their therapeutic potential for clinical use [3], and were the first AMPs to be commercially manufactured as antibiotics [4]. In the case of humans and other living creatures, which are constantly exposed to the threat of microbial infection, it had long been known that protection against these infections was provided by the adaptive immune system. However, this left the question as to why plants and insects, which lack an adaptive immune system, also remain free from infections for most of the time. The answer to this question is now known to be that similarly to prokaryotes, eukaryotes also produce AMPs and, historically, some sources attribute the discovery of eukaryotic AMPs to early work on plants [5] when in 1896 it was shown

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that a substance lethal to bread yeast was present in wheat flour [6]. At the end of the 1920s, lysozyme was identified by Alexander Fleming and is considered by some authors to be the first reported instance of a peptide with antimicrobial activity [7]. However, the mechanism of action used by lysozyme is now known to be enzymatic destruction of the bacterial cell wall, placing it at its time of discovery in a different category to AMPs, which utilize non-enzymatic mechanisms of antimicrobial activity [8, 9]. In 1928, Fleming discovered penicillin [10] and in the 1940s, along with Howard Florey and Ernst Chain, he brought the therapeutic use of penicillin to fruition, which led these three men to share the 1945 Nobel Prize for Medicine [11]. With the advent of penicillin and streptomycin in 1943, began the "Golden Age of antibiotics," which led to a rapid loss of interest in the therapeutic potential of natural host antibiotics such as lysozyme and the importance of this immune defense strategy [12, 13]. However, in 1942, the antimicrobial substance that had previously been detected in wheat flour [6] was isolated from wheat endosperm (*Triticum aestivum*) and found to be a peptide that inhibited the growth of a variety of phytopathogens, such as *Pseudomonas solanacearum* and *Xanthomonas campestris* [14]. Later named purothionin in the mid-1970s [15, 16], this peptide is now known to be a member of the family of thionins, which are AMPs distributed across the plant kingdom [5]. At the time this work was undertaken there was also the realization that the "Golden Age of antibiotics" had ended and with the rise of multidrug-resistant microbial pathogens in the early 1960s, an awakened interest in host defense molecules was prompted [17, 18]. It is this point in time that some sources consider to be the true origin of research into AMPs [19], beginning with studies that were conducted in the 1950s and 1960s, when it was shown that cationic proteins were responsible for the ability of human neutrophils to kill bacteria via oxygen-independent mechanisms – clearly not activity associated with the adaptive immune system [20, 21]. In 1962, in what some consider to be the first description of an animal AMP [22], bombinin was reported in the orange speckled frog *Bombina variegate* [23]. Also in the 1960s, the antimicrobial protein, lactoferrin, was isolated from milk [24] and small antimicrobial molecules were observed to be induced in the hemolymph of wax moth larvae after challenging with *Pseudomonas aeruginosa* [25]. In the late 1970s and 1980s several groups reported a number of AMPs and antimicrobial proteins from leukocytes [26], including what are now known to be α-defensins from rabbits [27–29] and humans [30]. Along with purothionin described above, these defensins were among the first cysteine-stabilized AMPs to be reported (Chapter 2). In 1981, in what are now generally considered as landmark studies, Boman *et al.* injected bacteria into the pupae of the silk moth, *Hyalophora cecropia*, and isolated the inducible cationic antimicrobial proteins, P9A and P9B, from the hemolymph of these pupae (Chapter 2) [31]. Soon after, these peptides were sequenced, characterized, and renamed as the more familiar "cecropins," thereby constituting the first major α-helical AMPs to be reported [32]. In 1987, another landmark study occurred when Zasloff *et al.* (1987) isolated and characterized cationic AMPs from the African clawed frog, *Xenopus laevis*, and, reflecting their defense role, named these peptides magainins after the Hebrew word for "shield" [33]. A few years

later, β-defensins and θ-defensins, which differ from α-defensins with respect to their cysteine pairings, were characterized after isolation, respectively, from bovine granulocytes [34] and leukocytes of the rhesus monkey [35]. In the mid-1990s, Brogden *et al*. identified the first anionic AMPs in *X. Laevis* [36] and characterized several other such peptides in ruminants, including sheep and cattle [37]. Ironically, also in the early 1990s, evidence began to accumulate that led to the current view that lysozyme possesses antimicrobial activity involving non-enzymatic mechanisms that are similar to AMPs, thereby substantiating the view that it was one of the first of these peptides to be discovered [8]. Based on these results, a number of investigators considered the possibility that AMPs may play a role in the defense systems of organisms lacking an adaptive immune system [38]. In the mid-1990s, this was confirmed for the fruit fly, *Drosophila melanogaster*, when it was shown that the deletion of a gene encoding an AMP rendered the insect susceptible to a massive fungal infection [39]. Since these earlier studies, AMPs have been extensively studied not only in plants [40, 41] and insects [42–44], but also other invertebrate organisms that lack an adaptive immune system [45–48] although most of the current understanding of AMPs has been obtained from studies on those isolated from amphibian skin secretions, which is a rich source of these peptides [49–52]. In combination, these studies have established that AMPs exist in virtually all multicellular organisms [53] and it is increasingly being recognized that that these peptides play an important role in the immune system of mammals, including humans [54–57]. These peptides have been identified at most sites of the human body normally exposed to microbes such as the skin and mucosae [54, 55], and are produced by a number of blood cell types, including neutrophils, eosinophils, and platelets [58–60]. However, as research into the expression of AMPs progressed, it became clear that the production of these peptides may be either constitutive or induced by inflammation or injury [38]. Typically, for example, α-defensins and dermcidin (the precursor of AMPs involved in skin defense) tend to be produced constitutively, whereas the majority of β-defensins are inducible [61–63]. Moreover, although particular AMPs may predominate at specific body sites only a small minority are exclusively produced by a certain cell type or tissue and each tissue has its own spectrum of AMPs that may vary in composition depending upon the prevailing physiological conditions [55]. For example, peptides derived from dermcidin are the major AMPs in human sweat but show differing profiles between the body sites of a given individual in response to exercise [64]. One question that has puzzled investigators since the discovery of AMPs is the fact that the minimal inhibitory concentrations (MICs) required for their *in vitro* antimicrobial activity are generally much higher than the physiological concentrations of these peptides found *in vivo* [65]. Two major explanations proposed for this observation are that at sites of inflammation, AMPs can accumulate at high local concentrations sufficiently above their MIC to exert their antimicrobial effect or that these peptides may act synergistically with other AMPs [65]. Over the last decade, these synergistic effects have been demonstrated for a variety of AMPs [66], including those that are structurally similar and from the same host organism, such as magainin and PGLa, which is another α-helical

peptide from *X. Laevis* [67, 68], and those that are structurally dissimilar and from differing host organisms, such as LL-37, an α-helical human peptide, and indolicidin, an extended bovine peptide (Chapter 2) [69]. Studies over the last decade have also established that some organisms produce AMPs as suites of closely related peptides that synergize to produce a broad spectrum of antimicrobial activity, such as maximins, which are α-helical AMPs produced in the brains of amphibians [70], and cyclotides, which are cyclic cystine knot AMPs produced in the leaves, flowers, stems, and roots of various plants [71, 72]. As more has been learnt about AMPs, it has become somewhat arbitrary as to their precise definition. For example, perforin and the complement component C9 are large proteins of approximately 60 kDa that under physiological conditions insert into membranes and form pores as result of highly regulated immune processes, and are thus not classified as AMPs [73, 74]. In contrast, lactoferrin, which is around 80 kDa, is generally included as an AMP, based on the fact that it is ubiquitous in various body fluids and utilizes a non-specific mode of antimicrobial action similar to other AMPs (Chapter 5) [75, 76]. Moreover, some "AMPs" do not appear to exert direct antimicrobial activity such as the PLUNC (palate, lung, nasal epithelium clone) proteins that appear to primarily play a role in neutralizing endotoxins, promoting the agglutination of bacteria, and modulating cytokine production [77]. Nonetheless, it is now well established that the production of AMPs is a defense strategy used across eukaryotes, evidenced by the list of databases dedicated to these peptides that have appeared almost every year over the last decade (Table 1.1). Examination of these databases shows that in excess of 2000 AMPs have now been listed and the number of these peptides being reported is increasing rapidly [87, 89]. The availability of these databases has allowed comparisons to be made between AMPs based on a variety of criteria, most often structure–function relationships and mechanisms of antimicrobial action, which are discussed in later chapters of this book. However, two less well discussed aspects of AMPs are reviewed in the remainder of this chapter, namely their ancient origins and evolution along with their functional promiscuity, encompassing a number of biological roles in addition to antimicrobial activity.

1.2 AMPs: Evolutionarily Ancient Molecules

Since the discovery of magainins in *X. laevis* [33], it has become clear that the skin secretions of many anurans include a spectrum of AMPs that ranges between 10 and 20 members [92–94] along with a variety of other bioactive peptides, including neuropeptides, pheromones, and neuronal nitric oxide synthase inhibitors [95–97], which has led to the availability of sequence data for these molecules at the levels of protein [91] and DNA [98–100]. Taken with the ubiquity of AMPs across the anuran suborders [94], this has facilitated investigation into the evolutionary history of these peptides, particularly those from frogs of the Ranidae and Hylidae families [96, 101–107]. In a seminal work, one of the earliest investigations

Table 1.1 Representative databases dedicated to AMPs.

Year	Database	Website	Content	Key reference
2002	AMSDb	http://www.bbcm.univ.trieste.it/~tossi/amsdb.html	Animal/plant AMPs	–
2002	SAPD	http://oma.terkko.helsinki.fi:8080/~SAPD	Synthetic AMPs	[78]
2003	NAD	http://www.nih.go.jp/~jun/NADB/search.html	General AMPs	–
2004	A/OL	http://www.atoapps.nl/AOLKnowledge/	Antimicrobial compounds	–
2004	Peptaibol	http://www.cryst.bbk.ac.uk/peptaibol/home.shtml	Fungal AMPs	[79]
2006	CyBase	http://researcht.imb.uq.edu.au/cybase	Plant AMPs	[80]
2006	PenBase	penbase.immunaqua.com	Shrimp AMPs	[81]
2007	BACTIBASE	bactibase.pfba-lab.org	Bacterial AMPs	[82]
2007	Defensins	defensins.bii.a-star.edu.sg	Defensins across eukarya	[83]
2007	AMPer	http://marray.cmdr.ubc.ca/cgi-bin/amp.pl	Animal/plant AMPs	[84]
2008	PhytAMP	phytamp.pfba-lab-tun.org	Plant AMPs	[85]
2008	RAPD	http://faculty.ist.unomaha.edu/chen/rapd/index.php	Recombinant AMPS	[86]
2009	APD2	http://aps.unmc.edu/AP	Natural AMPs	[87]
2010	CAMP	http://www.bicnirrh.res.in/antimicrobial	General AMPs	[88]
2012	YADAMP	www.yadamp.unisa.it	General AMPs	[89]
2012	DAMPD	http://apps.sanbi.ac.za/dampd	General AMPs	[90]
2012	DADP	http://split4.pmfst.hr/dadp/	Amphibian AMPS	[91]

into the phylogeny of these peptides considered the evolutionary relationships between AMPs from hylids of South America (HSAs) and hylids of Australia (HAs) along with ranids from Asia, North America, and Europe (RANAEs) [102]. Essentially, this latter study derived the amino acid sequences for precursor proteins of caerins, which are AMPs from *Litoria caerulea*, a member of the HAs [96, 102], and then aligned these sequences with those of precursor proteins from various HSAs and RANAEs [102], which have previously been shown to belong to a single family, the pre-prodermaseptins [52]. This alignment revealed that the precursor proteins from these three groups of frogs possessed highly conserved

N-terminal pre-prosequences of approximately 50 residues, including a 22-residue signal peptide and an acidic spacer region, that was linked to a hyper variable C-terminal domain. This domain corresponded to progenitor AMPs with great diversity in length, sequence, net positive charge, and antimicrobial spectra [102]. The strong conservation of these N-terminal pre-prosequences allowed molecular phylograms to be constructed, which showed that nucleotide sequences of pre-prodermaseptins from HSAs, HAs, and RANAEs formed three separate clusters. Further analysis of these phylograms indicated a key result: the genes encoding the pre-prodermaseptins in these clusters arose from a common ancestral locus, which subsequently diversified by several rounds of duplication and divergence of loci. Most of these duplication events appeared to have occurred in a species ancestral to the ranids and hylids, although gene duplication in *L. caerulea* appeared to have taken place after the divergence of HSAs and HAs [102]. To provide a temporal framework for the origins and evolution of the pre-prodermaseptin genes, data from phylogenetic analyses [101, 102] were used in conjunction with the historical biogeography of hylids and ranids, which is strongly linked with tectonic events that occurred during fragmentation of the supercontinent Gondwana to eventually form the modern day continents [108, 109]. This historical reconstruction showed that these genes arose before the isolation of India and South America from Africa in a pan-Gondwanan land mass, and therefore originated from an ancestral gene in excess of 150 million years old [101, 102]. Moreover, given that hylids and ranids belong to the Neobatrachia, which diverged from Archeobatrachia in early Jurassic times [110, 111], and that pre-prodermaseptins have not been detected in this latter suborder [112, 113], these observations suggested that the ancestral gene of HSAs, HAs, and RANAEs may be up to around 200 million years old [101]. Further analysis of cDNA from pre-prodermaseptins suggested that duplications of this ancestral gene accompanied by accelerated mutations in the AMPs progenitor region and the action of positive selection all appeared to be mechanisms that contributed to the hypervariability of their C-terminal domain, and hence the great diversity of modern day AMPs from HSAs, HAs, and RANAEs [102]. Interestingly, there was evidence to suggest that the diversity of these AMPs may have in part resulted from random substitutions involving the operation of a mutagenic error-prone DNA polymerase [102] similar to that reported for some bacteria [114–116]. Since the initial study [102], these results have been supported and extended by later studies, which have been facilitated by the growing repository of cDNA for AMPs of anuran species [101, 106, 107, 113]. For example, recent phylogenetic analyses have shown that the signal sequences of AMPs are highly conserved not only within lineages of the Neobatrachia, but also within those of Bombinatoridae and Pipidae from the Archeobatrachia. Although high divergence between the signal sequences of these three lineages was observed, there was evidence to suggest that the genes encoding AMPs in anurans had evolved convergently on at least three occasions in evolutionary time [113]. In another study, which compared the signal sequences of a range of bioactive peptides from anurans, phylogenetic analyses showed that caerulein neuropeptides produced by *Litoria* spp. have a different evolutionary origin to the

pre-prodermaseptins found in these frogs [105]. This latter study also showed that the profile of bioactive peptides produced by individual frogs from a range of species was sufficiently characteristic to form the basis of a diagnostic technique that was able to differentiate between subspecies and different population clusters of the same species and thereby provide insight into anuran evolutionary relationships. Use of this technique showed that the bioactive peptide profile of *L. caerulea* from mainland Australia differed strongly to that of the same species present on offshore islands that had been isolated for around 10 000 years, indicating that evolutionary change can be effected in a relatively short time in evolutionary terms [105]. Taken overall, these studies have led to the consensus view that the panopoly of AMPs produced by hylids, ranids, and other frogs represents the successful evolution of a defense system that maximizes host protection against rapidly changing microbial biota while minimizing the potential development of microbial resistance to these peptides [52, 101, 102].

In contrast to the peptides discussed above, which are produced by organisms of a single taxonomic order, defensins are AMPs that are produced by creatures across the eukaryotic kingdoms [48, 117–120]. In vertebrates these peptides have been identified in fish [121, 122], amphibians [92, 93, 96], reptiles [123, 124], rodents [125, 126], monotremes [127, 128], birds [129–132], marsupials [133], and mammals [134–136], including humans and other primates [137–139]. The defensins of vertebrates fall within the α-, β-, and θ-defensin groups described above, and are generally cationic, amphiphilic peptides that contain around 15–50 amino acid residues, including a conserved motif based on six cysteine residues that form three intramolecular disulfide bonds (Chapter 2). Both α- and β-defensins adopt triple-stranded antiparallel β-sheet configurations but whereas the former peptides form disulfide bonds via the linking of Cys1–Cys6, Cys2–Cys4, and Cys3–Cys5, the latter peptides form these bonds through links between Cys1–Cys5, Cys2–Cys4, and Cys3–Cys6 (Figure 1.1) [137, 138]. In contrast, θ-defensins are cyclized molecules of 18 residues that appear to be the product of a head-to-tail ligation of two truncated α-defensins and are the only known circular peptides of mammalian origin [140]. Defensins are also found in plants [141–143], fungi [144, 145], and invertebrates, such as insects [120, 146, 147], arachnids, including spiders [148, 149], ticks [150, 151], and scorpions [152, 153], crustaceans, including crabs [154–156] and lobsters [157], and bivalvia, including clams [158, 159] and mollusks [160–163]. These invertebrate peptides commonly adopt the cysteine-stabilized α-helical and β-sheet (CSαβ) fold, which consists of a single α-helix that is connected to a β-sheet formed from multiple antiparallel strands depending upon the number of disulfide bridges in the molecule [141, 164–167].

It has been proposed that all defensins evolved from a single precursor based on similarities in sequences, structures, modes of action, and the inter-functionality of these peptides derived from different kingdoms [120, 146, 168]. For example, plant defensins were found to be structurally similar to their insect counterparts [169] while some fungal defensins displayed high levels of sequence homology to those found in invertebrates [164, 166]. The identification of defensins in lower eukaryotes by these latter studies led to the suggestion that the ancestral gene of

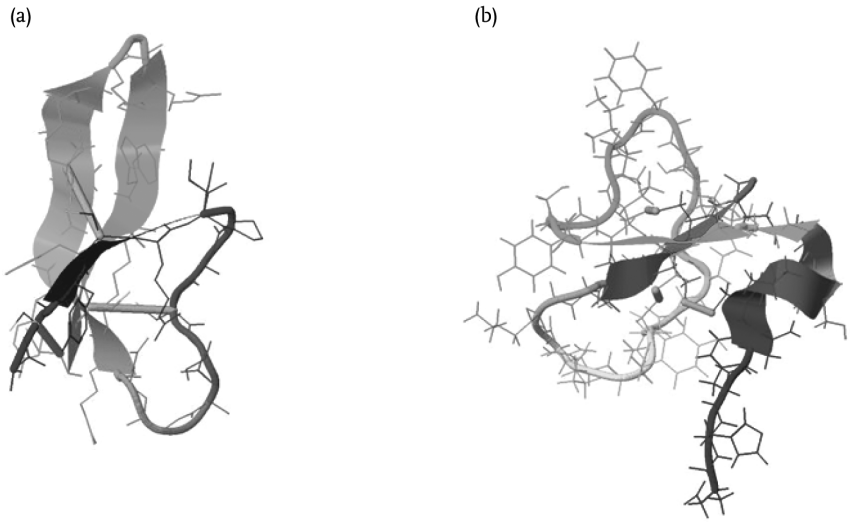

Figure 1.1 Structure of human defensins: three-dimensional structures of human α-defensin 1 (a) and β-defensin 1 (b). Both α- and β-defensins adopt triple-stranded antiparallel β-sheet configurations; however, whereas the former peptides form disulfide bonds via the linking of Cys1–Cys6, Cys2–Cys4, and Cys3–Cys5, the latter peptides form these bonds through links between Cys1–Cys5, Cys2–Cys4, and Cys3–Cys6.

these peptides existed before the fungal and insect lineages of the eukaryotic domain diverged, and may be therefore at least 1 billion years old [164, 166]. Consistent with these observations a conserved structural motif, the γ-core motif, was identified in cysteine-stabilized AMPs, including defensins, from organisms across the phylogenetic spectrum [170–172]. This motif was composed of two antiparallel β-sheets with an interposed short turn region and, based on its evolutionary lineage, it was proposed that defensins from mammals, plants, fungi, and invertebrates emerged from a common ancestor that can be traced back to prokaryotic origins and was therefore in the region of at least 2.6 billion years old [172, 173]. Strongly supporting this proposal, AMPs were identified in the myxobacteria *Anaeromyxobacter dehalogenans* and *Stigmatella aurantiaca*, which showed strong similarities in sequence and structure to fungal defensins, and it was suggested that these bacterial peptides may represent the ancestors of eukaryotic defensins [174, 175]. It has previously been hypothesized that myxobacteria played a central role in the endosymbiotic origins of the early eukaryotic nuclear genome [176, 177], and it was proposed that the transfer of myxobacterial genes encoding these ancestral defensins may have mediated the immune defense of early eukaryotes and thereby the lineage of modern day defensins [174, 175].

The evolutionary relationship between defensins from vertebrates and invertebrates is far from clear, but based on common sequence and structural features,

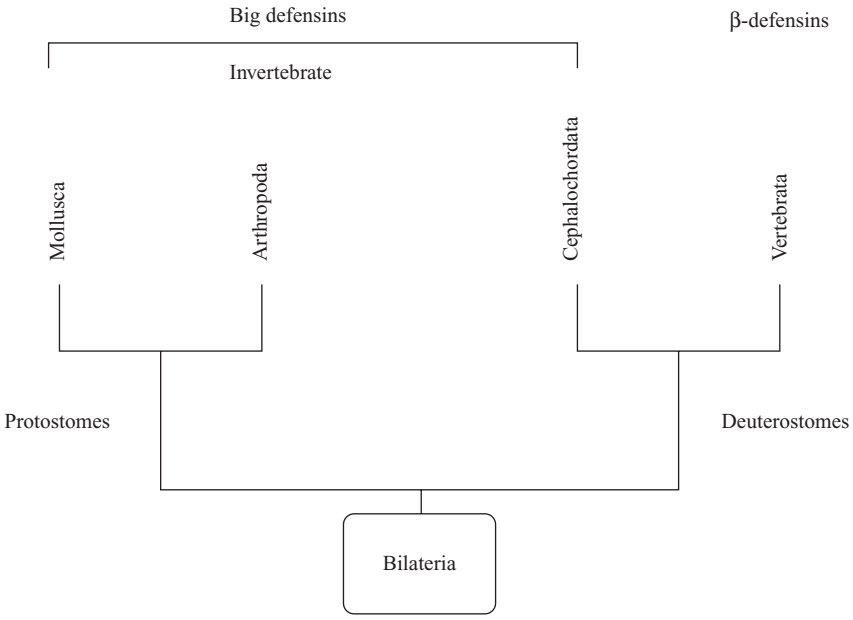

Figure 1.2 Evolution of β-defensins: phylogenetic tree indicating evolutionary relationships of bilateral animals that led to the emergence of β-defensins in the vertebrate lineage. This tree shows that the evolutionary history of these peptides to be traced back to Bilateria, indicating that they are in the region of at least 500 million years old [178–180].

there is evidence to suggest that some of the former peptides may have their origins in the "big defensins" (BDs), which have been identified in invertebrates, but not in vertebrates (Figure 1.2) [138, 181]. BDs are a family of evolutionarily conserved AMPs that were initially isolated from crustaceans [154–156], later identified in mussels [159, 160, 182–184], and most recently described in amphioxus [185], which are the closest invertebrate ancestors to vertebrates [178, 186, 187]. The presence of BDs in mollusks, which are Protostomes, and amphioxus, which are Deuterostomes (Cephalochordata), enables the evolutionary history of these peptides to be traced back to their common ancestor, Bilateria (Figure 1.2), indicating that they are in the region of at least 500 million years old [178–180]. BDs are between 79 and 94 residues in length, and comprise an N-terminal region that mediates activity against Gram-positive bacteria along with a C-terminal region that is active against Gram-negative bacteria [154, 181]. This C-terminal region has been shown to form a three-stranded β-sheet stabilized by three disulfide bridges that exhibits close structural homology to a number of a mammalian β-defensins, which also show selective activity against Gram-negative bacteria [138, 156, 181]. Taken with the fact that BDs and mammalian β-defensins show similar gene structures, the similarities in structure and bioactivity possessed by these two groups of AMPs suggested an evolutionary relationship with

β-defensins emerging from BDs through exon shuffling or intronization of exonic sequences [181, 188]. In relation to other mammalian defensins, α-defensins have only been detected in certain animals [189], such as humans [138] and horses [134], but were found to be absent from others, such as cattle [190] and dogs [191]. The underlying reasons for the selective expression of α-defensins in mammals is not well understood but clearly, these AMPs arose after mammals diverged from other vertebrates [192]. Primarily founded on the physical proximity of α- and β-defensins on the chromosome as well as similarities between their structures and biological activities, it is generally accepted that the former AMPs have evolved from the latter peptides via gene duplication and subsequent positive diversifying selection [126, 189, 193]. Based on the fact that they had only been identified in therians [194], it had been suggested that α-defensins were at least 130 million years old, arising before the divergence of placental mammals and marsupials [136]. However, the recent identification of α-defensins in the duck-billed platypus pushed their emergence back to before the divergence of monotremes and therians [128], which occurred around 210 million years ago [195]. It is generally believed that mutated α-defensin genes gave rise to θ-defensin genes [138], and these peptides appear to be only expressed in the rhesus macaque monkey [196] and baboons [197, 198]. In humans and their closest primate relatives, including chimpanzees, bonobos, and gorillas, θ-defensin genes exist as pseudogenes and although they are transcribed, a premature termination codon in the signal sequence precludes their translation and to date these peptides have not been reported to occur naturally in human cells [140, 199, 200]. Given that humans evolved from orangutans over 7 million years ago, and that these latter primates have both intact and silenced copies of θ-defensin genes, it has been proposed that the premature stop codon found in some of these genes may have originated at this fork in evolution [201]. Moreover, based on the evolutionary divergence of Hominoidea from Cercopithecoidea, this would suggest that θ-defensins are at least 30 million years old [202, 203]. Interestingly, the pseudogenes of human θ-defensins, or retrocyclins, have been successfully expressed in human cells by using aminoglycosides to bypass the premature stop codon of their mRNA [201, 204] and have been shown to have potent antiviral activity [205], particular against HIV [204, 206, 207]. Given that viruses have evolved in the absence of selective pressures from retrocyclins, it has been proposed that restoration of the endogenous production of these peptides in human cervicovaginal tissues by the application of aminoglycoside-based topical microbicides may help prevent the sexual transmission of HIV [201, 204].

It is clear that AMPs possess an ancient history, and positive selection appears to be the major evolutionary driver of the structural and functional diversity observed for these peptides in the case of both vertebrates [208, 209] and invertebrates [210, 211], engendering AMPs with an ability to combat new or altered microbial pathogens [212, 213]. Indeed, it has been suggested that AMPs and microbial resistance mechanisms have coevolved, leading to a transient host–pathogen balance that has shaped the existing repertoire of these peptides found in nature [57, 170, 172, 173, 214]. For example, plants and humans are susceptible

to different pathogens and thus their defensins are exposed to different selective pressures. As plants are generally much more susceptible to infection by fungi compared to other microbes, fungal pathogenicity would select for survivors with antifungal defensins. In contrast, humans are generally much more susceptible to infection by bacteria than fungi and thus bacterial pathogenicity would select for survivors with antibacterial defensins [215]. However, positive selection also appears to be an important factor in retaining duplicated genes and promoting the acquisition of a novel or more specialized function [216], which can provide the host with an evolutionary advantage as observed with the diversification of defensins [217–219]. For example, some plant defensins have no antifungal action, and, rather, inhibit the activity and synthesis of α-amylase, which is a digestive enzyme found in the gut of insects. It has been proposed that that the inhibition of α-amylase activity renders plant material indigestible and thus helps plants to resist feeding insects [141, 220]. In the case of vertebrates, it has been shown that β-defensins have evolved via gene duplication and diversification to become the major components of venom in the duck-billed platypus, believed to be the only venomous monotreme [127, 221]. Interestingly, some species of snakes and lizards produce venoms that include peptides derived from the duplication of β-defensins, and share many features with platypus venoms via convergent evolution [128, 222]. It is also interesting to note that the expression of platypus genes encoding venom defensins was detected in non-venom tissues, suggesting that these peptides may play other biological roles [223], and consistent with this suggestion, non-antimicrobial biological activities have been demonstrated for β-defensins in the case of a number of vertebrates [125, 137, 139, 224, 225]. This functional promiscuity of β-defensins represents an evolutionary advantage for mammals [181], which has been demonstrated for other AMPs [75, 226–231] and is now discussed below using defensins as a major example.

1.3 AMPs: Multifunctional Molecules

As described above, mammalian defensins were first isolated due to their antimicrobial properties [138, 232]; in other cases, however, AMPs were initially recognized for other functions and shown to possess antimicrobial activity at a later date [55]. For example, lactoferrin was originally recognized primarily for its ability to bind iron and later studies demonstrated that the protein was able to kill bacteria via iron-independent mechanisms of membrane interaction that were typical of AMPs [233]. The idea that peptides could serve both as AMPs and modulators of the immune system came from studies on α-melanocyte stimulating hormone (α-MSH). This peptide was well recognized for its endogenous melanogenic properties and ability to control inflammation via immunomodulation, promoting the generation of anti-inflammatory cytokines, and downregulating proinflammatory cytokines [234–237]. However, the widespread distribution of α-MSH in many barrier cells such as keratinocytes, fibroblasts, and melanocytes, and in various

immune cells, including neutrophils, monocytes, and macrophages, suggested the potential for a wider role in host defense [238–240]. Confirming this suggestion, α-MSH is now generally included within the AMP's classification based on its potent broad-spectrum antimicrobial activity [241, 242], which includes membranolytic action against bacteria [243–245]. The first hints that established AMPs such as defensins may possess functional promiscuity came from comparative studies between these peptides and chemokines, which play a role in the induction of leukocyte trafficking. These two groups of peptides were originally thought to have distinct roles in the innate immune response, but it was found that their expression levels could be upregulated in response to microbial challenge; further studies showed that many chemokines were able to exert direct antimicrobial activity via mechanisms similar to AMPs while some defensins were capable of activating specific chemokine receptors and exerting chemoattractant activity [246, 247]. Such studies led to the realization that AMPs are also multifunctional peptides that serve a variety of biological roles, including immunomodulatory functions in host defense that can supplement their direct antimicrobial activity and lead to the resolution of infection [231, 248].

1.3.1 Defensins as Effectors of Immunity

The human β-defensins (HBDs) 1–4 are the most studied mammalian defensins and are primarily expressed in various epithelial tissues, including airway epithelia, urogenital tissues, nasolacrimal duct, mammary gland, testes, and epididymis along with some immune cells, such as monocytes and macrophages [232, 249–258]. The best known role of HBDs and other mammalian β-defensins as effectors of innate immunity is direct action against Gram-positive and Gram-negative bacteria, fungi, viruses, and parasites, which has been extensively reviewed elsewhere (Chapter 2) [38, 125, 139, 232, 251, 257]. However, in addition to their direct antimicrobial activities, HBDs play a critical role in regulating inflammation, which is essentially a protective response by the host's immune system to eliminate injurious stimuli, such as infection by pathogenic microbes, and to initiate healing at the inflamed tissue site [137, 139]. As a contribution to regulating inflammation, HBDs function as chemoattractants for a variety of immune cells [224, 232] and in a landmark study, Yang *et al.* demonstrated the recruitment of immature dendritic cells and $CD4^+$ memory T cells by a concentration gradient of HBD1 and 2 [259]. The chemotactic activity of HBD3 towards immature dendritic cells has also been demonstrated, along with that of several murine defensins, which have been shown to chemoattract immature murine dendritic cells [224]. In combination, these results clearly suggested that HBDs and other defensins are able to serve as modulators of the adaptive immune response to infection with their initiation into this process being marked by the recruitment of immature dendritic cells to sites of microbial entry into the host. This recruitment leads to the uptake, processing, and presentation of microbial antigens by mature dendritic cells with the subsequent induction of the antigen-specific immune system [257,

260, 261]. HBDs have also been show to exert chemotactic activity towards phagocytes, which include monocytes and macrophages, and form part of the front-line effector cells involved in innate defense against invading pathogenic microbes [262–266]. For example, it has been shown that HBD1 is chemotactic for monocytes while HBD2–4 are variously chemotactic for monocytes, macrophages, and mast cells [139, 267], which in the latter case can indirectly promote phagocyte recruitment [224, 257]. Once recruited to the site of infection, phagocytes become activated and work in conjunction with a range of effector molecules to internalize and kill infecting microbes. Moreover, the processing of microbes by both immature and mature dendritic cells is phagocytic, and hence contributes to innate immunity by directly killing pathogens. In addition, dendritic cells, particularly upon activation, produce numerous mediators, including cytokines, chemokines and AMPs, that participate in innate immunity [224, 257, 261, 268]. Taken together, these results indicated that HBDs can serve as modulators of both the innate and adaptive immune response to infection [125, 139, 232]. HBDs are able to function as proinflammatory mediators of the immune response by indirect mechanisms as shown by a recent study, which demonstrated that these peptides are able to suppress the apoptosis of neutrophils [269] and prolonging the lifespan of these cells is an inflammatory event that contributes to host defense against invading microbes. It was found that HBDs, particularly HBD3, exerted their suppressive effect on neutrophil apoptosis through binding to CCR6, which is a chemokine receptor. The resulting activation of this receptor led to a series of antiapoptotic events, including the upregulation of antiapoptotic proteins and the downregulation of proapoptotic proteins [269]. However, in many cases HBDs directly promote a proinflammatory immune response by binding to various cell receptors [137]. For example, the ability of HBDs 2 and 3, and to a lesser extent HBD1, to chemoattract immature dendritic cells and $CD4^+$ memory T cells is facilitated by interaction with CCR6 [259, 270], which is a G-protein-coupled receptor (GPCR) [271, 272]. In the case of HBD3, a number of studies have investigated the structure–function relationships underpinning these interactions, and found that both the N-terminal tertiary structure and specific residues proximal to this terminus were important to the receptor binding and chemotactic activity of the peptide [273–275]. These data were strongly supported by the results of structure–function studies on the murine ortholog of HBD3, DEFB14 [274–276], which also chemoattracts cells that express CCR6 along with macrophages from both mice and humans [275, 276]. However, the chemotactic activity of DEFB8, a murine β-defensin, towards immature dendritic cells and $CD4^+$ T cells [277] along with that of HBD3 and 4 towards macrophages was found to be independent of CCR6, suggesting the use of different receptors [273]. In the case of HBD3 this appears to be the chemokine receptor CCR2 [278, 279], which is also a GPCR [280, 281]. The mechanisms used by β-defensins to mediate chemotaxis are poorly understood [139] and are generally beyond the scope of this chapter; however, currently, three alternative models have been proposed [65]. According to the "alternate ligand model," β-defensins directly bind to a specific chemokine receptor, resulting in the initiation of receptor signaling. This model appears to include

chemotaxis mediated by the binding of HBDs to CCR6 where these peptides serve as alternate ligands to the chemokine macrophage inflammatory protein-3α (MIP-3α), which is the natural agonist of this receptor. The ability of HBDs to bind CCR6 appeared to be based on similarities between the three-dimensional structures of HBDs and MIP-3α, including the presence of topological motifs that could mediate receptor recognition and the surface distribution of cationic residues on these molecules [232, 246]. The "membrane disruption model" proposes that β-defensins modify the membrane microdomain associated with the chemokine receptor, which indirectly changes its function to either signal without a ligand or to become unresponsive to binding by its specific ligand. In contrast, the "trans-activation model" suggests that β-defensins stimulate the release of a membrane-bound growth factor, which then binds and activates its high-affinity receptor (Figure 1.3) [65].

In addition to binding chemokine receptors, β-defensins have also been shown to interact with antigen-presenting cells (APCs) via Toll-like receptors (TLRs),

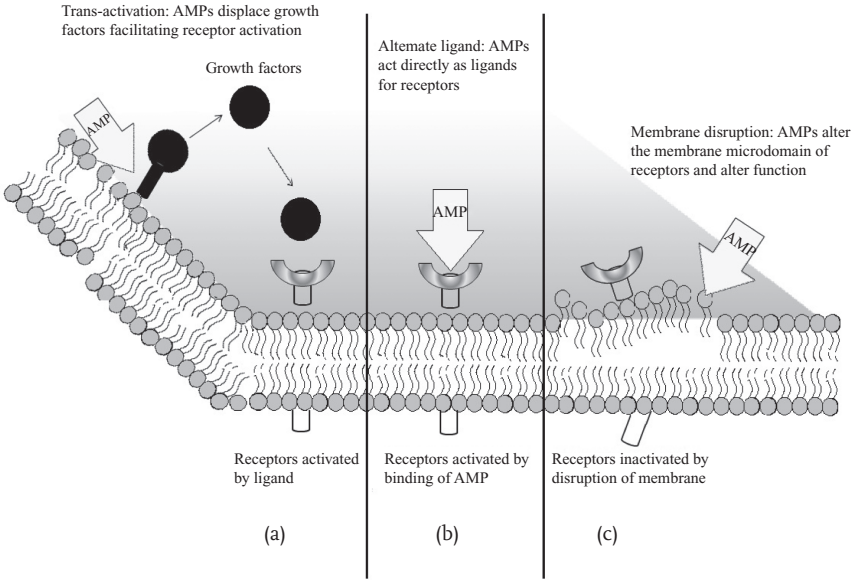

Figure 1.3 Models for the interaction of β-defensins with cell surface receptors: putative models for the interaction of defensins with chemokine receptors (adapted from [65]). According to the "trans-activation model" (a), AMPs stimulate the release of a membrane-bound growth factor, which then binds to its high-affinity receptor with activation resulting. In the "alternate ligand model" (b), AMPs bind directly to the receptor, which results in the initiation of signaling. The "membrane disruption model" (c) proposes that AMPs modify the membrane microdomain associated with the receptor, which indirectly leads to a change in receptor function. This functional change allows the receptor to either signal without a ligand or become insensitive to binding by its specific ligand.

which are integral membrane glycoproteins that recognize microbial components [282, 283]. For example, it has been reported that the activation of APCs by HBD3 is mediated by binding of the peptide to TLR1 and TLR2 and the subsequent activation of the MyD88 (myeloid differentiation primary response gene 88)-dependent signaling pathway [284]. MyD88 is an adapter molecule that is recruited by activated TLRs in order to propagate a signal [283]. Studies on MBD2, which is a murine ortholog of HBD2, showed that interactions between the peptide and TLR4 mediated the upregulation of costimulatory proteins on immature dendritic cells, including CD40, CD80, and CD86 [285], which play a role in the maturation of these cells [286]. MBD2 was found to stimulate the maturation of immature dendritic cells, which led to an adaptive immune response, including the production of proinflammatory cytokines [285, 287]. Most recently, the induction of proinflammatory cytokines by HBD3 was demonstrated in human macrophages by the enhanced expression of a variety of gene transcripts, including those for tumor necrosis factor-α, interleukin (IL)-1α, IL-6, and IL-8 [279]. In human monocytes, the increased expression of proinflammatory cytokines was detected at the protein level for IL-1α, IL-6, and IL-8, which appeared to be induced by the binding of HBD3 to TLR1 and TLR2 [288]. A number of studies have shown that HBD2–4 can stimulate human keratinocytes to increase the production of a variety of proinflammatory mediators, including IL-6, IL-8, IL-10, IL-20, and MIP-3α [289–291]. In some cases, there was evidence to suggest that the induction of these mediators was dependent on the interaction of HBDs with a GPCR [289]. The use of a GPCR was also implicated in the ability of HBD2–4 to enhance the expression of the pruritogenic cytokine, IL-31, in a variety of human mast cells, which constituted a novel source of this cytokine. These studies also showed that the expression of IL-31 was elevated in psoriatic skin mast cells, which led to the suggestion that the HBD-stimulated production of pruritogenic factors by mast cells provided a novel mechanism by, which human AMPs may contribute to inflammatory reactions and suggested a role for these HBDs in the pathogenesis of psoriasis and other skin disorders [292].

Collectively, the studies described above demonstrate that HBDs and other β-defensins are able to bind a variety of cell surface receptors and enhance the adaptive and innate immune response. However, studies over recent years have suggested that these peptides are also able to attenuate the immune response by suppressing a proinflammatory response [137, 293]. The ability of HBDs and other β-defensins to exert an immunosuppressive effect on proinflammatory responses induced by bacterial and viral components has been suggested by investigations both *in vitro* [137, 294–297] and *in vivo* [293, 298, 299]. In general, the mechanisms underpinning these immunosuppressive effects are unclear but recent work has suggested that HBDs and other β-defensins may combine both pro- and anti-inflammatory capacities with the balance of these effects determined by the levels of expression of the HBD peptides. For example, HBDs may be produced at high levels at the site of microbial infection, resulting in a proinflammatory response and the chemotactic recruitment of immune cells such as macrophages, as described above. However, as the microbial threat is nullified and the levels of

HBDs present at the site of infection decreases, the ability of these peptides to act as proinflammatory suppressors contributes to the resolution of infection [137].

1.3.2 Defensins and Wound Healing

A number of studies showed that HBD1–4 was expressed in skin substitutes prepared from keratinocyte cultures and split skin grafts from healthy and burned donors [300–302]. It has also been shown that a number of these HBDs stimulate the migration and proliferation of epidermal keratinocytes, which led to the suggestion that these peptides may promote cutaneous wound healing [289]. Consistent with this suggestion, the expression of HBD2 appeared to be upregulated in acute and chronic wounds while the presence of the peptide was not detected in healthy skin [303, 304]. It has been shown that the expression of HBD2 is significantly induced by epidermal growth factor (EGF) when costimulated with the cytokine, IL-1α [305]. It has previously been shown that this defensin is upregulated by IL-1α in the human corneal epithelium in response to ocular surface injury and it was suggested that the presence of the peptide in the regenerating corneal epithelium may indicate that it plays a direct role in the wound-healing process [306]. HBD2 also appears to play a role in the restitutive migration of epithelial cells and wound healing of the mucosal barrier in the intestine [307], while other studies have shown that, similarly to vascular endothelial growth factor, the peptide is able to promote the migration and wound healing of endothelial cells, manifested by the formation of capillary-like tubes with human umbilical vein endothelial cells [308]. More recent studies have shown that a high-glucose environment reduces HBD2 expression in keratinocytes via the downregulation of STAT protein signaling, which led to the observation that reduced expression of the peptide would have severe negative effects on the wound healing process under diabetic conditions, including impaired keratinocyte migration and suboptimal neovascular formation [309]. HBD3 is highly expressed in keratinocytes, especially at wound sites in response to the growth factors such as EGF, transforming growth factor-α, and insulin growth factor-1 [310–312], and it has been shown that the peptide promotes the proliferation and migration of keratinocytes through phosphorylation of the EGF receptor and STAT proteins [289]. Consistent with these findings, studies on murine models showed that the expression of rat β-defensin 3 (RBD3) was enhanced at both the mRNA and protein levels after skin wounding. However, this study also showed that when the skin of diabetic rats was wounded, RBD3 induction was negligible and similar observations were made for HBD3 expression in human keratinocytes under various glucose-treatment conditions, indicating that both defensins were dysfunctional under hyperglycemic conditions [313]. Reinforcing the importance of HBD3 to the process of wound healing, several studies demonstrated that gene transfer of the peptide to infected excisional wounds on the backs of diabetic pigs enhanced wound closure by around 25% compared to controls [314, 315].

1.3.3 Defensins and Canine Coat Color

CBD103 is a canine β-defensin with potent antibacterial activity that is widely expressed in the skin of dogs, thereby participating in the innate defense of the host [191, 316–318]. However, in addition to its immune-related functions, a number of recent studies have suggested that CBD103 may play a role in determining the color of some dog coats [137, 319]. A number of genes and their protein products were known to mediate the pigmentation of dog coats although interactions between the melanocortin 1 receptor (Mc1r) and its antagonist, the agouti signal peptide (ASP) appeared to be the primary determinants of this process [320]. Essentially, constitutive signaling by Mc1r located on the surface of melanocytes controls the production of eumelanin, or black pigment, and the binding of ASP to the receptor antagonizes this signaling, resulting in the production of pheomelanin, or red/yellow pigment [319, 321]. Differential signaling and genetic variation in both ASP and Mc1r was able to explain much of the variation found in canine coat color [319], but it had long been known that these interactions could not account for the dominant black coat color observed in breeds such as Labrador and Golden retrievers [322]. In response, recent studies showed that this dominant black phenotype was associated with an allele located at the K locus of the canine genome [323, 324], which encoded a variant of CBD103 that lacked an N-terminal glycine residue [325]. This mutant defensin was found to act as a neutral antagonist to Mc1r, thereby preventing ASP from associating with the receptor and allowing unrestricted signaling to synthesize black pigment [326]. Interestingly, through mating with domestic dogs, this dominant mutation has been identified in North American wolves and occurs at high frequency in those of these animals that inhabit forested locations where dark color would provide an advantage, demonstrating a molecular signature of positive selection [327]. Most recently, evidence has been presented suggesting that orthologs of CBD103 may be linked to the determination of coat color phenotypes in cattle [328] and function as novel Mc1r agonists in the regulation of melanocyte responses in humans [326, 329].

1.4 Discussion

In this chapter, the history of AMPs has been described, and the discovery of these peptides provided an explanation as to why plants and insects, which lack an adaptive immune systems, are able to resist infections. This chapter has shown that the starting point for the history of AMPs is popularly taken to be the 1980s based on the first descriptions of cecropins in 1981 [32] and magainins in 1987 [33]. However, there is some debate as to the validity of this assertion, which appears to be not without foundation, given that in 1962 AMPs were reported in the European frog, *B. variegate* [23], predating the first reports of magainins by around 25

years. However, while accreditation for the discovery of AMPs is an academic matter, the subsequent explosive increase in their rate of identification and characterization (Table 1.1) has provided some very tangible benefits in relation to the understanding and utilization of these peptides. The most obvious of these benefits is the promise shown by these peptides to act as lead molecules for development as novel antimicrobial agents and as anticancer agents (Chapter 6). However, the availability of databases of AMPs with detailed descriptions of their structural and antimicrobial properties has provided other benefits, such as the construction of models to describe their membrane interactions, which is a key factor in the ability of these peptides to kill microbes (Chapters 5). Such understanding can also provide insight into other lipid–peptide interactions within biological systems. The availability of databases of AMPs with structural information has also contributed to the development of theoretical techniques to analyze the properties of AMPs, including the prediction of their membrane interactive potential (Chapter 4) along with phylogenetic analysis of their evolutionary relationships as discussed above. This chapter has shown that AMPs have an evolutionarily ancient history with pre-prodermaseptins from frogs of the Ranidae and Hylidae families, originating from an ancestral gene over 200 million years old. Moreover, these AMPs have evolved to give modern frogs profiles of these peptides that are sufficiently characteristic to aid the taxanomic classification of these creatures and link their biodiversity to their recent evolutionary history. In the case of vertebrate β-defensins, these peptides appear to have evolved from the C-terminal domain of ancestral invertebrate defensins with an evolutionary history that can be traced back to bilateran organisms that lived over half a billion years ago (Figure 1.2).

An emerging theme from the voluminous literature on AMPs is the multifunctional nature of these peptides. In the case of β-defensins, these peptides contribute to host defense by direct antimicrobial action and by their ability to modulate both the innate and adaptive immune systems. These peptides have been shown to promote the proinflammatory response via indirect mechanisms such as inhibiting the apoptosis of immune cells and directly through their ability to bind to immune cell receptors and induce the chemoattraction of these cells to sites of microbial infection, thereby facilitating pathogen clearance. There is also increasing evidence that β-defensins are able to suppress the proinflammatory response, thereby contributing to the resolution of infection. Increasingly, other biological functions for β-defensins are being reported and here we have briefly described several of these functions, including the ability of these peptides to influence canine coat color and to contribute to wound healing. However, beyond the scope of this chapter, β-defensins have also been reported to play roles in mammalian fertility and development along with conditions ranging from psoriasis and atopic dermatitis to cancer and Crohn's disease, which have been extensively reviewed elsewhere [19, 125, 137, 139, 251, 330]. Taking this chapter as a whole, it is clear that AMPs are ancient molecules; however, given their evolutionary significance and functional promiscuity, they would appear to have a promising future in areas ranging from phylogeny to therapeutics.

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