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MULTI-AUTHOR REVIEW

Structural diversity and species distribution of host-defense peptides in frog skin secretions

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Abstract Cationic peptides that adopt an amphipathic α-helical conformation in a membrane-mimetic environment are synthesized in the skins of many frog species. These peptides often display cytolytic activities against bacteria and fungi consistent with the idea that they play a role in the host's system of defense against pathogenic microorganisms, but their importance in the survival strategy of the animal is not clearly understood. Despite the common misconception that antimicrobial peptides are synthesized in the skins of all anurans, the species distribution is sporadic, suggesting that their production may confer some evolutionary advantage to the organism but is not necessary for survival. The low potency of many frog skin antimicrobial peptides is consistent with the hypothesis that cutaneous symbiotic bacteria may provide the major system of defense against pathogenic microorganisms in the environment with antimicrobial peptides assuming a supplementary role in some species.

Keywords Antimicrobial peptide · Frog skin · Host defense · Archaeobatrachia · Neobatrachia

Introduction

Skin secretions from many species of Anura (frogs and toads) contain a wide range of compounds with biological activity, often in very high concentrations, that have excited interest because of their potential for drug development [1, 2]. Among these substances are peptides with broad-spectrum antibacterial and antifungal activities and the ability to permeabilize mammalian cells. It is generally assumed that these peptides constitute a component of the system of innate immunity that defends the animal against invasion by pathogenic microorganisms [3], and they may also play a role in protecting the animal from ingestion by predators. Frog skin antimicrobial peptides comprise between 8 and 48 amino acid residues and a comparison of their amino acid sequences reveals the lack of any conserved domains that are associated with biological activity. However, with few exceptions, these peptides are cationic, generally with a molecular charge between +2 and +6 at pH 7, and contain at least 50% hydrophobic amino acids.

Circular dichroism and NMR studies have shown that they lack stable secondary structure in aqueous solutions, but have the propensity to form an amphipathic alpha-helix in the environment of a phospholipid vesicle or in a membrane-mimetic solvent such as 50% trifluoroethanol/water [4]. There is no single mechanism by which peptides produce cell death but their action invariably involves a non-specific interaction with the bacterial cell membrane rather than binding to a specific receptor (reviewed in [5]).

The frog skin antimicrobial peptides may be grouped together in peptide families on the basis of limited similarities in amino acid sequence [6, 7]. Skin secretions from a single species frequently contain several members of a particular family that are presumed to have arisen from multiple duplications of an ancestral gene [8]. The molecular heterogeneity of the peptides within a particular family is considerable with a peptide from one species rarely being found with an identical amino acid sequence in another, even when those species are quite closely related

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phylogenetically. The variation in primary structure is reflected in a wide variability in antimicrobial potencies and specificities for different microorganisms. This multiplicity may provide a broader spectrum of defense against the range of pathogenic microorganisms encountered in the environment [9] but firm evidence to support this claim is lacking.

In the period following the first unambiguous identification of peptides with antimicrobial activity in skin secretions of the African clawed frog *Xenopus laevis* (Pipidae) [10], several hundred cell-penetrating peptides have been isolated from skin secretions and/or skin extracts from species belonging to both the primitive Archaeobatrachia and the more highly derived Neobatrachia. Despite extensive investigation of their antimicrobial properties in the laboratory, the precise biological function of the dermal peptides in the wild and their contribution to the system of host defense in anurans remains unclear. This article reviews the distribution of host-defense peptides among the various anuran families and attempts to assess how important are skin antimicrobial peptides in the protecting the organism against pathogens encountered in the environment. This article uses the species names and taxonomic recommendations of Frost [11] and follows recent guidelines for antimicrobial peptide nomenclature [12–14].

Distribution of skin antimicrobial peptides in the anuran families

For at least part of their life cycle, anurans are of necessity confined to a warm and moist environment that is conducive to the growth of potentially harmful bacteria and fungi. It is not surprising, therefore, that over the course of evolution these animals have developed a system of host defense that will protect the skin from invasion by pathogenic microorganisms. What is not clear, however, is the extent to which the synthesis of dermal antimicrobial peptides is necessary to the survival strategy of the organism or confers an evolutionary advantage to the species. It is a common misconception that all frogs synthesize and release cytotoxic peptides into their skin secretions. The distribution of dermal antimicrobial peptides among anuran families is sporadic, and this article will attempt to differentiate between those anuran families that contain species that have been shown to produce host defence peptides in their skin secretions and those families that contain species that do not. It must be pointed out, however, that production of antimicrobial peptides in some species is seasonal and influenced by thyroid hormone status [15], and may also be inhibited by environmental factors such as exposure to bacteria [16] and pesticides [17]. Thus, a report that a particular species does or does not synthesize skin antimicrobial peptides may not be correct under all circumstances.

Distribution of antimicrobial peptides within the Archaeobatrachia

Among the phylogenetically more ancient Archaeobatrachia, cationic α-helical peptides with the ability to inhibit growth of bacteria and fungi have been identified in species belonging to the families Leiopelmatidae, Alytidae, Bombinatoridae, and Pipidae. In contrast, such antimicrobial peptides have not been detected in those species examined to date that belong to the families Pelobatidae and Scaphiopodidae [18]. An overview of the structures and distribution of skin host-defence peptides in frogs classified in the Archaeobatrachia is provided in Table 1.

Leiopelmatidae

The tailed frog *Ascaphus* sp. Stejneger, 1899 occupies a uniquely important position in amphibian phylogeny as the most primitive extant anuran [19]. Originally classified alone in the family Ascaphidae as the sister group to the clade of all other living frogs, it is now united with the New Zealand frogs of the genus *Leiopelma* Fitzinger, 1861 in the family Leiopelmatidae [11]. Tailed frogs occupy two well-separated ranges in the northwest region of North America—the Cascade Mountains and coastal region from British Columbia south to northern California, and an inland range in the northern Rocky Mountains and the Blue and Wallowa Mountains. Although traditionally regarded as a single species, the coastal and inland groups may be distinguished on the basis of morphological characters and by comparison of nucleotide sequences of mitochondrial genes so that animals from the inland range are now regarded as a separate species, the Rocky Mountain tailed frog *A. montanus* [20].

Eight peptides with broad-spectrum antimicrobial activity, termed ascaphin 1–8, were isolated from norepinephrine-stimulated skin secretions of *A. truei* from the coastal range [21]. The peptides are structurally similar to each other, suggesting an origin that involves multiple duplications of an ancestral gene (Fig. 1). Ascaphin-1, -3, -5, -7, isolated from tailed frogs from the inland range, show differences in amino acid sequence compared with the corresponding peptides from *A. truei* consistent with the assignment of this population to a separate species [22]. Orthologs of ascaphin-2, -6 and -8 were not detected in the secretions from *A. montanus*.

The ascaphins show broad spectrum antimicrobial activity with greatest activity against Gram-negative bacteria [21]. Ascaphin-8, in particular, shows possibilities

Table 1 Distribution of antimicrobial peptides in skin secretion of frogs of the Archaeobatrachia

Anuran family	Representative genera	Peptide family	Representative peptide	Amino acid sequence
Leiopelmatidae	*Ascaphus*	Ascaphin	Ascaphin-8	GFKDLLKGAAKALVKTVLF[a]
Alytidae	*Alytes*	Alyteserin-1	Alyteserin-1a	GLKDIFKAGLGSLVKGIAAHVAN[a]
		Alyteserin-2	Alyteserin-2a	ILGKLLSTAAGLLSNL[a]
Bombinatoridae	*Bombina*	Bombinin	Bombinin	GIGGALLSAAKVGLKGLAKGLAEHFAN[a]
		Bombinin H	Bombinin H1	IIGPVLGMVGSALGGLLKKI[a]
Pipidae	*Xenopus*	Magainin	Magainin-1	GIGKFLHSAKKFGKAFVGEIMNS
		Peptide glycine-leucine amide	PGLa	GMASKAGAIAGKIAKVALKAL[a]
		Caerulein precursor fragment	CPF-1	GLASFLGKALKAGLKIGAHLLGGAPQQ
		Xenopsin-precursor fragment	XPF-1	GWASKIGQTLGKIAKVGLQGLMQPK

[a] C-terminal alpha-amidation

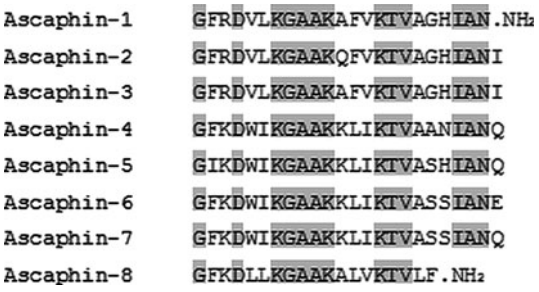

Fig. 1 Antimicrobial peptides isolated from skin secretions of the tailed frog *Ascaphus truei* in the family Leiopelmatidae. The *shaded* amino acid residues are conserved in all peptides

for development into a therapeutically valuable anti-infective agent. The peptide inhibited with relatively high potency (minimum inhibitory concentration MIC < 25 μM) the growth of a range of clinical isolates of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* strains [23]. The therapeutic potential of ascaphin-8, especially for systemic application, is limited by toxicity against mammalian cells (LC_{50} against human erythrocytes $= 55$ μM), but non-hemolytic analogs that retain potency against microorganisms have been designed [24].

Alytidae

In common with most anuran species, the taxonomy of the frogs of the Alytidae family has undergone major revision in recent years. Traditionally, species such as the midwife toad *Alytes obstetricans* were included in the family Discoglossidae that was divided into the four genera *Alytes*, *Discoglossus*, *Bombina*, and *Barbourula.* However, the monophyletic status of the Discoglossidae has been called into question and current taxonomic recommendations restrict the taxon Alytidae to the two genera *Alytes* (5 species) and *Discoglossus* (6 species). *Bombina* (6 species) is united with *Barbourula* (2 species) in the family Bombinatoridae [11].

Two families of structurally related C-terminally α-amidated antimicrobial peptides have been identified in norepinephrine-stimulated skin secretions of *A. obstetricans* (Fig. 2) [25]. The alyteserin-1 peptides show limited structural similarity to the ascaphins from the skins of frogs of the family Leiopelmatidae whereas the alyteserin-2 peptides show limited sequence identity with bombinin H6, present in the skins of frogs of the family Bombinatoridae. The alyteserin-1 peptides show selective growth inhibitory activity against Gram-negative bacteria while displaying low hemolytic activity against human erythrocytes. Alyteserin-1c is particularly potent against multidrug-resistant strains of the nosocomial pathogen *Acinetobacter baumannii* [26]. The alyteserin-2 peptides preferentially inhibit the growth of Gram-positive bacteria such as *Staphylococcus aureus* and also display low hemolytic activity [25].

Bombinatoridae

The six species of fire-bellied toads of the genus *Bombina* are widely distributed in Eurasia. Antimicrobial peptides have been isolated from skin secretions of the European species *B. bombina* and *B. variegata* and from the Asian species *B. orientalis* and *B. maxima* (reviewed in [27, 28]).

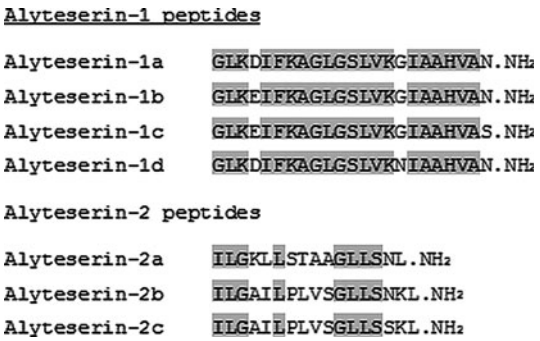

Fig. 2 Antimicrobial peptides isolated from skin secretions of the midwife toad *Alytes obstetricans* in the family Alytidae

On the basis of structural similarity, these peptides may be divided into two families: bombinins and bombinins H (Fig. 3). Bombinin from *B. bombina* was one of the first biologically active peptides to be identified in frog skin secretions on the basis of its strong hemolytic activity [29]. Subsequently, orthologous peptides termed bombinin-like peptides (BLP) were isolated from *B. variegata* [30] and *B. orientalis* [31]. BLPs were also isolated from the Chinese frog *B. maxima* that were somewhat incongruously termed maximins [32].

Determination of the nucleotide sequences of the biosynthetic precursors of the bombinins indicated that the genes encoded a second C-terminally alpha-amidated, cationic antimicrobial peptide with 20 amino acid residues termed bombinin H [33, 34]. Two shorter, less cationic bombinin H components with 17 amino acid residues were identified in *B. orientalis* skin secretions [35]. Bombinin H3 and H4 from *B. variegata* and bombinin H6 from *B. orientalis* are unusual in that isoforms have been found that contain D-alloisoleucine instead of the gene-encoded L-isoleucine and D-leucine instead of L-leucine at position 2 in the molecules [27, 36]. The enzyme responsible for effecting this post-translational isomerization has been isolated from frog skin [37]. In addition, a peptide termed maximin H5 (ILGPVLGLVSDTLDDVLGIL.NH_2) was predicted from the nucleotide sequence of a cDNA from

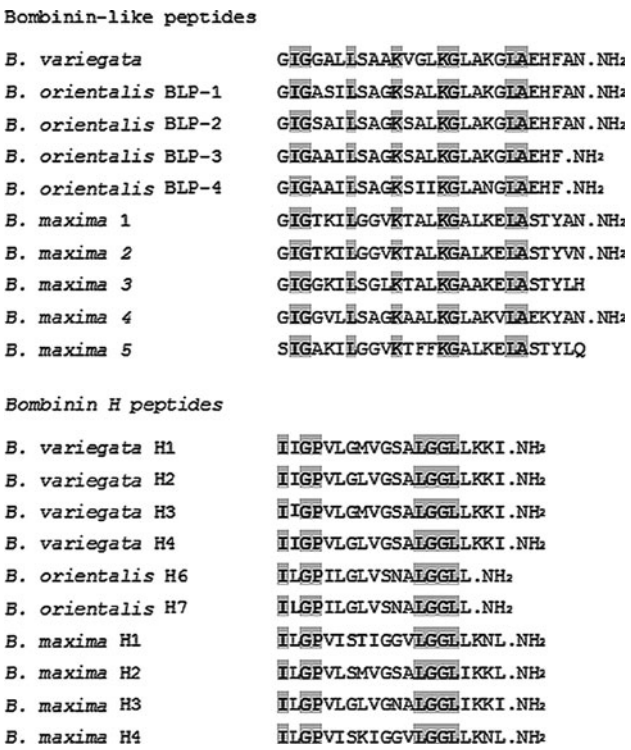

Fig. 3 Antimicrobial peptides isolated from skin secretions of species in the genus *Bombina* in the family Bombinatoridae. The residues shown in *bold* are D-amino acids

B. maxima that is unusual in that it does not contain a basic amino acid residue [38].

The bombinin-like peptides BLP-1 and BLP-3 show potent, broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria and against the opportunistic yeast pathogen, *Candida albicans* together with low hemolytic activity, whereas bombinin H2 and bombinin H4 are less potent against microorganisms but more hemolytic [27]. Interestingly, the analogs containing a D-amino acid were more potent and showed a more rapid rate of killing than the all L-amino acid peptides.

The antimicrobial activity of the more hydrophobic bombinin H6 and H7 was low except in the case of *Aeromonas hydrophilia,* the most common causative agent of the generally fatal red leg disease in frogs [35]. A synthetic replicate of the anionic maximin H5 showed weak antimicrobial activity against *S. aureus* only [38].

Pipidae

African clawed frogs in family Pipidae currently comprise 32 species distributed in five genera (*Hymenochirus*, *Pipa*, *Pseudhymenochirus*, *Silurana*, and *Xenopus*) [11]. The cytogenetics of the *Silurana/Xenopus* genera is complex [39]. *S. tropicalis* with 2n = 20 chromosomes is the only diploid species. A putative whole genome duplication within the *Silurana* lineage has given rise to the tetraploid species *S. epitropicalis*, and a duplication within the *Xenopus* lineage has produced multiple tetraploid species with 2n = 36 chromosomes. At least one further genome duplication event within the tetraploid lineage has given rise to five characterized octoploid species (*X. amieti, X. andrei, X. boumbaensis, X. vestitus,* and *X. wittei*). Further independent polyploidizations involving the combination of tetraploid and octaploid genomes have produced the dodecaploid species *X. ruwenzoriensis* and *X. longipes* (2n = 108) [40].

X. laevis was the first amphibian species in whose skin peptides with antimicrobial activity (magainin-1 and -2) were unambiguously identified [10]. Subsequent analysis of *X. laevis* skin secretions has led to the isolation and characterization of peptide glycine-leucine amide (PGLa) and additional antimicrobial peptides with varying potencies and specificities that are derived from the post-translational processing of the biosynthetic precursors of caerulein and xenopsin [41, 42] (Fig. 4). A comparison of the amino acid sequences of procaerulein, promagainin, and proxenopsin, deduced from the nucleotide sequences of cDNAs, reveals significant structural similarity in the N-terminal regions of the precursors suggesting that the peptides may have evolved from a common ancestral gene by a series of duplication events [43]. Orthologs of magainin-1 and -2, PGLa, caerulein-precursor fragment (CPF),

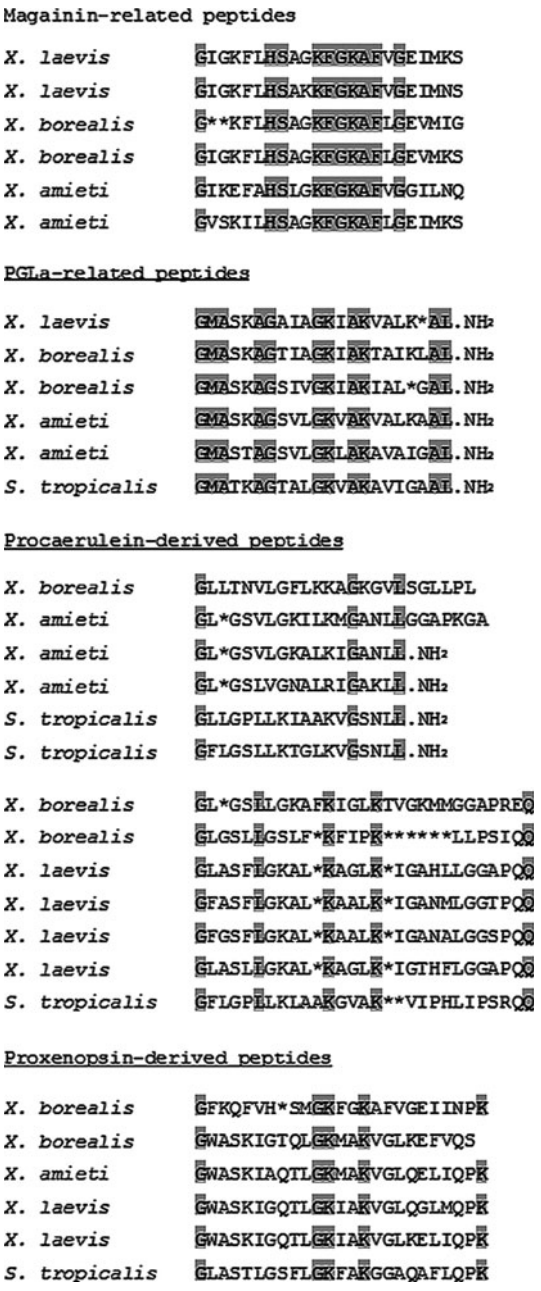

Fig. 4 Antimicrobial peptides isolated from skin secretions of species in the genera *Xenopus* and *Silurana* in the family Pipidae. In order to maximize structural similarity, amino acid deletions, denoted by *asterisks*, have been introduced into some sequences

and xenopsin-precursor fragment (XPF) have been identified in skin secretions of the tetraploid frog, *X. borealis* [44] and the octoploid frog, *X. amieti* [45]. A total of seven host-defense peptides were isolated from skin secretions of the diploid frog *S. tropicalis* [46]. Peptide XT-5 is probably orthologous to PGLa, XT-1, XT-6 and XT-7 are orthologous to CPF, and XT-2, XT-3, and XT-4 are orthologous to XPF. As is apparent from Fig. 4, evolutionary pressure to conserve the primary structures of the antimicrobial peptides from the clawed frogs has not been strong and the sequences of the procaerulein- and proxenopsin-related peptides are particularly variable.

Although magainin-2 has only very low potency against Gram-positive bacteria, a lysine-substituted analog, termed pexiganan, shows potent broad-spectrum antimicrobial activity and low toxicity against mammalian cells and has been studied extensively as a topical therapy, particularly for infected foot ulcers in diabetic patients [47]. The CPF-related peptide XT-7 shows moderate hemolytic activity against human erythrocytes ($LD_{50} = 140$ μM), limiting its therapeutic applicability, but the analog [G4K]XT-7 is non-hemolytic ($LC_{50} > 500$ μM) and retains potent and broad-spectrum antimicrobial activity. Proton NMR spectroscopy has demonstrated that the reduced toxicity of the analog correlates with a decrease in helicity as well as an increase in cationicity [48].

Despite the fact that the skins of species belonging to the genera *Silurana* and *Xenopus* produce multiple cationic α-helical peptides, such components were not detected in the skin of the Surinam toad *Pipa pipa* that also belongs to the family Pipidae [18]. This observation illustrates that, even within a particular anuran family, species belonging to one particular genus may synthesize dermal antimicrobial peptides whereas species belonging to other genera within the family do not.

Distribution of antimicrobial peptides within the Neobatrachia

Among the more highly derived Neobatrachia, cationic α-helical antimicrobial peptides have been identified in the skins of species belonging to the families Dicroglossidae, Hylidae, Hyperoliidae, Leptodactylidae, Myobatrachidae, and Ranidae. Such peptides have not been detected in those species examined to date that belong to the families Bufonidae, Ceratophyridae, Eleutherodactylidae, Microhylidae, Pyxicephalidae, and Rhacophoridae [18]. An overview of the distribution of skin host-defense peptides in frogs classified in the Neobatrachia is provided in Table 2. Histone H2B with cytolytic properties has been isolated from the skin of the tree frog *Rhacophorus schlegelii* (Rhacophoridae) [49] and skin secretions of the tomato frog *Dyscophus guineti* (Microhylidae) contain a Kunitz-type protease inhibitor with weak antimicrobial activity [50], but it is unclear whether these peptides play a significant role in host defense.

Myobatrachidae

The water frogs of Australia and New Guinea belonging to the family Myobatrachidae currently comprise 85 species

Table 2 Distribution of antimicrobial peptides in skin secretion of frogs of the Neobatrachia

Anuran family	Representative genera	Peptide family	Representative peptide	Amino acid sequence
Myobatrachidae	*Uperoleia*	Uperin	Uperin-3.1	GVLDAFRKIATVVKNVV[a]
	Crinia	Signiferin	Signiferin 2.1	IGHLIKTALGMLGL[a]
		Riparin	Riparin 2.1	IIEKLVNTALGLLSGL[a]
Hylidae: Phyllomedusinae	*Phyllomedusa*	Dermaseptin	Dermaseptin-B1	AMWKDVLKKIGTVALHAGKAALGA VADTISQ[a]
		Dermatoxin	Dermatoxin B1	SLGSFLKGVGTTLASVGKVVSDQFG KLLQAGQG
		Phylloseptin	Phylloseptin-H1	FLSLIPHAINAVSAIAKHN[a]
		Phylloxin	Phylloxin-B1	GWMSKIASGIGTFLSGMQQ[a]
		Plasticin	Plasticin-B1	GLVTSLIKGAGKLLGGLFGSVTGGQS
Hylidae: Pelodryadinae	*Litoria*	Aurein	Aurein 2.1	GLLDIVKKVVGAFGSL[a]
		Caerin	Caerin 1.1	GLLSVLGSVAKHVLPHVVPVIAEHL[a]
		Citropin	Citropin 1.1	GLFDVIKKVASVIGGL[a]
		Dahlein	Dahlein 1.1	GLFDIIKNIVSTL[a]
		Maculatin	Maculatin 1.1	GLFGVLAKVAAHVVPAIAEHF[a]
Hylidae: Hylinae	*Pseudis*	Pseudin	Pseudin-2	GLNALKKVFQGIHEAIKLINNHVQ
	Hyla	Hylaseptin	Hylaseptin P1	GILDAIKAIAKAAG
	Hypsiboas	Raniseptin	Raniseptin-1	AWLDKLKSLGKVVGKVALGVAQNY LNPQQ
Leptodactylidae	*Leptodactylus*	Ocellatin	Ocellatin-1	GVVDILKGAGKDLLAHLVGKISEKV[a]
Hyperoliidae	*Kassina*	Kassinatuerin-1	Kassinatuerin-1	GFMKYIGPLIPHAVKAISDLI[a]
Dicroglossidae	*Hoplobatrachus*	Tigerinin	Tigerinin 1	FCTMIPIPRCY[a]
Ranidae	*Rana*	Brevinin-1	Brevinin-1PLa	FFPNVASVPGQVLKKIFCAISKKC
		Brevinin-2	Brevinin-2GRa	GLLDTFKNLALNAAKSAGVSVLNSLS CKLSKTC
		Esculentin-1	Esculentin-1PLa	GLFPKINKKKAKTGVFNIIKTVGKEAG MDLIRTGIDTIGCKIKGEC
		Esculentin-2	Esculentin-2PLa	GLFSILKGVGKIALKGLAKNMGKMGL DLVSCKISKEC
		Ranatuerin-1	Ranatuerin-1CBa	SMLSVLKNLGKVGLGFVACKINKQC
		Ranatuerin-2	Ranateuerin-2PLa	GIMDTVKNVAKNLAGQLLDKLKCK ITAC
		Temporin	Temporin-PLa	FLPLVGKILSGLI[a]
		Palustrin-2	Palustrin-2PLa	GFLSTVKNLATNVAGTVLDTIRCKVT GGCRP
		Japonicin-1	Japonicin-1 J	FFPIGVFCKIFKTC
		Japonicin-2	Japonicin-2CHa	FVLPLLGILPKELCIVLKKNC
		Nigrocin-2	Nigrocin-2GRa	GLLSGILGAGKHIVCGLSGLC
		Ranacyclin	Ranacyclin-T	GALRGCWTKSYPPKPCK[a]

[a] C-terminal alpha-amidation

arranged in 13 genera [11]. At this time, antimicrobial peptides have been identified in extracts of skin glands of species belonging to the *Uperoleia* and *Crinia* genera (Fig. 5). A family of structurally related peptides, termed the uperins, has been isolated from the Australian frogs *Uperoleia mjoberii* [51] and *Uperoleia inundata* [52] that are active against Gram-positive bacteria. From the species *Crinia signifera*, two peptides differing by a single amino acid, termed signiferin 2.1 and signiferin 2.2, were identified that are also active against Gram-positive bacteria [53]. A peptide with some structural similarity to the signiferins, termed riparin 2.1, was isolated from *C. riparia* [54].

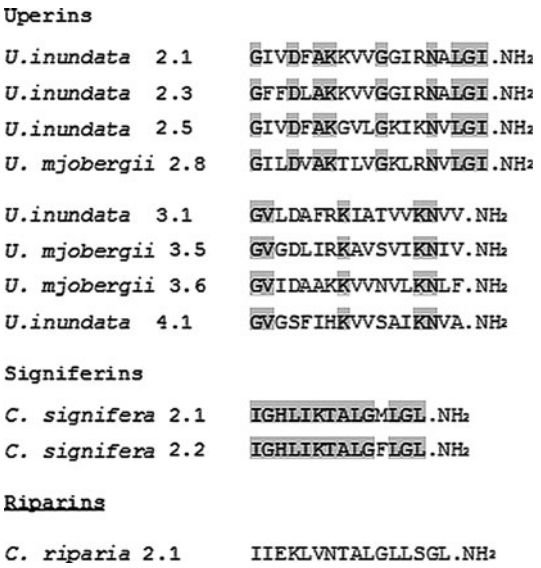

Fig. 5 Antimicrobial peptides isolated from extracts of the dorsal skin glands of species in the genera *Uperoleia* and *Crinia* in the family Myobatrachidae

Hylidae

The extensive and widely distributed family Hylidae is divided into the subfamilies Phyllomedusinae (59 species), Pelodryadinae (196 species), and Hylinae (636 species) [11]. The Central and South American treefrogs, particularly species in the genera *Agalychnis*, *Hylomantis*, *Pachymedusa,* and *Phyllomedusa* in the subfamily Phyllomedusinae, have proved to be a rich source of antimicrobial peptides. The diversity in structure and properties of these peptides has been the subject of several detailed reviews [7, 12, 55] and so this article will present only an overview. As indicated in Table 2, the antimicrobial peptides may be grouped into five major families on the basis of amino acid sequence similarity. These are the dermaseptins, phylloseptins, plasticins, dermatoxins, and phylloxins, but a number of "orphan" peptides have been described that are not included in these groups. The members of the different families differ appreciably in both primary structures and biological activities, but strong conservation of the amino acid sequences of the signal peptide and N-terminal proregions of the biosynthetic precursors of the peptides demonstrates that they are related evolutionarily [7].

The dermaseptin family has been studied in the greatest detail and members have been isolated from the skins of a range of species (*Phyllomedusa sauvagii, P. bicolor, P. oreades, P. distincta, P. hypochondrialis, Pachymedusa dacnicolor, Agalychnis annae, A. callidryas*, and *Hylomantis lemur*) (reviewed in [7, 11]). The dermaseptins vary appreciably both in length and amino acid sequence but generally contain a Trp residue at position 3 and a conserved motif (Ala-Ala-Xaa-Lys-Ala-Ala-Leu-Xaa-Ala) in the central region of the molecule. The peptides differ appreciably in their cytolytic activities. For example, dermaseptins-S1, -S3, and -S5 from *P. sauvagii* show broad-spectrum antimicrobial activity with relatively high potency against a range of Gram-positive and Gram-negative bacteria, but are only weakly hemolytic, whereas dermaseptin-S4 also shows broad-spectrum antibacterial activity, but is very strongly hemolytic [56]. Studies with truncated analogs of dermaseptin-S3 have shown that the (1–16) fragment retains full antimicrobial potency [57].

Peptides from the plasticin family contain multiple copies of the GXXXG motif and are characterized by a high degree of conformational flexibility. For example, plasticin-DA1 adopts a predominantly helical conformation when bound to anionic 1,2-dimyristoylphosphatidylglycerol phospholipid vesicles but a β-sheet structure when bound to zwitterionic dimyristoylphosphatidylcholine vesicles (56). The plasticins may be divided into two classes on the basis of their cytolytic activities. The strongly cationic peptides that contain multiple lysine residues (plasticin-B1 and -S1) show potent, broad-spectrum antimicrobial activity and will lyse erythrocytes whereas the weakly cationic or neutral plasticins (plasticin-A1, -C1, -C2, and DA1) are hemolytic only [55].

Australian treefrogs belonging to the genus *Litoria* (Pelodryadinae) also synthesize a wide range of antimicrobial peptides whose primary structures and biological activities have been comprehensively reviewed [58, 59]. As summarized in Table 2, the *Litoria* peptides may be arranged in five families on the basis of structural similarity. These are the aureins, caerins, citropins, dahleins, and maculatins. There is very little structural similarity between the active peptides from the Phyllomedusinae and the Pelodryadinae, but the amino acid sequences of the signal peptide and N-terminal proregions of the biosynthetic precursors of the aureins and the dahleins are quite similar to the corresponding regions of the precursors of the dermaseptins suggesting a common evolutionary origin [7, 60]. Citropin 1.1 (GLFDVIKKVASVIGGL.NH_2) and aurein 1.2 (GFLDIIKKIAESF.NH_2) adopt well-defined alpha-helical conformations in a membrane-mimetic solvent, and structure-activity studies have demonstrated that C-terminal alpha-amidation and the Lys^7–Lys^8 residues are necessary for the observed high antimicrobial potency [61]. The larger broad-spectrum antimicrobial peptide caerin 1.1 (GLLSVLGSVAKHVLPHVVPVIAEHL.NH_2) adopts a conformation in which two amphipathic helical regions are linked by a more flexible hinge region [62]. Replacement of the Pro^{15} and Pro^{19} residues in this region by Ala results in a reduction in activity.

Despite the fact that frogs in the subfamilies Phyllomedusinae and Pelodryadinae are prolific producers of dermal antimicrobial peptides, synthesis of such components among species belong to the subfamily Hylinae is much more restricted. Four structurally related peptides, termed pseudin 1–4, were isolated from an extract of the skin of the South American paradoxical frog *Pseudis paradoxa* (Hylinae) although these peptides were not detected in norepinephrine-stimulated skin secretions [63]. Pseudin-2 showed the highest antimicrobial potency particularly against Gram-negative bacteria but had very low hemolytic activity. The peptide was bactericidal against *E. coli* but bacteriostatic against *S. aureus* [64].

Skin secretions from several North American species belonging to the genera *Hyla, Hypsiboas, Osteopilus,* and *Pseudacris* in the sub-family Hylinae have been shown not to contain host-defence peptides [18]. However, a 14 amino acid residue cationic α-helical peptide, termed hylaseptin P1, that showed broad-spectrum antimicrobial activity but low cytotoxic activity against mammalian cells was isolated from electrically-stimulated skin secretions from the Brazilian treefrog *Hyla punctata* [65]. Similarly, a family of antimicrobial peptides with low hemolytic activity, termed the raniseptins, that share some structural similarity with the dermaseptins, were identified in skin secretions of another Brazilian treefrog, *Hypsiboas raniceps* [66].

Leptodactylidae

The family Leptodactylidae (currently 99 species) has been divided into four genera: *Hydrolaetare*, *Leptodactylus*, *Paratelmatobius*, and *Scythrophrys*, but both morphological and molecular investigations have indicated that the Leptodactylidae probably does not constitute a monophyletic group [11]. Antimicrobial peptides have been isolated from skin secretions of *L. fallax*, *L. ocellatus*, *L. laticeps*, *L. pentadactylus*, *L. syphax* and *L. validus* (reviewed in [14]) (Fig. 6). All species produce one or more structurally related peptides that were initially designated by species name (fallaxin, pentadactylin, etc.). This nomenclature did not indicate that the peptides were evolutionarily related, and so it has been proposed [14] that all members of the family are termed ocellatins, after the first peptide to be identified [67]. The upper case initial letter of the species is used to indicate their origin and isoforms are designated by numbers. Thus, fallaxin becomes ocellatin-F1. Members of the ocellatin family show growth-inhibitory activity against Gram-negative bacteria but are inactive or only weakly active against Gram-positive bacteria and yeasts such as *C. albicans*. The hemolytic activity of the ocellatins is very low.

Fig. 6 Antimicrobial peptides isolated from skin secretions of species in the genus *Leptodactylus* in the family Leptodactylidae

More recently, glycine-leucine-rich peptides have been isolated from skin secretions of *L. laticeps* [68] and *L. pentadactylus* [69] that show limited structural similarity to the plasticins, previously identified only in phyllomedusid frogs of the family Hylidae. Like the plasticins, the peptide from *L. laticeps* adopts a random coil conformation in water, a *β*-sheet structure in methanol, and an alpha-helical conformation in 50% trifluoroethanol-water. This component lacked antimicrobial activity [68], but the peptide from *L. pentadactylus*, unexpectedly in view of its low cationicity, was active against Gram-negative bacteria [69].

Hyperoliidae

The taxonomy of the African frogs of the Hyperoliidae (currently 208 species) is complex with the family comprising 19 genera [11]. Antimicrobial peptides have been identified only in the skins of running frogs belonging to the genus *Kassina* (currently 16 species). A 21 amino acid residue peptide, termed kassinatuerin-1 (GFMKYIGPLIPH AVKAISDLI.NH_2), was isolated from an extract of the skin of *K. senegalensis* and showed potent growth inhibitory activity against both *E. coli* and *S. aureus* (MIC less than 10 μM) but was strongly hemolytic [70]. A structurally related peptide, kassinatuerin-2 (FIQYLAPLIPHAVKAIS-DLI.NH_2) was also isolated from the extract in high yield but was devoid of antimicrobial activity against these microorganisms. In contrast, kassinatuerin-2 orthologs were identified in the skin of *K. maculata* that were active against *S. aureus* only [71].

Dicroglossidae

The widely distributed Dicroglossidae family is subdivided into the subfamilies Dicroglossinae (148 species) and

Occidozyginae (22 species) [11]. Four small (11–12 amino acids), structurally related C-terminally α-amidated peptides with broad spectrum antimicrobial activity, termed the tigerinins, were identified in the skin of the Indian frog *Hoplobatrachus tigerinus* belonging to the Dicroglossinae [72] (Fig. 7). This species was formerly classified as *Rana tigerina* in the family Ranidae. The tigerinins exist as a mixed population of unordered and β-turn conformations. More recently, cDNAs encoding biosynthetic precursors of tigerinins from the Chinese frog *Fejervarya cancrivora* (formerly *Rana cancrivora*), also in the subfamily Dicroglossinae, have been characterized [73], and a tigerinin-related peptide has been isolated from an extract of the skin of *Hoplobatrachus rugulosus* (formerly *Rana rugulosa*) (unpublished data), but unexpectedly these peptides were not C-terminally alpha-amidated. Amidation has been shown to be necessary for potent antimicrobial activity in the tigerinins [74]. Among species belonging to the Dicroglossidae family, expression of antimicrobial peptide genes in the skin appears to be species-specific, as those frogs studied to-date belonging to the genus *Limnonectes*, also classified in the subfamily Dicroglossinae, do not produce dermal host-defense peptides [18].

Ranidae

The taxonomy of frogs belonging to the extensive family Ranidae, distributed worldwide, except for the polar regions, southern South America and most of Australia, has undergone a series of major revisions in recent years, but many issues remain to be resolved. Current recommendations by Frost [11] divide the 342 species currently in the family Ranidae into 16 genera with the genus *Rana* being retained for a more restricted group of 48 species from Eurasia and North America. However, other established taxonomists have claimed that these assignments are arbitrary, and phylogenetic analysis based upon maximum likelihood analysis of two mitochondrial and three nuclear genes has divided the ranids into four monophyletic genera: *Amolops*, *Huia*, *Rana*, and *Staurois*, with all North American frogs placed in the genus *Rana* [75].

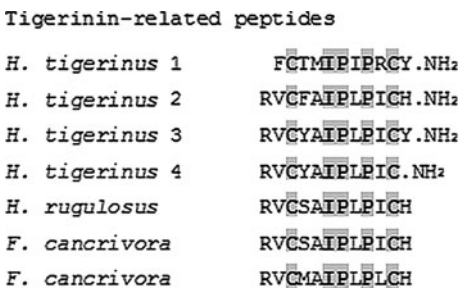

Fig. 7 Antimicrobial peptides isolated from skin secretions of species in the genera *Hoplobatrachus* and *Fejervarya* in the family Dicroglossidae

The repertoire of antimicrobial peptides in the skin secretions is extremely diverse and has been the subject of comprehensive reviews [6, 13, 58, 76]. As shown in Table 2, the peptides contain a disulfide-bridged cyclic domain of varying sizes. The brevinin-1, brevinin-2, esculentin-1, esculentin-2, ranatuerin-1, palustrin-2, and nigrocin-2 families contain a 7-membered ring that has been referred to as the "Rana box". Peptides of the ranatuerin-2 family contain a 6-membered ring, japonicin-2 peptides contain an 8-membered ring, and the ranacyclins contain an 11-membered ring. The C-terminal α-amidated peptides of the temporin family are acyclic. In this context, a peptide family constitutes a group of peptides with common structural features that have been found in more than one species. There have been several reports of "orphan" peptides that have been described in only one species, such as palustrin-1 (ALFSILRGLKKLGKMGQAFVNCEIYKKC) from *Lithobates palustris* [77] and the amolopins from *Amolops loloensis* [78]. Peptides of the brevinin-1 and temporin families have the widest distribution, being found in the majority of Eurasian and New World species. Esculentin-1, esculentin-2, and palustrin-2 have a more restricted distribution in both Eurasian and North American species. To date, peptides of the brevinin-2 family have been found only in Eurasian frogs, but brevinin-2-related peptides, lacking the C-terminal cyclic domain, have been isolated from North American ranids [76]. Peptides of the ranatuerin-1 family have been identified only in the skins of North American bullfrogs of the Aquarana species group. Ranatuerin-2 peptides are found in most New World species, but the distribution in Eurasian frogs is much more restricted. Japonicin-1, japonicin-2, and nigrocin-2 have only been found in Asian species.

As there are no well-defined structural motifs that determine antimicrobial activity, assignment of a newly identified peptide to a particular family is to some extent arbitrary. For example, although most members of the brevinin-1 family comprise 24 amino acid residues and contain a C-terminal disulfide-bridged cyclic heptapeptide domain, several peptides that have been classified in that family are acyclic and contain residue deletions within the molecule. Cladistic analysis has shown that "ranalexins" present in skin secretions of North American bullfrogs of the Aquarana group represent brevinin-1 peptides that have undergone a 4 amino acid residue internal deletion [76]. Similarly, molecular cloning studies have demonstrated that melittin-related peptides found in the skins of certain Japanese brown frogs are not evolutionarily related to peptides in bee venom and they have been assigned to the brevinin-1 family [79]. The amino acid sequence of brevinin-1 has proved useful in cladistic analyses to elucidate

evolutionary relationships between species of North American ranids [76] and Japanese brown frogs [80]. Brevinin-1 peptides generally show potent and broad-spectrum antimicrobial activities, but their therapeutic potential is limited by high hemolytic activity.

Because of their small size (8–21 amino acid residues) and ease of synthesis, the temporins have received attention as lead compounds for development into therapeutically valuable anti-infective agents [81]. Most temporins show potent growth-inhibitory activity only against Gram-positive bacteria, but temporin-L (FVQWFSKFLGRIL.NH_2) from *R. temporaria* [82] and temporin-1DRa (HFLGTLVNLAKKIL.NH_2) from *R. draytoniii* [83], which bear a net positive charge of +3 at pH 7, are active against clinically relevant Gram-negative species such as *E. coli* and *Pseudomonas aeruginosa*, and against the opportunistic yeast pathogen *C. albicans*. Analogs of temporin-DRa with increased cationicity but decreased helicity (for example, [V7K]temporin-DRa) retained the high solubility and potent, broad-spectrum antimicrobial activity of the naturally occurring peptide but were appreciably (up to tenfold) less hemolytic [83].

Biological significance of host defense peptides in frog skin

There have been relatively few studies in which the activity of frog skin peptides have been tested against pathogens common in the animal's environment compared with studies involving readily available microorganisms that have relevance to human rather than amphibian disease. Brevinin 2-related peptide (B2RP), first isolated from skin secretions of the mink frog *L. septentrionalis* [84], bombinin-like peptide BLP-1 from *B. orientalis* [35], and mixtures of temporin A + temporin L, and temporin B + temporin L [85], were active against natural bacterial strains recovered from frog skin, including *A. hydrophilia*. The emergence in almost all parts of the world of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* has led, or has contributed, to widespread declines in frog populations [86]. A number of studies have shown that purified cytolytic peptides from the skins of a wide range of species will inhibit the growth of both *B. dendrobatidis* zoospores and mature cells under laboratory conditions (reviewed in [87]). However, a recent review has concluded that the correlation between documented susceptibility of species to chytridiomycosis and the ability to synthesize antimicrobial peptides in the skin is only fair [88].

Ranaviruses (family Irodoviridae) are another group of pathogens that are responsible for amphibian mortalities and, to a lesser extent, population declines. Several frog skin peptides that were first identified on the basis of their ability to inhibit the growth of bacteria have subsequently been shown to inactivate viruses. These include brevinin-1, effective in inactivating herpes simplex virus type 1 and type 2 [89], and caerin 1.1, caerin 1.9, and maculatin 1.1 that completely inhibit HIV infection of T cells within minutes of exposure to virus at concentrations that were not toxic to target cells [90]. Esculentin-2P and ranatuerin-2P, from *L. pipiens*, dermaseptin-B1 from *P. bicolor,* and temporin A rapidly inactivated frog virus 3, a potentially pathogenic iridovirus infecting anurans [91, 92]. These observations are consistent with, but of course do not prove, the hypothesis that antimicrobial peptides in frog skin play a role in protecting the animal against pathogenic viruses.

The growth inhibitory potencies of frog skin antimicrobial peptides against microorganisms vary substantially and, in some instances, are so low as to cast doubt on their role as protective agents. Reported MIC values against reference strains of *E. coli* and *S. aureus* vary in the range 1–300 μM. However, the literature abounds with examples in which the synthetic chemist has prepared analogs of naturally occurring peptides containing one or more amino acid substitutions that show greatly increased antimicrobial potencies [83, 88]. The reader is entitled to ask why natural selection has not acted to produce similar appropriate amino acid substitutions in the frog skin peptides in order to optimize antimicrobial potency and promote more effective killing of invading pathogens. One possible explanation for this apparent paradox lies in the hypothesis that symbiotic bacteria on the skin may make the major contribution to innate immune defenses against pathogenic microorganisms [93]. For example, evidence was obtained that bacterial isolates from the skin of *R. muscosa* [94] and the antifungal bacterial species *Janthinobacterium lividum* found on *R. muscosa* skin [95] inhibit growth of the chytrid fungus *B. dendrobatidis* and reduce morbidity and mortality in infected animals. Thus, in those species that produce antimicrobial peptides, natural selection may have acted to attenuate potency so that symbiotic cutaneous microbes are able to survive in the anti-bacterial environment of the skin secretions

Summary

It is not possible at this time to draw a definitive conclusion regarding the importance of frog skin peptides in defending the host against infection by environmental pathogens. It is apparent that production of antimicrobial peptides in the skin is restricted to certain families and to certain genera within families with no obvious correlation between peptide synthesis and the habitat of the frog and its potential

exposure to environmental pathogens. Even among species that do produce dermal cytotoxic peptides, their precise contribution to host defense in the wild remains unclear. The fact that these peptides are produced by the most primitive extant anurans, the tailed frogs, *Ascaphus* sp., as well as by highly derived neobatrachians, such as the Ranidae ("true frogs"), suggests that they offer definite evolutionary advantage to anurans, but their presence in skin secretions is not essential to survival. It is highly probable that antimicrobial peptides in the skin do represent a component of the system of innate immunity in the limited number of species that produce them. However, their contribution may be less than previously thought and may be secondary to the defenses provided by symbiotic bacteria.

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