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Research paper

Novel dermaseptin, adenoregulin and caerin homologs from the Central American red-eyed leaf frog, *Agalychnis callidryas*, revealed by functional peptidomics of defensive skin secretion

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Abstract

By integrating systematic peptidome and transcriptome studies of the defensive skin secretion of the Central American red-eyed leaf frog, *Agalychnis callidryas*, we have identified novel members of three previously described antimicrobial peptide families, a 27-mer dermaseptin-related peptide (designated DRP-AC4), a 33-mer adenoregulin-related peptide (designated ARP-AC1) and most unusually, a 27-mer caerin-related peptide (designated CRP-AC1). While dermaseptin and adenoregulin were originally isolated from phyllomedusine leaf frogs, the caerins, until now, had only been described in Australian frogs of the genus, *Litoria*. Both the dermaseptin and adenoregulin were C-terminally amidated and lacked the C-terminal tripeptide of the biosynthetic precursor sequence. In contrast, the caerin-related peptide, unlike the majority of *Litoria* analogs, was not C-terminally amidated. The present data emphasize the need for structural characterization of mature peptides to ensure that unexpected precursor cleavages and/or post-translational modifications do not produce mature peptides that differ in structure to those predicted from cloned biosynthetic precursor cDNA. Additionally, systematic study of the secretory peptidome can produce unexpected results such as the CRP described here that may have phylogenetic implications. It is thus of the utmost importance in the functional evaluation of novel peptides that the primary structure of the mature peptide is unequivocally established – something that is often facilitated by cloning biosynthetic precursor cDNAs but obviously not reliable using such data alone.
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Keywords: Phyllomedusinae; Antimicrobial peptides; Mass spectrometry; Cloning; Precursor

1. Introduction

The defensive secretions produced by specialized skin glands in many anuran amphibians are rich sources of bioactive peptides with several hundred being present in some species [1,2]. In view of the fact that most species have not thus far been subjected to critical and systematic study, these complex mixtures present the peptide chemist with unique and challenging model systems for systematic study with a high degree of probability for the discovery of novel active natural peptides with potential as cellular biological/biochemical tools or as novel drug leads [3–5].

Peptidomics represents the quest for total peptide inventory of a cell/tissue/organ/organism or in the present context, that of a defensive skin secretion. Functional peptidomics implies a holistic approach to the study of the components of such a system by integrating peptide primary structural data, with that generated by biosynthetic precursor cDNA cloning and physiological/pharmacological experiments alluding to function.

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The antimicrobial peptides of amphibian defensive skin secretions are unquestionably the most studied group of secreted peptides to date with much data having been accumulated on primary structures, cloned biosynthetic precursor organizations, physicochemical attributes, molecular modeling, mode of action and use as taxonomic clues [3,6,7]. While a considerable body of data has been generated on such peptides in phyllomedusine frogs, almost all of this has been reported from species of the genus, *Phyllomedusa*, with little information available on the genus, *Agalychnis*, to which the red-eyed leaf frog belongs. *Phyllomedusa* frogs have been shown to produce several different families of antimicrobial peptides including dermaseptins, adenoregulins, phylloxins, dermatoxins and phylloseptins [8]. Here we present data on three novel antimicrobial peptides, a dermaseptin-related peptide (DRP-AC4), an adenoregulin-related peptide (ARP-AC1) and a caerin-related peptide (CRP-AC1), from the skin secretion of this species (*Agalychnis callidryas*) generated through a functional genomic approach involving establishment of bioactivity, determination of primary structure and deduction of respective biosynthetic precursor sequences from cloned skin secretion-derived cDNAs. While dermaseptins and adenoregulins have been found previously in phyllomedusine frogs, the caerins have hitherto only been associated with frogs of the genus, *Litoria*, from Australasia [9].

2. Materials and methods

2.1. Acquisition of skin secretions

Adult red-eyed tree frogs, *Agalychnis callidryas* of the Costa Rican strain (both sexes; snout-to-vent length 4–6 cm) were housed in a purpose-designed terrarium under a 12 h/12 h light/dark cycle and were fed multivitamin-loaded crickets three times per week. Animals were kept under these conditions for at least 3 months prior to experimentation. Skin secretions were obtained by transdermal electrical stimulation after the method of Tyler et al. [10], washed from the skin with de-ionized water, snap-frozen in liquid nitrogen, lyophilized and stored at −20 °C prior to analysis.

2.2. Gel permeation chromatography

A 35 mg sample (dry weight) of *Agalychnis callidryas* skin secretion was reconstituted in 3 ml of 2 M acetic acid, clarified by centrifugation, and applied directly to a 90 × 1.6 cm column of Sephadex G-50 (fine), equilibrated in 2 M acetic acid and eluted at a flow rate of 10 ml/h. Fractions (2.5 ml) were collected at 15 min intervals and the column had previously been calibrated with Blue Dextran (V_o) and potassium dichromate (V_t).

2.3. Reverse-phase HPLC

Five hundred microlitre aliquots of gel permeation chromatographic fractions that displayed antimicrobial activity (#22 through #34), were pooled and subjected to reverse-phase HPLC fractionation using a Thermoquest gradient HPLC system fitted with a Vydac semi-preparative C-18 column (30 × 1 cm). Bound peptides were eluted with a linear gradient formed from 0.05/99.5 (v/v) TFA/water to 0.05/19.95/80.0 (v/v/v) TFA/water/acetonitrile in 80 min at a flow rate of 1 ml/min. Fractions (1 ml) were collected at minute intervals and the effluent absorbance was continuously monitored at λ214 nm. Samples (100 μl) were removed from each fraction in triplicate, lyophilized and stored at −20 °C prior to secondary antimicrobial testing. Additional purification of the peptides in the fractions displaying antimicrobial activity was achieved, where necessary, using a Phenomenex C-8 analytical column (250 × 4.6 mm) on a Thermoquest gradient HPLC system. The column was equilibrated in 0.1% TFA/water (solution A) and elution of the peptides was achieved by increasing the concentration of solution B (0.1% TFA in 70% acetonitrile/water) from 0 to 100% B in 80 min at a flow rate of 1 ml/min. Peaks were hand collected and the molecular masses of peptides in the fractions were determined using MALDI-TOF mass spectrometry. Samples (50 μl) were removed from each fraction in triplicate, lyophilized and stored at −20 °C prior to antimicrobial analysis.

2.4. Antimicrobial assays

Antimicrobial activity of the peptides was monitored by incubating reconstituted lyophilized samples of HPLC purified fractions on Luria–Bertani (LB)-agarose plates using an inhibition zone assay as described by Hultmark et al. [11]. Standard bacterial and fungal strains were used in these assays. Gram-positive *Micrococcus luteus* NCT C2665, Gram-negative *Escherichia coli* K12D31, and the fungus *Candida albicans* CB5562v, were employed and were established non-pathogenic strains. To study the bactericidal effect, 2 μl of each fraction, following lyophilization and reconstitution in phosphate-buffered saline (10 μl), were added to 2-mm diameter holes punched in the surface of the agar plate. The plates were then incubated at 37 °C overnight. The diameters of the inhibition zones were subsequently measured. Doubling dilutions of cecropin B were prepared ranging from 0.08 to10 μg/ml and were included as positive controls. Note that this assay was not employed to determine minimal inhibitory concentrations but rather to qualitatively identify those peptides with antimicrobial activity.

2.5. Structural analyses

Each antibacterial peptide was purified to homogeneity from initial fractions by a second reverse-phase HPLC fractionation with collection of the major absorbance peak by hand. The primary structures of the antibacterial peptides were deduced by automated Edman degradation using an Applied Biosystems 491 Procise sequencer, following identification by LC/MS. The limit for detection of phenylthiohydantoin amino acids was 0.2 pmol. The molecular masses of the purified peptides were verified using matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry

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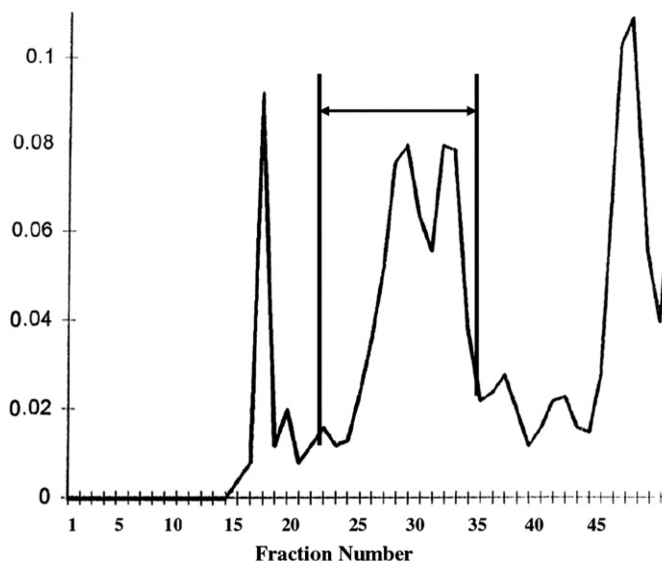

Fig. 1. Gel permeation chromatogram (absorbance 280 nm) of skin secretion from red-eyed leaf frog, *Agalychnis callidryas*. The fractions containing antimicrobial activity on first pass screening using zonal growth inhibition (#22 through #34) are indicated.

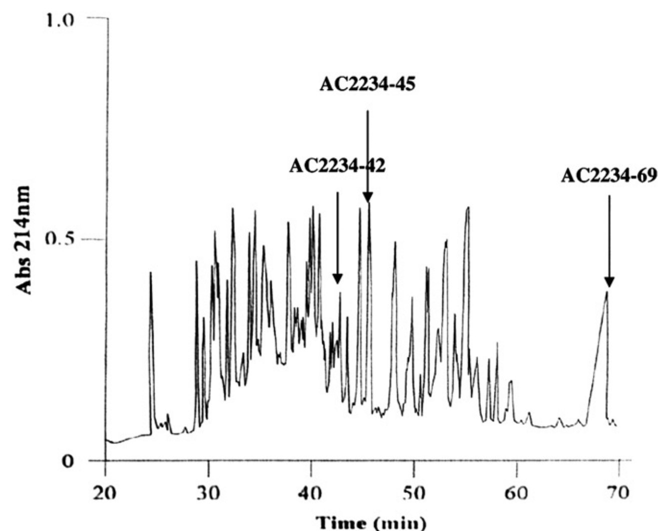

Fig. 2. Reverse-phase HPLC chromatogram of pooled gel permeation chromatographic fractions (#22 through #34) of *Agalychnis callidryas* skin secretion that contained antimicrobial activity. The fraction number codes that contained major activities are indicated.

(MALDI-TOF MS) on a linear time-of-flight Voyager DE mass spectrometer (Perseptive Biosystems), in positive detection mode using alpha-cyano-4-hydroxycinnamic acid as the matrix. Internal mass calibration of the instrument with known standards (neurotensin − 1671.92 Da; magainin 2 − 2465.33 Da; human insulin − 5807.60 Da) established the accuracy of mass determination as ±0.1%.

2.6. Cloning of antimicrobial peptide cDNAs

A 5 mg sample of lyophilized skin secretion was dissolved in 1 ml of cell lysis/mRNA protection buffer supplied by Dynal Biotec, UK. Polyadenylated mRNA was isolated by the use of magnetic oligo-dT beads as described by the manufacturer (Dynal Biotec, UK). The isolated mRNA was subjected to 5′ and 3′-rapid amplification of cDNA ends (RACE) procedures to obtain full-length antimicrobial peptide precursor nucleic acid sequence data using a SMART-RACE kit (Clontech UK) essentially as previously described [12–14]. Briefly, the 3′-RACE reactions employed a nested universal (NUP) primer (supplied with the kit) and degenerate sense primers (S1: 5′-GGIATGTGGWSIAARATHAA-3′, S2: 5′-CNYTNGGNAAYATGGCNAA-3′, S3: 5′-GGIATGTGGGGIACIGTITT-3′) that were complementary to the N-terminal amino acid sequences, GMWSKIK − of ARP-AC1 and GMWGTVF- of CRP-AC1 and to the internal amino acid sequence, − TLGNMAK −, of DRP-AC4. The 3′-RACE reactions were purified and cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated sequencer. The sequence data obtained from the 3′-RACE product was used to design a common and only partially degenerate antisense primer (AS: 5′-GGCACAATYATTGATAATTGTWCKCCTT-3′) to a conserved site within the 3′-non-translated region of both ARP-AC1 and DRP-AC1-encoding transcripts and a specific antisense primer (AS2: 5′-GGCACAATTGTTGATAATTGAGCTT-3′) to a defined site within the 3′ non-translated region of the CRP-AC1-encoding transcript. 5′-RACE was carried out using these primers in conjunction with the NUP primer and resultant products were purified, cloned and sequenced.

3. Results

3.1. Isolation and structural characterization of novel antimicrobial peptides

Antimicrobial activity was identified in gel permeation chromatographic fractions #22 through #34 (Fig. 1). Subsequent reverse-phase HPLC fractionation of these, resolved three major active fractions (Fig. 2 and Table 1). Fractions eluting at 42, 45 and 69 min, that contained more than one peptide as determined by mass spectrometry, were subjected to further purification on a Phenomenex column to achieve a single mass product (Fig. 3a–c). Ultimately, these three novel peptides were structurally characterized (Table 2) and bioinformatic analysis indicated that they belonged to dermaseptin, adenoregulin and caerin families, respectively (Fig. 4).

Table 1
Antimicrobial activities of peptides in reverse-phase HPLC fractions of *A. callidryas* skin as measured by zonal growth inhibition

Peptide fraction code	Zone of inhibition on *E. coli* seeded plates (mm^2)	Zone of inhibition on *M. luteus* seeded plates (mm^2)
AC2234-42	125	125
AC2234-45	190	85
AC2234-69	0	230
Cecropin B (10 μg/ml)	81	52

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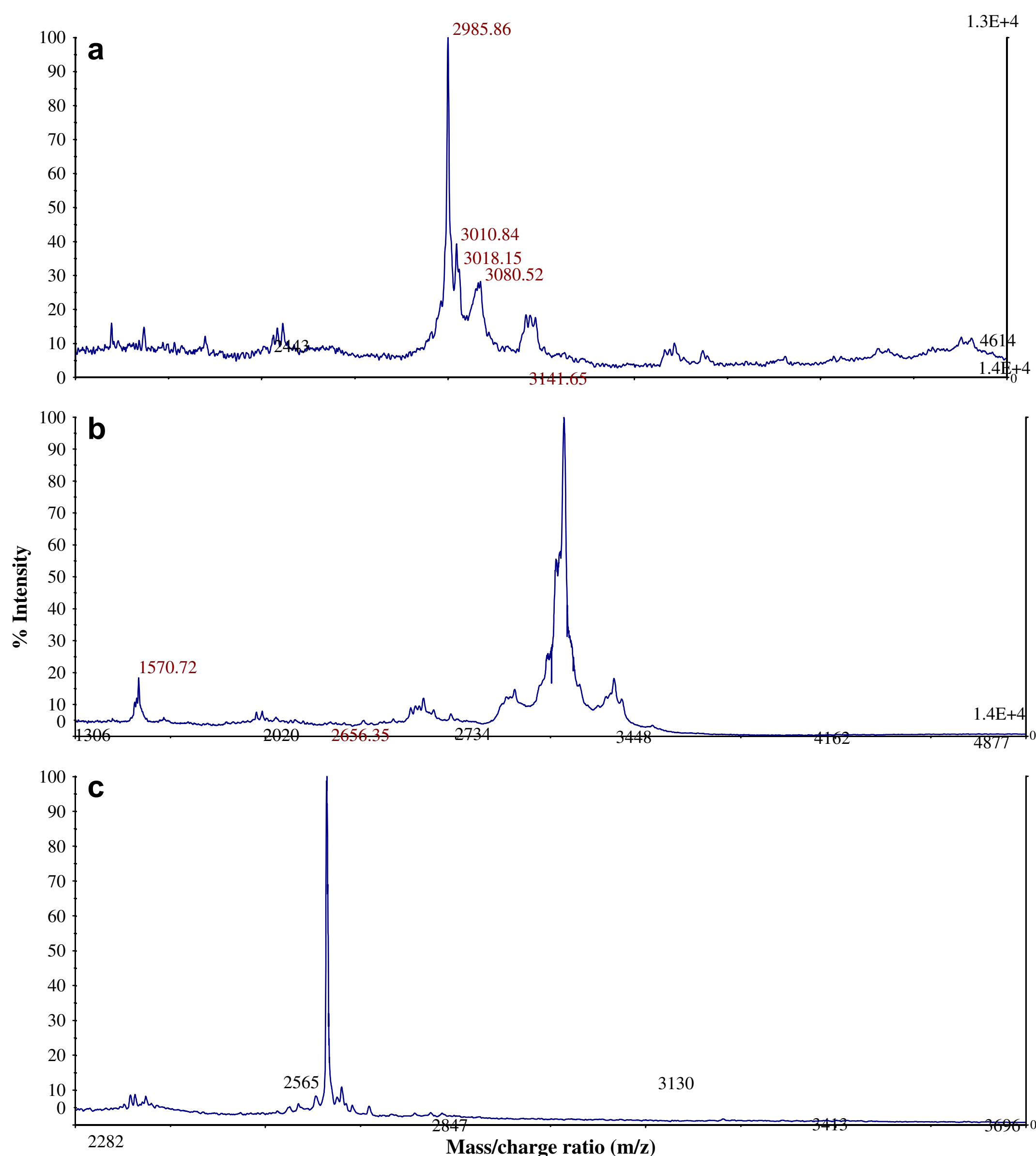

Fig. 3. MALDI-TOF mass spectra of fractions (a) AC2234-42, (b) AC2234-45 and (c) AC2234-69, respectively.

These peptides were named dermaseptin-related peptide *Agalychnis callidryas* 4 (DRP-AC4), adenoregulin-related peptide *Agalychnis callidryas* 1 (ARP-AC1) and caerin-related peptide *Agalychnis callidryas* 1 (CRP-AC1), respectively, in accordance with previously established nomenclature for skin antimicrobial peptides from this species [8].

3.2. Molecular cloning of novel antimicrobial peptide cDNAs

The cDNAs encoding the biosynthetic precursors of each of the three novel antimicrobial peptide homologs identified through primary structural characterization were consistently

Table 2
Molecular masses and primary structures of three novel antimicrobial peptides identified in reverse-phase HPLC fractions of skin secretions from the red-eyed tree frog, *Agalychnis callidryas*

Peptide	Original fraction	Mass observed (Da)	Mass calculated (Da)	Primary structure
CRP-AC1	42	2985.86	2984.50	GMWGTVFKGIKTVAKHLLPHVFSSQQS
ARP-AC1	45	3141.65	3140.71	GMWSKIKEAGKAAAKAAAKAAGKAALDVVSGAI-NH_2
DRP-AC4	69	2656.35	2655.09	SLLSTLGNMAKAAGRAALNAITGLVNQ-NH_2

CRP-AC1 – caerin-related peptide; ARP-AC1 – adenoregulin-related peptide; DRP-AC4 – dermaseptin-related peptide.

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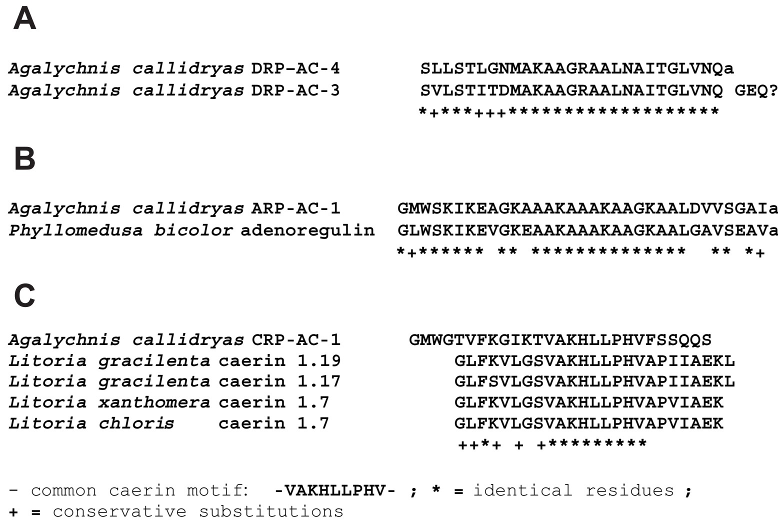

Fig. 4. The primary structures of the three novel antimicrobial peptides from *Agalychnis callidryas* skin secretion compared with their closest homologs in the NCBI database. Fully conserved residues are indicated with asterisks and conservative substitutions with crosses.

cloned (represented in at least five clones each) from the skin secretion library (Fig. 5). The organization and domain topology of each of the biosynthetic precursors encoding DRP-AC4, ARP-AC1 and CRP-AC1, respectively, are illustrated in Fig. 6 and compared with their closest homologs present in the NCBI database. Fig. 4 compares the structures of the novel peptides described here with the most structurally-related mature peptides present in the NCBI database. Note from this figure that the most closely-related homologs to the mature CRP-AC1 peptide are caerins from *Litoria gracilenta*, *xanthomera* and *chloris* but with respect to biosynthetic precursor comparison in Fig. 6, that of caerin 1.12 from *Litoria caerulea* has been represented. The reason for this is that the biosynthetic precursors encoding the most structurally-similar caerins shown in Fig. 4 have not thus far been cloned. This may in part explain the more significant differences that are apparent in this comparison (CRP-AC1 with the caerins) when compared to the precursors of the DRPs and ARPs but also note that this comparison is between frogs that occur on different continents and are placed in separate major taxa. The nucleotide sequences have been deposited in EMBL Nucleotide Sequence Database under the accession codes AM944840 (CRP-AC1), AM944841 (ARP-AC1) and AM944843 (DRP-AC4).

4. Discussion

Although the red-eyed leaf frog, *Agalychnis callidryas*, is a species that is readily available in the herpetology pet trade due to its striking beauty and relative ease of captive breeding, few studies have been reported to date on the spectrum and structures of its defensive skin secretion peptides.

Tachykinins and other biologically-active peptides, including bradykinins, caeruleins and dermorphins, have been isolated and sequenced from methanolic skin extracts of this species and the presence of low amounts of sauvagine was implied by effects of such extracts on diuresis in a rat model [15]. More recently, the putative primary structures of three dermaseptin-related peptides, named DRP-AC1 through 3, have been deduced from cloned skin library-generated

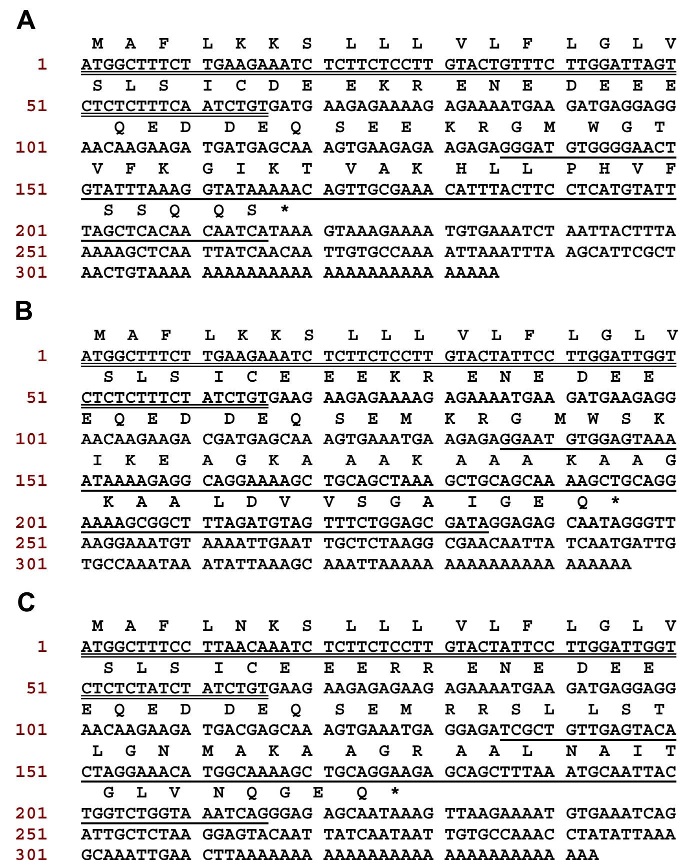

Fig. 5. Nucleotide sequences of clones encoding the biosynthetic precursors of (A) CRP-AC1, (B) ARP-AC1 and (C) DRP-AC4. Putative signal peptides are double-underlined, mature peptides are single underlined and stop codons are indicated by asterisks.

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A

                 ←--------1---------→  ←---------2-------→  3  ←-------------4------------→

DRP-AC-3         MAFLKKSLLLVLFLGLVSLSIC EEEKRENEDEEEQEDDEQSEM RR SVLSTITDMAKAAGRAALNAITGLVNQGEQ

DRP-AC-4         MAFLNKSLLLVLFLGLVSLSIC EEERRENEDEEEQEDDEQSEM RR SLLSTLGNMAKAAGRAALNAITGLVNQGEQ

                 **** ***************** ***+***************** ** *+***+++**********************

B

Adenoregulin     MAFLKKSLFLVLFLGLVSLSIC EEEKRENEDEEEQEDDEQSEM KR GLWSKIKEVGKEAAKAAAKAAGKAALGAVSEAVGEQ

ARP-AC-1         MAFLKKSLLLVLFLGLVSLSIC EEEKRENEDEEEQEDDEQSEM KR GMWSKIKEAGKAAAKAAAKAAGKAALDVVSGAIGEQ

                 ********+************* ********************* ** *+****** ** **************  ** *+***

C

Caerin 1.12      MAFLKKSLFLVLFLGLVSLSIC EEEKRQ-EDEDEHEEEGENQEEGSEE KR GLFGILG------SVAKHVLPHVVPVIAEHS

CRP-AC-1         MAFLKKSLLLVLFLGLVSLSIC DEEKR– ENEDEEEQEDDEQ---SEE KR GMWGTVFKGIK--TVAKHLLPHVFS--SQQS

                 ********+************* +****  *+*** *+* + *   *** ** *+++* +       +****+****    +++*
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Fig. 6. Comparative domain topologies of translated biosynthetic precursors of (A) DRP-AC4 and DRP-AC3, (B) ARP-AC1 and adenoregulin and (C) CRP-AC1 and caerin 1.12. Domain 1 – putative signal peptide, 2 – acidic amino acid residue-rich spacer peptide, 3 – dibasic propeptide convertase processing site, 4 – mature peptide. Conserved residues are indicated in each comparison by asterisks and conservative substitutions by plus signs. With the caerins (group C), gaps have been inserted to maximize alignments.

biosynthetic precursor cDNAs [8]. These peptide families, when occurring together in a frog's skin are quite typical of the phyllomedusids even though the primary structures of individual members may exhibit slight variations [8].

However, methanolic extraction, while providing laudable service to the discovery of biologically-active peptides in amphibian skin for many years, is not appropriate for the application of peptidomic techniques as many skin secretion peptides will not be soluble in this medium and hence will be unrepresented and many components will arise from other skin structures rather than from the stored secretion – a fact that will result in much wasted effort. Stimulated skin secretion, obtained by the method of Tyler et al. [10], remains the best technique to obtain as complete a defensive secretion as is possible for peptidomic analysis with virtually no contamination with molecules from other sources apart from mucus glands. In lyophilized form and stored under vacuum at low temperatures, this material can be used for many years if not indefinitely.

Several years ago, our research group published the observation, which has been confirmed in many subsequent reports [12–14], that the transcriptomes of the peptide-synthesizing granular glands are preserved in this lyophilized material. This fact permits parallel study of peptidomes and transcriptomes in amphibian skin secretions without resort to sacrifice of donor animals and has been used to effect in the present study.

Identification of three peptides with antimicrobial activity, none of which corresponded in molecular mass to previously reported DRP-AC1 through 3 peptides [8] prompted their subsequent primary structural characterization. Amino acid sequences indicated that they were novel peptides from this species. The first peptide exhibited a high degree of primary structural similarity to the previously described DRP-AC3 and was named DRP-AC4 in accordance with established nomenclature [8]. However, this peptide was C-terminally amidated at the Gln (Q) residue at the –4 position from the C-terminus in the putative DRP-AC3 whose structure had not been deduced from sequencing of mature peptide in secretion but rather deduced from cloned skin cDNA that encoded the biosynthetic precursor [8]. The cDNA encoding the biosynthetic precursor of DRP-AC4 was cloned from a skin secretion cDNA library and found to be structurally very similar to that reported for DRP-AC3 throughout all domains (signal peptide, acidic spacer and mature peptide). It is thus highly likely that the mature DRP-AC3 peptide is C-terminally truncated and amidated. These data clearly demonstrate the need to confirm putative mature peptide structures deduced from cloned cDNAs by identification and primary structural characterization of the mature peptide as occurring in the natural secretion.

The second antimicrobial peptide was likewise subjected to amino acid sequencing and found to be a C-terminally amidated 33-mer exhibiting a high degree of structural similarity to adenoregulin from the skin secretion of *Phyllomedusa bicolor* – an antimicrobial peptide with additional biological activity in modulating adenosine binding to its cognate receptor [16]. It was named ARP-AC1 in accordance. This is the first report of an adenoregulin homolog in the skin secretion of a frog of the genus *Agalychnis*. In contrast to the situation with the *Agalychnis* DRPs 1–3, the mature 33-mer adenoregulin peptide was isolated and structurally-characterized from skin secretion and its biosynthetic precursor cloned from *P. bicolor* skin [8]. The mature adenoregulin was C-terminally amidated at the valyl residue at position –4 from the C-terminus and was truncated from the precursor sequence by the tripeptide, GEQ. ARP-AC1 exhibited both of these modifications but terminated in an isoleucinamide. The post-translational

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processing and modifications of both highly homologous adenoregulin precursors are thus identical in both *P. bicolor* and *A. callidryas*.

The third peptide that displayed antimicrobial activity was more enigmatic. This peptide was a 27-mer, was not C-terminally amidated and was most closely structurally-related to caerins from Australasian tree frogs – caerins 1.17 and 1.19 from *Litoria gracilenta*, caerin 1.7 from *Litoria xanthomera* and caerin 1.7 from *Litoria chloris* [9]. It was named CRP-AC1 in accordance. CRP-AC1 was found to be structurally-similar to a peptide named dermaseptin AA-3–4 whose structure was deduced from a cDNA that was cloned from the skin of *Agalychnis annae* [17]. However, this peptide is not a dermaseptin. When trawled in the National Centre for Biotechnology Information database not a single dermaseptin sequence occurs in the top 100 hits despite the fact that two caerins do – caerin 1.4 from *Litoria gilleni*/*Litoria caerulea* and caerin 1.2 from *Litoria caerulea* [9]. Likewise, for CRP-AC1, not a single dermaseptin occurs in the top 100 hits despite the fact that five/seven top hits are caerins from *Litoria gracilenta*, *xanthomera* and *chloris* [9]. We therefore contend that CRP-AC1 represents a novel prototype antimicrobial peptide from phyllomedusine frogs – a finding that is not inconsistent with taxonomic suggestions and evidence that both Neotropical phyllomedusids and Australasian litorids are close phylogenetic relatives. One of the most compelling pieces of evidence to this end is provided by the presence of pterorhodin, a red melanosomal pigment found in the unique large melanosomes of phyllomedusine leaf frogs and some Australasian tree frogs, including litorids, but not in any vertebrates [18]. Thus the presence of a novel defensive skin peptide, CRP-AC1 in a phyllomedusine frog, that possesses a common structural motif with analogous peptides of litorid tree frogs, is not surprising.

The vast peptide-based defensive arrays that are contained within amphibian skin secretions will continue to provide the basis for many interesting discoveries that are relevant to a multitude of different disciplines within the biological and biomedical sciences.

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