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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.

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