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Post-secretory events alter the peptide content of the skin secretion of *Hypsiboas raniceps*

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ABSTRACT

A novel family of antimicrobial peptides, named raniseptins, has been characterized from the skin secretion of the anuran *Hypsiboas raniceps*. Nine cDNA molecules have been successfully cloned, sequenced, and their respective polypeptides were characterized by mass spectrometry and Edman degradation. The encoded precursors share structural similarities with the dermaseptin prepropeptides from the *Phyllomedusinae* subfamily and the mature 28–29 residue long peptides undergo further proteolytic cleavage in the crude secretion yielding consistent fragments of 14–15 residues. The biological assays performed demonstrated that the Rsp-1 peptide has antimicrobial activity against different bacterial strains without significant lytic effect against human erythrocytes, whereas the peptide fragments generated by endoproteolysis show limited antibiotic potency. MALDI imaging mass spectrometry *in situ* studies have demonstrated that the mature raniseptin peptides are in fact secreted as intact molecules within a defined glandular domain of the dorsal skin, challenging the physiological role of the observed raniseptin fragments, identified only as part of the crude secretion. In this sense, stored and secreted antimicrobial peptides may confer distinct protective roles to the frog.

Among the most relevant compounds secreted by frog skin, the antimicrobial peptides have been characterized for their ability to disrupt membranes of a wide range of microorganisms [1]. Much insight into their biosynthetic pathway has come from the description of the gene organization of the dermaseptin peptide family [2]. These peptides are endogenously expressed within the dorsal granular gland cells as large precursor molecules organized at the N-terminal region as a 22-residue signal peptide, followed by a 22–23-residue acidic propeptide domain and a single copy of the biologically active peptide at the C-terminal domain [2]. This preproprecursor is subject to proteolytic processing before being stored in the gland granules. Furthermore, the dermaseptin precursor molecule also shares a high amino acid sequence identity with precursors for temporins, brevinins, and esculentins from the *Ranidae* family [3]. The diversity of biologically active peptides secreted with a conserved precursor molecule prompted the investigation as to whether the same gene organization is also present in the genome of the *Hylanae* subfamily of frogs. To date, the biochemical characterization of the peptide content of the skin secretions of *Hylanae* frogs is sparse. Few studies have highlighted the presence of antimicrobial peptides, such as hylaseptin isolated from *Hyla punctata* [4] and hylins isolated from *Hyla biobeba* [5]. Building on these initial findings, a novel family of dermaseptin-related peptides, termed raniseptins, has been characterized from the hylid *Hypsiboas raniceps* [6]. Moreover, when scrutinizing the secretome of *H. raniceps* using mass spectrometry, peptides derived from the internal cleavage of the raniseptin transcript at a conserved site were also characterized. The antimicrobial activities of the raniseptin peptides and truncated peptides were tested against Gram-negative and Gram-positive bacteria, together with the cytotoxic effect against mammalian cells in order to evaluate the occurrence of endoproteolysis as a possible physiological event. In addition, MALDI imaging mass spectrometry (IMS) was applied to the dorsal skin of *H. raniceps* to investigate whether the mature raniseptin peptide was in fact secreted as a single molecule or whether it was cleaved when still present in the glandular ducts. From these studies, it was also possible to assess that a degree of co-localization existed *in situ* among the mature raniseptin peptides and hence, that the glands of the dorsal skin possess a degree of specialization capable of producing distinct peptide families.

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Material and methods

Amphibian skin secretions. Adult specimens of *H. raniceps* were collected in São Domingos, Goiás, 13°23′41″S, 46°19′35″W, Brazil, under IBAMA license number 240/2005—CGFAU/LIC. Frog secretion was obtained by mild electric stimulation of the granular skin glands, collected in distilled water as a soluble extract, frozen, and lyophilized. Sadly for the course of Science and differently from all previous publications of the group, this work had to be conceived under strict compliance with the recent Brazilian Provisional Amendment regulating the access to genetic resources (No. 2186-16, Resolutions No. 28-29).

Gene cloning, cDNA sequencing, and peptide purification. The protocol for gene cloning and cDNA sequencing has been described elsewhere [7]. The crude extract was dissolved in 0.1% aqueous trifluoroacetic acid (TFA) and submitted to reverse-phase high-performance liquid chromatography (RP-HPLC) as previously described [7]. Alternatively, the crude extract was fractioned by nano-HPLC (NanoCapLC®, Waters Co) using a Symetry® C18 5 μm reverse-phase column (150 × 0.32 mm) (Waters Co) for 240 min at 1 μL/min flow rate.

Peptide sequencing. Monoisotopic molecular masses and purity of the peptides were determined by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF/MS) on an Ultraflex II (Bruker Daltonics). Alternatively, MS acquisition was performed with a NanoLC-Q-TOF Ultima spectrometer (Waters Co) in W mode with positive ion selection. Capillary voltage was set to 2.8 kV and cone voltage to 30 V. The peptides of interest were automatically selected for fragmentation during the Nano-LC tandem MS/MS experiments. Additionally, automatic N-terminal sequencing of peptides was performed on a Protein and Peptide PPSQ-23 Sequencer (Shimadzu Co).

Solid phase peptide synthesis. The peptides were manually synthesized by the solid phase approach using the Fmoc/t-butyl chemistry [8]. An Fmoc-Gln-NovaSyn® TGT resin was used for the syntheses of Rsp-1 and the C-terminal domain. Alternatively, a Fmoc-PAL-PEG-polystyrene resin (NovaBiochem) was used for the synthesis of the amidated N-terminal segment. Cleavage and final deprotection were conducted with a trifluoroacetic acid:thioanisole:ethanedithiol:triisopropylsilane (91.5:5:2.5:1, v:v:v:v) solution for 90 min at room temperature. Peptide purification was performed through RP-HPLC with a Vydac 218TP1022 preparative column and purity was assessed by MALDI-TOF/MS.

Antimicrobial activity and hemolysis assay. The antimicrobial activities of the raniseptin peptides and conventional antibiotics (ampicillin and chloramphenicol) were determined against the following bacterial strains: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29313 and *Xanthomonas axonopodis* pv. *citri*. The human pathogenic bacteria were cultured in Mueller–Hinton medium, whereas *X. citri* was grown in nutrient yeast glycerol (NYG) medium. The minimal inhibitory concentrations (MICs) were determined in 96-well plates by growing the initial bacterial inoculum ($\sim 10^5$ colony forming units/mL) with twofold serial dilutions of peptide or antibiotic. The absorbance of the cells was measured at 600 nm following a 12 h incubation period at 37 °C or alternatively a 48 h period at 28 °C for *X. citri*.

Freshly collected blood in K_3-EDTA coated tubes was rinsed three times with PBS buffer. The red blood cells (RBC) were then diluted tenfold to yield an RBC suspension of $\sim 10^8$ RBC/mL. The cell suspension was incubated for 1 h at 37 °C in 96-well plates with twofold serial dilutions of peptide. The release of hemoglobin was monitored at 414 nm. Complete hemolysis was determined by the addition of 0.1% Triton X-100 to the cell suspension.

MALDI imaging mass spectrometry (MALDI IMS MS). The preparation of the dorsal frog skin fragment (~ 1 cm^2) for MALDI IMS analysis has been described elsewhere [7]. The molecular ion profiling was obtained using an Ultraflex II and automatic scanning steps were separated by 50 μm and a total of 20 laser shots were used at each position. The data set was converted to the BioMAP format (Novartis) using the AnalyzeThis software [9]. BioMAP was used to generate intensity-based ion maps of the molecular components ranging from 600 to 4000 Da and global mass spectrum for the total ion content. Co-localization images of the ions detected were also generated with BioMAP and calculation of the co-localized areas was generated with an *in-house* script running under the ImageJ software [10]. The script estimates the number of superimposed pixels between two images. To validate the co-localization scores derived between different pairs of raniseptin ions, the two most intense ions in the global mass spectrum of the skin fragment, termed Ref-1 and Ref-2, were chosen as controls and were also co-localized with the raniseptin peptides. These two ions correspond to peptides which have been *de novo* sequenced by MS/MS techniques and which do not belong to the raniseptin peptide family (unpublished data).

Results and discussion

cDNA molecules encoding the raniseptin peptides

Nine cDNA molecules, each encoding a single copy of the raniseptin peptides were successfully sequenced (Fig. 1). An example of a full-length cDNA molecule, together with the translated amino acid sequence of the preproraniseptin is shown in Fig. 1A. The structure of the raniseptin preproprecursor is composed of an N-terminal signal peptide, followed by a 22-residue acidic propiece, terminating at a dibasic processing site consisting of two Arg residues (Fig. 1B). This organization resembles closely that of the precursor molecules of the *Phyllomedusinae* frogs [3]. Not only do they share an overall high sequence similarity (*ca.* 90%), but the mature raniseptins display around 80% sequence similarity with various dermaseptins of the *Phyllomedusa* genus. As previously demonstrated, the existence of a common ancestral precursor gene coding for antimicrobial peptides in *Phyllomedusinae*, *Pelodryadinae*, and *Raninae* frogs [11], now also holds true for antimicrobial peptides present in the *Hylinae* subfamily.

Nevertheless, the mature raniseptins are in addition, consistently processed between the conserved residues Gly-14 and Lys-15 as evidenced by mass spectrometry (*vide infra*). This proteolytic cleavage site was previously described in fragments of magainin and caerulein peptides isolated from the skin secretion of *Xenopus laevis* [12]. The enzyme was later characterized as a metalloprotease which cleaves Xaa-Lys bonds, where Xaa is Ala, Gly, Leu, or Lys [13]. In this respect, the endopeptidase recognizes an α-helical structure upon peptide binding comprising at least 12 amino acids and a hydrophobic face with at least four non-polar residues [13]. An additional cleavage site in Rsp-1, between Gly-11 and Lys-12, would in principle meet these criteria. However, the presence of Lys-5 within the hydrophobic domain would impair the reaction as demonstrated by Resnick and co-workers [13]. Despite this fact, conclusive evidence substantiating the presence of such an enzyme in *Hylinae* frogs is the subject of ongoing research.

Peptide purification and identification

The peptide content present in the secretion of *H. raniceps* was fractioned with two independent procedures: standard RP-HPLC and nano-LC/MS. A comparison of both chromatograms obtained is shown in Fig. 2. The combination of both purification strategies