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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

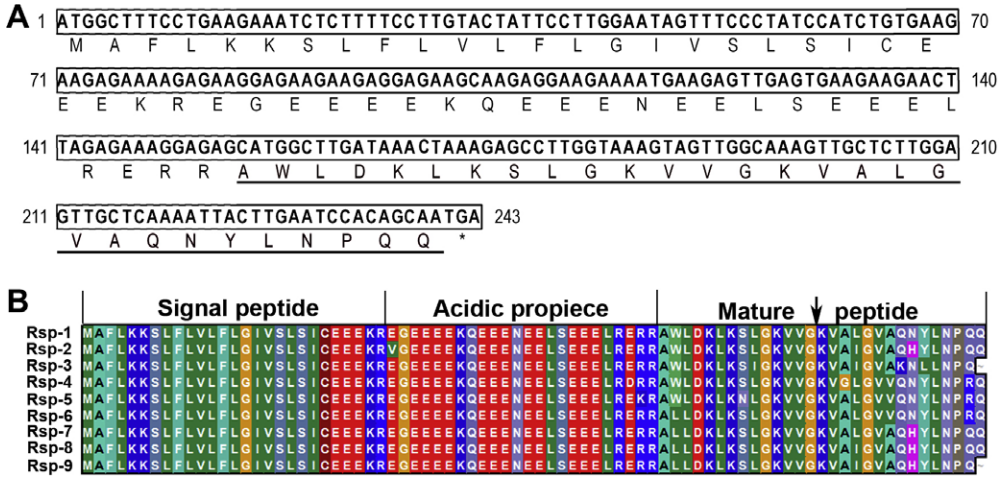

Fig. 1. (A) Full-length cDNA sequence of the encoded Rsp-1 precursor molecule (UniProt KB P86037). The dashed line underlines the mature Rsp-1 peptide sequence and the arrow indicates the secondary proteolytic cleavage site. (B) Amino acid sequence alignment of the translated preproraniseptins peptides, Rsp1–8.

with the subsequent application of either MALDI-TOF MS/MS or ESI Q-TOF MS/MS analyses enabled a comprehensive identification of several peptides of which, at least four full-length raniseptin peptides and eight raniseptin half-peptides as indicated in the legend of Fig. 2. *De novo* peptide sequencing of all these peptides confirmed their presence in the secretion and a typical MS/MS spectrum of the raniseptins is shown in Fig. S1 (Supplementary material). The successful identification of the full-length raniseptins was only possible when low temperature and pH conditions were undertaken during the skin secretion extraction procedures. In fact, a clear depletion of mature raniseptins in different skin secretion stocks collected from *H. raniceps* was detected by RP-HPLC (Fig. S2). Since, there is evidence suggesting that the endopeptidase of *X. laevis* acts within minutes following vesicular disruption and subsequent secretion, this result is consistent with the notion that delaying the storage of the crude extract produces a higher number of peptide fragments [12].

Furthermore, although the Nano-LC/MS/MS technique automates much of the peptide identification procedure, the chromatographic run was not capable of discriminating the isobaric peptide pairs such as Rsp-1(1–14)/Rsp-3(1–14) and Rsp-2(15–29)/Rsp-7(15–29), and the mature peptides such as Rsp-7 and Rsp-8, which differ in terms of a single Leu to Ile substitution (Fig. 1B). However, when fractioned by analytical RP-HPLC, the fragment pairs were successfully separated with distinct elution times as shown in Fig. 2A. Further purification steps (Fig. S3) enabled the confirmation of their amino acid sequences by Edman degradation.

Biological assays

The *in vitro* antimicrobial properties of Rsp-1 were demonstrated against three human pathogenic bacterial strains, namely, *E. coli*, *P. aeruginosa*, and *S. aureus*. Rsp-1 was chosen for tests since its relative abundance, when compared to the other raniseptin peptides identified in the secretion, was the highest representing ~8% of the total peptide content (Fig. 2A). The MIC values determined against the Gram-negative *E. coli* and *P. aeruginosa* were 5 and 10 μM, respectively (Table 1) and 20 μM against the Gram-positive *S. aureus*. These values are in the same range as MIC values commonly determined for dermaseptins [7,14]. For comparison purposes, the MIC values of commercial antibiotics were determined, of which ampicillin and chloramphenicol showed variable specificities and potencies (Table 1). Moreover, the antimicrobial activities of the peptide fragments were also investigated. Rsp-1(1–14) showed much lower or no antimicrobial action when compared to the intact Rsp-1. The MIC values were 85 and 170 μM against *E. coli* and *P. aeruginosa* respectively, and innocuous against *S. aureus*, whereas Rsp-1(15–29) lacked antimicrobial potential

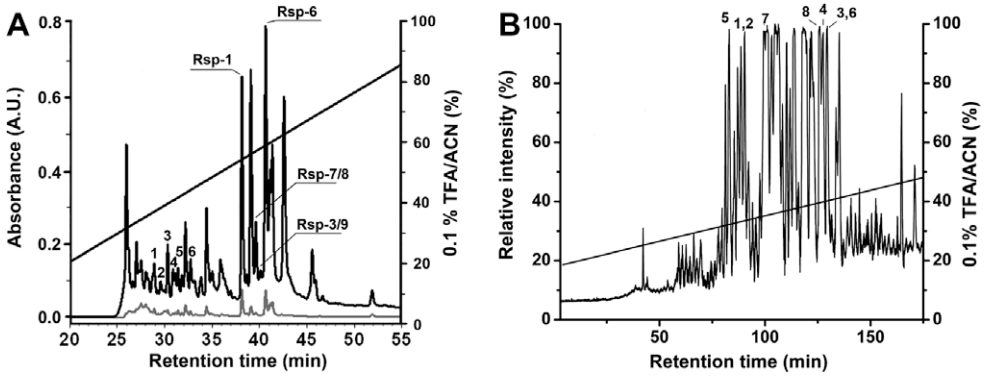

Fig. 2. (A) Analytical RP-HPLC chromatogram of the *H. raniceps* skin secretion crude extract. The absorbance was monitored at 216 and 280 nm (black and gray lines, respectively) in arbitrary units (A.U.). Fractions containing the full-length peptides Rsp-1, 6, 7/8, and 3/9 are shown. (B) CapLC/MS chromatogram of the *H. raniceps* skin secretion crude extract. The total ion intensity monitored during the LC/MS run has been normalized and has been reported as relative intensity of the ions. The numbers 1–8 in the chromatograms indicate the fractions where a corresponding raniseptin peptide half was identified, namely **1**: Rsp-2(15–29); **2**: Rsp-7(15–29); **3**: Rsp-3(1–14); **4**: Rsp-6(15–29); **5**: Rsp-1(15–29); **6**: Rsp-1(1–14); **7**: Rsp-3(15–28); **8**: Rsp-6(1–14). The gradient line across both chromatogram shows the increasing percentage of 0.1% TFA/ACN during the run.

Table 1
Antibacterial activity of Rsp-1, Rsp-1(1–14), Rsp-1(15–29) and commercial antibiotics.

Bacterial strain	MIC (μM)[a]				
	Rsp-1	Rsp-1 (1–14)	Rsp-1 (15–29)	Ampicillin	Chloramphenicol
E. coli	5	85	>156	46	395
P. aeruginosa	10	170	>156	>732	25
S. aureus	20	>170	>156	<11	25
X. citri	<2	5	>156	ND	ND

ND, not determined.
[a] MIC, average minimal peptide inhibitory concentration required for total bacterial growth inhibition.

altogether (Table 1). The fact that Rsp-1(1–14) displayed weak antimicrobial potential contrasts with other reports that have shown that the first eighteen N-terminal residues of a related dermaseptin S1 isolated from *Phyllomedusa sauvagei* are solely responsible for the antimicrobial activity of the peptide [15]. Although it can be argued that the fourteen residues of Rsp-1(1–14) are not sufficient to spam a lipid bilayer, other studies have shown that shorter fragments of dermaseptin S3, also synthesized with a C-terminal amidation, still retain full potency against Gram-negative bacteria [14]. In this sense, since a high positive charge content has been known to be important for antimicrobial activity, one possible explanation for the limited activity of Rsp-1(1–14) could be the presence of the negative charge of Asp-4, absent from the dermaseptins described. Further studies are necessary to verify this hypothesis.

Nevertheless, Rsp-1 and Rsp-1(1–14) were significantly active against *X. citri*, the causal agent of citrus canker, the determined MIC values were lower than 2 and 5 μM, respectively (Table 1). This result attests to the overall higher susceptibility of the phytopathogenic bacteria, providing an instance for possible biotechnology applications aiming at the engineering of resistant crop plants [16]. In order to investigate whether the proteolytic cleavage of Rsp-1 sustains a physiological role, Rsp-1(1–14) and Rsp-1(15–29) were assayed for synergism against the *X. citri* strain. However, the fragments did not mimic the activity of the full-length Rsp-1 *in vitro*. The MIC value of the peptide equimolar mixture was 10 μM. Considerations derived therefrom point to a possible role for this proteolytic enzyme in peptide turnover rather than an additional peptide activation event [12]. Furthermore, the cytotoxicity of all three peptides was also evaluated against human red blood cells. The Rsp-1 fragments produced no significant lytic effect (Fig. S4), whereas Rsp-1 induced 20% hemolysis at 80 μM (Fig. S4) which is consistent with values found for non-hemolytic dermarseptins [17].

MALDI Imaging MS

MALDI IMS techniques were applied to the study of the localization of the raniseptin peptides directly on the dorsal skin of *H. raniceps*. The undesirable liberation of the glandular secretion is prevented during excision of the skin, as demonstrated in similar studies [7,18]. The global mean MS spectrum acquired of the dorsal frog skin of *H. raniceps* is shown in Fig. 3a. The molecular ions corresponding to the mature Rsp-1, Rsp-3, and Rsp-7/8 sodium and potassium adducts of either peptide have been detected and are shown in the inset of Fig. 3a. The detected ions of the mature raniseptins were then mapped onto the skin fragment (Fig. 3b) to generate the localization profiles. Rsp-1, Rsp-3, and Rsp-7/8 peptides were co-localized in pairs as shown in Fig. 3e–g. Since primary visual inspection of their *in situ* spatial profiles alluded to the existence of a certain degree of co-localization, two reference peptides,

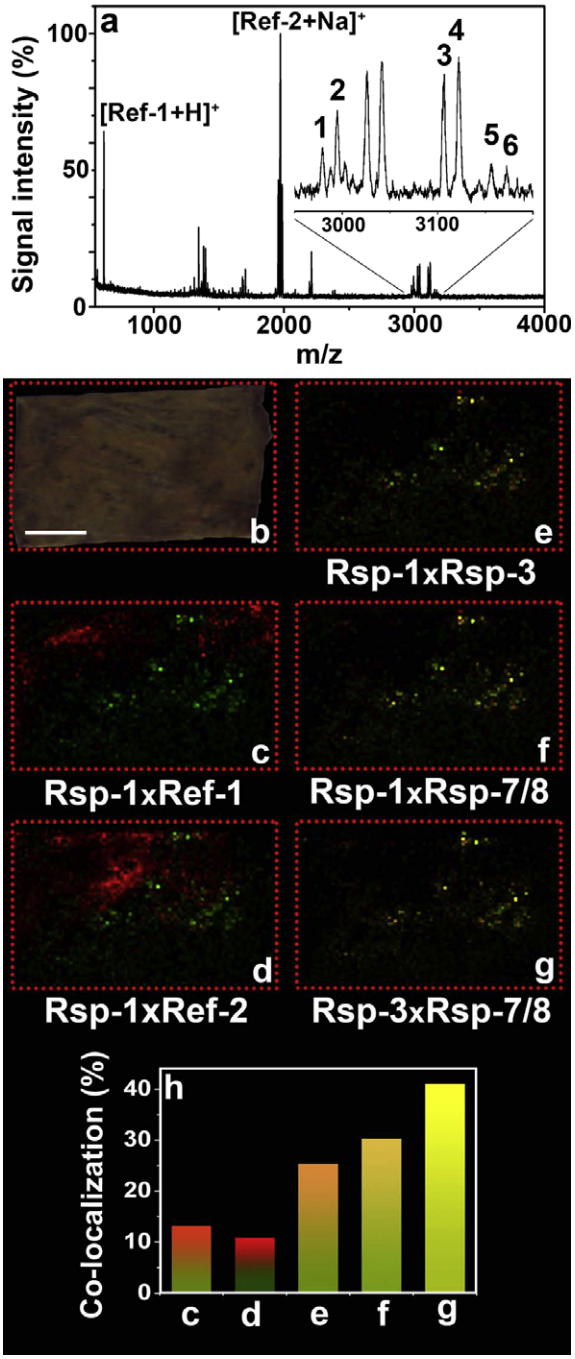

Fig. 3. (a) Global MS spectra acquired of the skin secretion of *H. raniceps*, showing the molecular masses corresponding to the reference peptides Ref-1 and Ref-2 sodium adducts. The inset shows the peaks corresponding to the molecular masses of the sodium and potassium adducts of Rsp-3 (peaks 1 and 2), Rsp-7/8 (peaks 3 and 4) and Rsp-1 (peaks 5 and 6). (b) Photographic image of the frog skin fragment set on a MALDI sample target. The red dotted rectangle defines the MS acquisition area and the white bar corresponds to 5 mm. (c) Co-localization profile of the molecular ion Ref-1 (red) with the molecular ion corresponding to the Rsp-1 sodium adduct (green). (d) Co-localization profile of the molecular ion Ref-2 (red) with the molecular ion corresponding to the Rsp-1 sodium adduct (green). (f–h) Co-localization profiles of the molecular ions which correspond to the mature raniseptin peptides, Rsp-1, Rsp-3, and Rsp-7/8 sodium adducts. The names of the peptide pairs, which were co-localized, are indicated under each image. (e) Histogram showing the percentage of co-localized areas between the pairs of peptides shown in the images (c,d) and (f–h). The histogram bars follow a color-coded gradient in which red and green colors indicate low co-localization values and yellow coloration indicates higher co-localization scores. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Ref-1 and Ref-2 (Fig. 3c and d), which do not belong to the raniseptin peptide family (unpublished results), were also co-localized with Rsp-1 (Fig. 3c and d). To demonstrate and quantify the different degrees of co-localization of the raniseptins and the reference peptides, a co-localization script was used to estimate the areas of the overlapping pixels within two images of choice (Fig. 3h). The scores derived show that the lowest co-localization value was obtained between the ions maps of the two reference peptides when superimposed with the ion map of the Rsp-1 peptide (*ca.* 15%). In contrast, the highest co-localized areas were found between the pairs of mature Rsp-1, Rsp-3, and Rsp-7/8 molecular ions, which cluster within the same region of the skin fragment with an average score of 30% co-localization (Fig. 3h). These results underline the initial assumption that distinct spatial glandular domains are in principle specialized in the synthesis and storage of different peptide families. However, the possibility of other peptides being stored with the raniseptins is not excluded.

Most importantly, the products of the internal cleavage of the raniseptins could not be detected *in situ*. From an evolutionary standpoint, the full-length peptides remain in its active form when stored in the granular glands, which in principle agrees with the existence of a first line of defense, of inducible expression upon contact with microorganisms [19]. However, since the secretion is liberated under stress conditions, mostly credited to predation threats rather than triggered by bacterial contact, post-secretory events such as rapid endoproteolysis presumably constitute a secondary attribute of innate immunity, responsible for altering the peptide and protein content of the crude extract, assuring peptide turnover, or alternatively by generating peptides with novel functions, better adapted to resist predator attack rather than bacterial infections. In fact, it is known that protein degradation can generate peptides with diverse biological functions, degrees of bitterness and/or unpleasant tastes [20].

In sum, the present work describes a novel family of antimicrobial peptides of *H. raniceps*, which shares a conserved cDNA organization common to hylid and ranid frogs, displays antimicrobial potential *in vitro* preferentially against Gram-negative bacteria and limited cytotoxicity against red blood cells. In addition, MALDI IMS demonstrated that the mature raniseptins although stored inside the skin glands in their intact form within a defined glandular domain, undergo rapid proteolytic cleavage upon secretion. Since the peptide fragments generated possess little or no antimicrobial activity, the raniseptin processing in *Hylinae* frogs presumably acts as a mechanism by which the protein and peptide content is altered to respond to different predatory attacks.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.10.102.

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