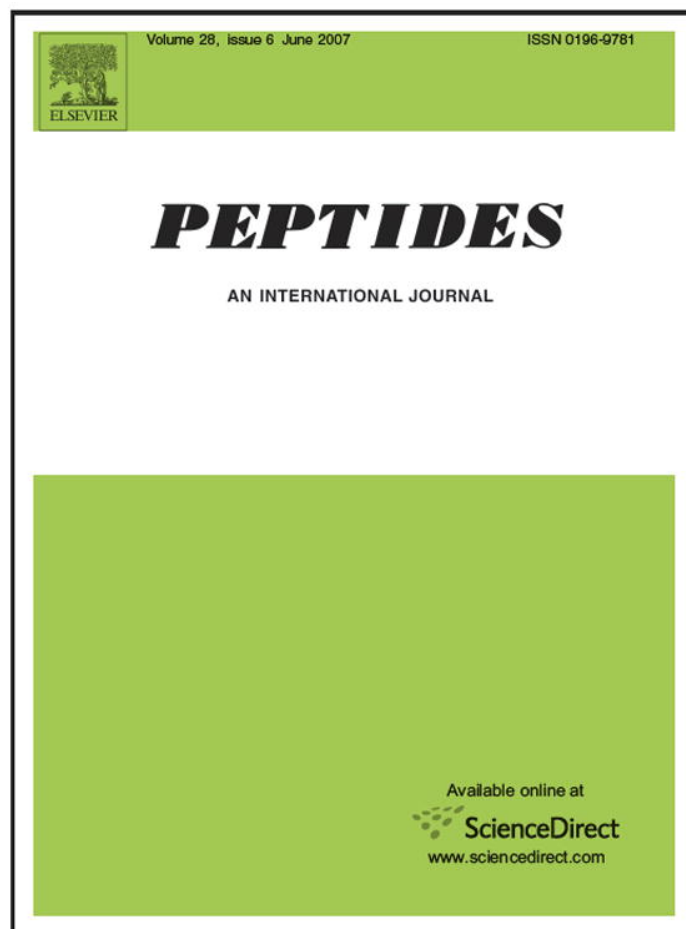


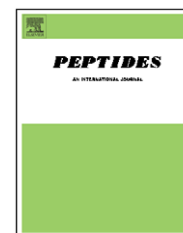
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The complex array of bradykinin-related peptides (BRPs) in the peptidome of pickerel frog (*Rana palustris*) skin secretion is the product of transcriptional economy

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ABSTRACT

Previous peptidomic analyses of the defensive skin secretion from the North American pickerel frog, *Rana palustris*, have established the presence of canonical bradykinin and multiple bradykinin-related peptides (BRPs). As a consequence of the multiplicity of peptides identified and their diverse primary structures, it was speculated that they must represent the products of expression of multiple genes. Here, we present unequivocal evidence that the majority of BRPs (11/13) identified in skin secretion by the peptidomic approach can be generated by differential site-specific protease cleavage from a single common precursor of 321 amino acid residues, named skin kininogen 1, whose primary structure was deduced from cloned skin secretion-derived cDNA. The organization of skin kininogen 1 consists of a hydrophobic signal peptide followed by eight non-identical domains each encoding a single copy of either canonical bradykinin or a BRP. Two additional splice variants, encoding precursors of 233 (skin kininogen 2) or 189 amino acid residues (skin kininogen 3), were also cloned and were found to lack BRP-encoding domains 5 and 6 or 4, 5 and 6, respectively. Thus, generation of peptidome diversity in amphibian defensive skin secretions can be achieved in part by differential protease cleavage of relatively large and multiple-encoding domain precursors reflecting a high degree of transcriptional economy.

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1. Introduction

Amphibian skin is arguably the most fundamental structure within this taxon in terms of environmental interaction both physiologically, with respect to respiration and excretion, and defensively, with respect to the pigmentation related to either camouflage or biohazard warning to potential predators [15]. In addition, specialized granular glands within the skin, in response to stressful stimuli, produce a noxious secretion that contains an abundance of bioactive molecules that mediate

the exquisite chemical defence of the animal. These chemical cocktails contain many classes of biochemical including biogenic amines, bufogenins, alkaloids, proteins and peptides [16,25]. Additionally, the polyadenylated mRNAs that encode the protein and peptide precursors are also present [6], a fact that facilitates parallel genomic and peptidomic/proteomic studies from crude secretions following collection and lyophilization.

Bradykinin (BK) is a peptide that mediates a wide range of physiological phenomena in mammals including vasodilation,

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hypotension, smooth muscle contraction, pain and inflammation [21], and analogs are present in the skin secretions of many frog and toad species including the most primitive extant frog *Ascaphus truei* [12]. The potent pain- and inflammation-inducing properties of BK make it an ideal deterrent to mammalian predators [13].

The specialized cells of the amphibian skin granular glands synthesize bioactive peptides that are structural homologs of many endogenous vertebrate regulatory peptides with subtle differences in primary structure likely being dictated by the peptide's target receptor in the discrete vertebrate predator [6,10]. The BRPs produced by frog skin generally differ from the canonical mammalian bradykinin in primary structure, with site-substituted, truncated and extended (both NH₂- and COOH-terminally) analogs being plentiful and often predominant in many species [13]. Specific examples include phyllokinin from *Phyllomedusa* sp. [7], maximakinin from *Bombina* sp. [8] and ranakinins from *Rana* sp. [26]. Canonical bradykinin was first discovered in the skin of *Rana temporaria* [14] and is now known to be present in the skin secretions of many additional species from different anuran taxa including *Phyllomedusa sauvagei* [7], *P. hypochondrialis hypochondrialis* [3], *Bombina maxima* [8], *B. orientalis* [9], *B. variegata* [10], *Agalychnis callidryas* [19], *Rana rugosa* [26] and *R. ridibunda* [20].

The pickerel frog (*Rana palustris*), whose skin is known to produce a particularly noxious and highly effective defensive skin secretion, is native to the eastern third of the North American continent. Two peptidomic studies on the defensive skin secretion peptides of this species have been reported [1,2]. In the first study, Basir et al. [1], a family of N- and C-terminally extended [Leu⁸]-bradykinin related peptides were identified in addition to canonical bradykinin. In the second, more comprehensive study [2], a family of bradykinin-related peptides (BRPs), designated Family E, were identified, in addition to several other families of non-BRPs.

All of these, *R. palustris* BRPs were discovered by a classical peptidomic strategy involving sequential high performance liquid chromatography, mass spectrometry and Edman degradation. However, as BK and BRPs occur in the defensive secretions of anurans independent of a kallikrein-kinin system, which is the means by which BK is produced in mammalian tissues [5,22], the lack of information on biosynthetic precursor structure, achieved by deduction from cloned cDNAs, makes interpretation of molecular events leading to primary structural diversity of products impossible. Previous studies addressing the cloning of amphibian skin kininogens, or preprobradykinins, in a variety of species [6-11,17,18,23,24], have shown that there is a plethora of different precursor structures with some encoding single BRP domains and others encoding multiple domains. Some species produce multiple splice variants while others have single transcripts. Within the multi-domain precursors, the primary structures of encoded BRPs can either be identical or highly variable. Prediction of BRP precursor structure within a given species is thus impossible based upon the data present in contemporary databases.

In the previous peptidomic reports on BRPs in *R. palustris* skin secretions, the authors speculatively suggested that some structurally related peptides were derived from a common precursor, while others were probably derived from three

different paralogous genes [1,2]. However, here we report the molecular cloning, primary structural identity and organization of a single multi-domain skin kininogen that encodes canonical bradykinin, [Leu⁸]-bradykinin related peptides and Family E BRPs identified in previous peptidomic studies. In addition, two splice variants were also cloned lacking several BRP-encoding domains. These data validate the scientific robustness and facilitation of data interpretation that can be achieved by performing peptidomic and transcriptomic studies in parallel in amphibian skin peptide studies.

2. Materials and methods

2.1. Acquisition of *R. palustris* skin secretion

Four adult specimens of pickerel frog (*R. palustris*), snout-to-vent length 5-6 cm, were housed in an appropriately designed terrarium at diurnal temperatures ranging from 16 to 24 °C and with a 12 h light:12 h dark cycle. They were sustained on a diet of multivitamin-loaded crickets fed three times per week. Under these conditions, the frogs remained healthy over a period of 4 years. Skin secretions were obtained by mild transdermal electrical stimulation (4 ms pulse width, 50 Hz, 5 V) using platinum electrodes for periods of 10 s applied to the moistened dorsal surfaces. Skin secretion, in the form of a milky white exudate, was profuse. This was rinsed from the skin surface using deionized water and collected into a clean chilled glass beaker. The washings from the four specimens were pooled and the entire procedure took less than 2 min. The contents of the chilled beaker were snap-frozen in liquid nitrogen and lyophilized in a Hetosicc 2-5 freeze-dryer (Heto, UK). The resultant white powder was carefully transferred to a clean 10 ml polypropylene tube, weighed, and stored at -20 °C. All procedures on living animals were carried out under appropriate licences from local and national bodies.

2.2. *In vitro* cDNA library construction from lyophilized skin secretion and molecular cloning of skin kininogen precursor cDNA

Five milligrams of lyophilized skin secretion were dissolved in 1 ml of cell lysis/mRNA stabilization solution (Dynal, UK). Polyadenylated mRNA was isolated using magnetic oligo-dT beads as described by the manufacturer (Dynal Biotech, UK). The isolated mRNA was subjected to 5' and 3'-RACE procedures to obtain full-length skin kininogen nucleic acid sequence data using a SMART-RACE kit (Clontech UK) essentially as described by the manufacturer. Briefly, the 3'-RACE reactions employed a nested universal primer (NUP) that was supplied with the kit and a sense primer (BK-S1; 5'-ATHMGIMGICCCICGIGTT-3') that was complementary to the amino acid sequence, IRRPPGF [1]. 3'-RACE reactions were gel-purified and cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated DNA sequencer. The sequence data obtained from these 3'-RACE products were used to design a gene-specific antisense primer: (BK-AS1: 5'-TCAATTCAAGATTAAACGATTGCTGG-3') to a region in the vicinity of the stop codon of the open-reading frame. 5'-RACE was carried out using this specific primer in

conjunction with the NUP and the generated product was gel-purified, cloned and sequenced as described above. Following acquisition of these data, the RACE reactions were amplified using a second gene-specific sense primer (BK-S2: 5'-ATGTT-CACCTTGAAGAAATCCCTGTT-3') designed to a site within the putative signal peptide domain, and a second antisense primer (BK-AS2, 5'-ACATCTGTTGTGTAGATAATTA-3') designed to a region of the 3'-non-translated region. Products were likewise gel-purified, cloned and sequenced as described previously. All identified skin kininogen nucleotide sequences were represented at least 10 times in the individual clones that were sequenced.

3. Results

Three distinct cDNAs were consistently cloned from the skin secretion-derived cDNA library using the strategy employed. These encoded skin kininogen precursors with deduced open-reading frames consisting of 321, 233 and 189 amino acid residues, respectively, that were named skin kininogens 1

through 3 (RPSK 1-3) (Figs. 1 and 2). While each contained an identical putative signal peptide, they differed in content of BRP encoding domains containing 8, 6 and 5, respectively. RPSK-2 and RPSK-3 were probable splice variants of RPSK-1 (Fig. 3). Table 1 illustrates the spectrum of BRPs detected in the skin secretion of *R. palustris* using a peptidomic approach in two previous studies [1,2], and with just two exceptions, all peptides could be generated by differential peptidase cleavage from the single precursor, RPSK-1. This skin kininogen contained two domains encoding canonical bradykinin (RPPGFSPFR), one domain encoding the nonadecapeptide, AGYSRVISLPAGLSPLRIA (A-19-A), two domains encoding the heptadecapeptide, AGYARVISLPAGLSPLR (A-17-R), one domain encoding the tridecapeptide, RVISLPAGLSPLR (R-12-FR), one domain encoding the tridecapeptide, RENSLPAGLSPLR (RE-12-R) and one domain encoding the pentadecapeptide, AGIRPPGFSPPLRIA (A-15-A), from which all of the [Leu⁸]-bradykinin-related peptides reported in Basir et al. [1], could be accounted for by sequential proteolysis of N- and C-terminals. The tridecapeptide, RVISLPAGLSPLR, reported by Basir et al. [2] could be generated from both A-19-A and A-17-R.

	<u>M</u>	<u>F</u>	<u>T</u>	<u>L</u>	<u>K</u>	<u>K</u>	<u>S</u>	<u>L</u>	<u>L</u>	<u>L</u>	<u>L</u>	<u>F</u>	<u>F</u>	<u>L</u>	<u>G</u>	<u>T</u>	<u>I</u>					
1	ATG	TTC	ACCT	TGA	AGAA	ATC	CCT	GTT	ACTC	C	TTT	CTT	TTC	TTG	GG	ACCAT						
	<u>S</u>	<u>L</u>	<u>S</u>	<u>L</u>	<u>C</u>	<u>E</u>	<u>Q</u>	<u>E</u>	<u>R</u>	<u>D</u>	<u>A</u>	<u>D</u>	<u>E</u>	<u>D</u>	<u>E</u>	<u>Y</u>						
51	CTC	CTT	ATCT	CTC	TG	TGA	AC	AAG	AG	GAGA	TG	CTG	AC	GAA	GAC	GAATATG						
	<u>A</u>	<u>G</u>	<u>D</u>	<u>A</u>	<u>K</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>V</u>	<u>K</u>	<u>R</u>	<u>A</u>	<u>G</u>	<u>Y</u>	<u>S</u>	<u>R</u>	<u>V</u>					
101	CAG	GGG	ACGC	TAA	AGCC	GAA	GAC	GT	TAAA	GAG	CAG	GATA	CTC	GAG	AGT	G						
	<u>I</u>	<u>S</u>	<u>L</u>	<u>P</u>	<u>A</u>	<u>G</u>	<u>L</u>	<u>S</u>	<u>P</u>	<u>L</u>	<u>R</u>	<u>I</u>	<u>A</u>	<u>P</u>	<u>A</u>	<u>S</u>						
151	ATC	AGT	CTCC	CAG	CTG	GGTT	GAG	CCC	ACTT	CGT	TATG	CAC	CAG	CGT	CTTC							
	<u>R</u>	<u>M</u>	<u>I</u>	<u>R</u>	<u>R</u>	<u>P</u>	<u>P</u>	<u>G</u>	<u>F</u>	<u>S</u>	<u>P</u>	<u>F</u>	<u>R</u>	<u>I</u>	<u>A</u>	<u>P</u>						
201	GAG	AAT	GATA	AG	AC	GGCC	AC	CTG	GGT	T	TTAG	CCC	ATT	TCG	T	ATTG	CACCAG					
	<u>A</u>	<u>S</u>	<u>T</u>	<u>L</u>	<u>K</u>	<u>R</u>	<u>D</u>	<u>A</u>	<u>D</u>	<u>E</u>	<u>D</u>	<u>E</u>	<u>Y</u>	<u>A</u>	<u>G</u>	<u>E</u>	<u>A</u>					
251	CAT	CT	TACTCT	CA	AG	AGAT	GCC	GAT	GAAG	ACG	AATATGC	AGG	GAAG	CT								
	<u>K</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>V</u>	<u>K</u>	<u>R</u>	<u>A</u>	<u>G</u>	<u>Y</u>	<u>S</u>	<u>R</u>	<u>V</u>	<u>I</u>	<u>S</u>	<u>L</u>	<u>P</u>					
301	AA	GCC	GAAG	ACG	T	TAAA	AG	AG	CAG	GATAC	T	C	GAG	AGT	G	A	T	CAG	T	CCC		
	<u>A</u>	<u>G</u>	<u>L</u>	<u>S</u>	<u>P</u>	<u>F</u>	<u>R</u>	<u>I</u>	<u>A</u>	<u>P</u>	<u>A</u>	<u>S</u>	<u>T</u>	<u>L</u>	<u>K</u>	<u>R</u>						
351	AG	CTG	GGTTG	AG	CC	ATTT	GT	ATT	GC	ACC	AG	CAT	CT	ACT	CT	CAAG	AG	AG	G	AG		
	<u>D</u>	<u>A</u>	<u>D</u>	<u>E</u>	<u>D</u>	<u>E</u>	<u>Y</u>	<u>A</u>	<u>G</u>	<u>E</u>	<u>A</u>	<u>K</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>V</u>	<u>K</u>					
401	AT	GCC	GATGA	AG	AC	GAATAT	GC	AG	GGG	GAAG	CT	AA	AG	CCG	A	AG	AC	GT	TAAA			
	<u>R</u>	<u>A</u>	<u>G</u>	<u>Y</u>	<u>A</u>	<u>R</u>	<u>V</u>	<u>I</u>	<u>S</u>	<u>L</u>	<u>P</u>	<u>A</u>	<u>G</u>	<u>L</u>	<u>S</u>	<u>P</u>	<u>L</u>					
451	AG	AG	CAGGAT	AC	G	CAGAGT	GAT	CAG	TCTC	CC	AG	CTG	GGT	TG	AG	CCC	ACT					
	<u>R</u>	<u>I</u>	<u>A</u>	<u>P</u>	<u>A</u>	<u>S</u>	<u>T</u>	<u>L</u>	<u>K</u>	<u>R</u>	<u>D</u>	<u>A</u>	<u>D</u>	<u>E</u>	<u>D</u>	<u>E</u>						
501	TC	G	TATTGCA	CC	AG	CATCTA	CT	CT	CAAG	AG	ATG	CCG	GAT	GA	AG	AC	GAAT					
	<u>Y</u>	<u>A</u>	<u>G</u>	<u>E</u>	<u>A</u>	<u>K</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>V</u>	<u>K</u>	<u>R</u>	<u>A</u>	<u>G</u>	<u>Y</u>	<u>A</u>	<u>R</u>					
551	AT	G	CAG	GGG	GA	CTAA	AGCC	GA	AG	AC	GTTA	AA	AG	AG	CAG	AT	AC	G	C	G	A	
	<u>V</u>	<u>I</u>	<u>S</u>	<u>L</u>	<u>P</u>	<u>A</u>	<u>G</u>	<u>L</u>	<u>S</u>	<u>P</u>	<u>L</u>	<u>R</u>	<u>I</u>	<u>A</u>	<u>P</u>	<u>A</u>	<u>S</u>					
601	GT	GAT	CAGTC	TCC	CAG	CTGG	GTT	GAG	CCCA	CTT	CGT	ATTG	CACC	AG	CATC							
	<u>T</u>	<u>L</u>	<u>K</u>	<u>R</u>	<u>D</u>	<u>A</u>	<u>D</u>	<u>E</u>	<u>D</u>	<u>E</u>	<u>Y</u>	<u>A</u>	<u>G</u>	<u>E</u>	<u>A</u>	<u>K</u>						
651	TAC	TCT	CAAG	AG	AG	ATGCAG	AT	GA	AG	ACGA	AT	ATG	CAG	GG	GA	AG	CTAA	AG				
	<u>A</u>	<u>E</u>	<u>D</u>	<u>V</u>	<u>K</u>	<u>R</u>	<u>A</u>	<u>R</u>	<u>Y</u>	<u>S</u>	<u>R</u>	<u>E</u>	<u>N</u>	<u>S</u>	<u>L</u>	<u>P</u>	<u>A</u>					
701	CC	GA	AG	ACGT	TAA	AG	AGCA	AG	ATA	CTCGA	GAG	AGA	ACAG	TCT	CC	CAG	CT					
	<u>G</u>	<u>L</u>	<u>S</u>	<u>P</u>	<u>L</u>	<u>R</u>	<u>I</u>	<u>A</u>	<u>P</u>	<u>A</u>	<u>S</u>	<u>T</u>	<u>L</u>	<u>K</u>	<u>R</u>	<u>D</u>	<u>A</u>					
751	GG	GTT	GAGCC	CA	CTT	CGTAT	TG	CA	CCAG	CA	TCT	ACT	CTCA	AG	AG	AG	ATGC					
	<u>D</u>	<u>E</u>	<u>D</u>	<u>E</u>	<u>Y</u>	<u>A</u>	<u>G</u>	<u>E</u>	<u>A</u>	<u>K</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>V</u>	<u>K</u>	<u>R</u>						
801	CG	AT	GAAGAC	GA	AT	ATGCAG	GG	GA	AG	CTAA	AG	CC	GAAG	AC	GT	TAA	AG	AG				
	<u>A</u>	<u>G</u>	<u>I</u>	<u>R</u>	<u>P</u>	<u>P</u>	<u>P</u>	<u>G</u>	<u>F</u>	<u>S</u>	<u>P</u>	<u>L</u>	<u>R</u>	<u>I</u>	<u>A</u>	<u>P</u>	<u>A</u>					
851	CAG	GG	GATCAG	AC	G	CCC	ACCT	GGG	T	TAG	CC	CA	CTT	CGT	AT	TG	CA	CC	AG	CG		
	<u>S</u>	<u>S</u>	<u>R</u>	<u>M</u>	<u>I</u>	<u>R</u>	<u>R</u>	<u>P</u>	<u>P</u>	<u>G</u>	<u>F</u>	<u>S</u>	<u>P</u>	<u>F</u>	<u>R</u>	<u>I</u>	<u>A</u>					
901	TCT	T	CGAGAA	TG	AT	CAGACG	CCC	AC	CTGG	TT	TAG	CC	CA	TTC	G	T	ATTG	CG				
	<u>P</u>	<u>A</u>	<u>I</u>	<u>V</u>	*																	
951	CCC	AG	CAATC	GT	TAA																	

Fig. 1 – Nucleotide sequence and amino acid residue translation of the open-reading frame of *Rana palustris* skin kininogen 1 (RPSK-1) deduced from cDNA cloned from a skin secretion-derived library. The putative signal peptide is double-underlined and sequences encoding peptides identified in previous peptidomic studies are single-underlined. The stop codon is indicated by an asterisk.

(A)

	<u>M</u>	<u>F</u>	<u>T</u>	<u>L</u>	<u>K</u>	<u>K</u>	<u>S</u>	<u>L</u>	<u>L</u>	<u>L</u>	<u>L</u>	<u>F</u>	<u>F</u>	<u>L</u>	<u>G</u>	<u>T</u>	<u>I</u>
1	ATGTTACCT	TGAAGAAATC	CCTGTTACTC	CTTTTCTTTC	TTGGGACCAT												
	<u>S</u>	<u>L</u>	<u>S</u>	<u>L</u>	<u>C</u>	<u>E</u>	<u>Q</u>	<u>E</u>	<u>R</u>	<u>D</u>	<u>A</u>	<u>D</u>	<u>E</u>	<u>D</u>	<u>E</u>	<u>E</u>	<u>Y</u>
51	CTCCTTATCT	CTCTGTGAAC	AAGAGAGAGA	TGCTGACGAA	GACGAATATG												
	<u>A</u>	<u>G</u>	<u>D</u>	<u>A</u>	<u>K</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>V</u>	<u>K</u>	<u>R</u>	<u>A</u>	<u>G</u>	<u>Y</u>	<u>S</u>	<u>R</u>	<u>V</u>
101	CAGGGGACGC	TAAAGCCGAA	GACGTTAAAA	GAGCAGGATA	CTCGAGAGTG												
	<u>I</u>	<u>S</u>	<u>L</u>	<u>P</u>	<u>A</u>	<u>G</u>	<u>L</u>	<u>S</u>	<u>P</u>	<u>L</u>	<u>R</u>	<u>I</u>	<u>A</u>	<u>P</u>	<u>A</u>	<u>S</u>	<u>S</u>
151	ATCAGTCTCC	CAGCTGGGTT	GAGCCCACTT	CGTATTGCAC	CAGCGTCTTC												
	<u>R</u>	<u>M</u>	<u>I</u>	<u>R</u>	<u>R</u>	<u>P</u>	<u>P</u>	<u>G</u>	<u>F</u>	<u>S</u>	<u>P</u>	<u>F</u>	<u>R</u>	<u>I</u>	<u>A</u>	<u>P</u>	
201	GAGAATGATA	AGACGCCAC	CAGGGTTTAG	CCCATTTTCGT	ATTGCACCAG												
	<u>A</u>	<u>S</u>	<u>T</u>	<u>L</u>	<u>K</u>	<u>R</u>	<u>D</u>	<u>A</u>	<u>D</u>	<u>E</u>	<u>D</u>	<u>E</u>	<u>Y</u>	<u>A</u>	<u>G</u>	<u>E</u>	<u>A</u>
251	CATCTACTCT	CAAGAGAGAT	GCCGATGAAG	ACGAATATGC	AGGGGAAGCT												
	<u>K</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>V</u>	<u>K</u>	<u>R</u>	<u>A</u>	<u>G</u>	<u>Y</u>	<u>S</u>	<u>R</u>	<u>V</u>	<u>I</u>	<u>S</u>	<u>L</u>	<u>P</u>
301	AAAGCCGAAG	ACGTTAAAAG	AGCAGGATAC	TCGAGAGTGA	TCAGTCTCCC												
	<u>A</u>	<u>G</u>	<u>L</u>	<u>S</u>	<u>P</u>	<u>F</u>	<u>R</u>	<u>I</u>	<u>A</u>	<u>P</u>	<u>A</u>	<u>S</u>	<u>T</u>	<u>L</u>	<u>K</u>	<u>R</u>	
351	AGCTGGGTTG	AGCCCATTTT	GTATTGCACC	AGCATCTACT	CTCAAGAGAG												
	<u>D</u>	<u>A</u>	<u>D</u>	<u>E</u>	<u>D</u>	<u>E</u>	<u>Y</u>	<u>A</u>	<u>G</u>	<u>E</u>	<u>A</u>	<u>K</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>V</u>	<u>K</u>
401	ATGCCGATGA	AGACGAATAT	GCAGGGGAAG	CTAAAGCCGA	AGACGTTAAA												
	<u>R</u>	<u>A</u>	<u>G</u>	<u>Y</u>	<u>A</u>	<u>R</u>	<u>V</u>	<u>I</u>	<u>S</u>	<u>L</u>	<u>P</u>	<u>A</u>	<u>G</u>	<u>L</u>	<u>S</u>	<u>P</u>	<u>L</u>
451	AGAGCAGGAT	ACGCGAGAGT	GATCAGTCTC	CCAGCTGGGT	TGAGCCCACT												
	<u>R</u>	<u>I</u>	<u>A</u>	<u>P</u>	<u>A</u>	<u>S</u>	<u>T</u>	<u>L</u>	<u>K</u>	<u>R</u>	<u>D</u>	<u>A</u>	<u>D</u>	<u>E</u>	<u>D</u>	<u>E</u>	
501	TCGTATTGCA	CCAGCATCTA	CTCTCAAGAG	AGATGCCGAT	GAAGACGAAT												
	<u>Y</u>	<u>A</u>	<u>G</u>	<u>E</u>	<u>A</u>	<u>K</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>V</u>	<u>K</u>	<u>R</u>	<u>A</u>	<u>G</u>	<u>I</u>	<u>R</u>	<u>R</u>
551	ATGCAGGGGA	AGCTAAAGCC	GAAGACGTTA	AAAGAGCAGG	GATCAGACGC												
	<u>P</u>	<u>P</u>	<u>G</u>	<u>F</u>	<u>S</u>	<u>P</u>	<u>L</u>	<u>R</u>	<u>I</u>	<u>A</u>	<u>P</u>	<u>A</u>	<u>S</u>	<u>S</u>	<u>R</u>	<u>M</u>	<u>I</u>
601	CCACCTGGGT	TTAGCCCACT	TCGTATTGCA	CCAGCGTCTT	CGAGAATGAT												
	<u>R</u>	<u>R</u>	<u>P</u>	<u>P</u>	<u>G</u>	<u>F</u>	<u>S</u>	<u>P</u>	<u>F</u>	<u>R</u>	<u>I</u>	<u>A</u>	<u>P</u>	<u>A</u>	<u>I</u>	<u>V</u>	
651	CAGACGCCCA	CCTGGGTTTA	GCCCATTTTCG	TATTGCCCCA	GCAATCGTTT												
	*																
701	AA																

(B)

	<u>M</u>	<u>F</u>	<u>T</u>	<u>L</u>	<u>K</u>	<u>K</u>	<u>S</u>	<u>L</u>	<u>L</u>	<u>L</u>	<u>L</u>	<u>F</u>	<u>F</u>	<u>L</u>	<u>G</u>	<u>T</u>	<u>I</u>
1	ATGTTACCT	TGAAGAAATC	CCTGTTACTC	CTTTTCTTTC	TTGGGACCAT												
	<u>S</u>	<u>L</u>	<u>S</u>	<u>L</u>	<u>C</u>	<u>E</u>	<u>Q</u>	<u>E</u>	<u>R</u>	<u>D</u>	<u>A</u>	<u>D</u>	<u>E</u>	<u>D</u>	<u>E</u>	<u>E</u>	<u>Y</u>
51	CTCCTTATCT	CTCTGTGAAC	AAGAGAGAGA	TGCTGACGAA	GACGAATATG												
	<u>A</u>	<u>G</u>	<u>D</u>	<u>A</u>	<u>K</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>V</u>	<u>K</u>	<u>R</u>	<u>A</u>	<u>G</u>	<u>Y</u>	<u>S</u>	<u>R</u>	<u>V</u>
101	CAGGGGACGC	TAAAGCCGAA	GACGTTAAAA	GAGCAGGATA	CTCGAGAGTG												
	<u>I</u>	<u>S</u>	<u>L</u>	<u>P</u>	<u>A</u>	<u>G</u>	<u>L</u>	<u>S</u>	<u>P</u>	<u>L</u>	<u>R</u>	<u>I</u>	<u>A</u>	<u>P</u>	<u>A</u>	<u>S</u>	<u>S</u>
151	ATCAGTCTCC	CAGCTGGGTT	GAGCCCACTT	CGTATTGCAC	CAGCGTCTTC												
	<u>R</u>	<u>M</u>	<u>I</u>	<u>R</u>	<u>R</u>	<u>P</u>	<u>P</u>	<u>G</u>	<u>F</u>	<u>S</u>	<u>P</u>	<u>F</u>	<u>R</u>	<u>I</u>	<u>A</u>	<u>P</u>	
201	GAGAATGATA	AGACGCCAC	CTGGGTTTAG	CCCATTTTCGT	ATTGCACCAG												
	<u>A</u>	<u>S</u>	<u>T</u>	<u>L</u>	<u>K</u>	<u>R</u>	<u>D</u>	<u>A</u>	<u>D</u>	<u>E</u>	<u>D</u>	<u>E</u>	<u>Y</u>	<u>A</u>	<u>G</u>	<u>E</u>	<u>A</u>
251	CATCTACTCT	CAAGAGAGAT	GCCGATGAAG	ACGAATATGC	AGGGGAAGCT												
	<u>K</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>V</u>	<u>K</u>	<u>R</u>	<u>A</u>	<u>G</u>	<u>Y</u>	<u>S</u>	<u>R</u>	<u>V</u>	<u>I</u>	<u>S</u>	<u>L</u>	<u>P</u>
301	AAAGCCGAAG	ACGTTAAAAG	AGCAGGATAC	TCGAGAGTGA	TCAGTCTCCC												
	<u>A</u>	<u>G</u>	<u>L</u>	<u>S</u>	<u>P</u>	<u>F</u>	<u>R</u>	<u>I</u>	<u>A</u>	<u>P</u>	<u>A</u>	<u>S</u>	<u>T</u>	<u>L</u>	<u>K</u>	<u>R</u>	
351	AGCTGGGTTG	AGCCCATTTT	GTATTGCACC	AGCATCTACT	CTCAAGAGAG												
	<u>D</u>	<u>A</u>	<u>D</u>	<u>E</u>	<u>D</u>	<u>E</u>	<u>Y</u>	<u>A</u>	<u>G</u>	<u>E</u>	<u>A</u>	<u>K</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>V</u>	<u>K</u>
401	ATGCCGATGA	AGACGAATAT	GCAGGGGAAG	CTAAAGCCGA	AGACGTTAAA												
	<u>R</u>	<u>A</u>	<u>G</u>	<u>I</u>	<u>R</u>	<u>R</u>	<u>P</u>	<u>P</u>	<u>G</u>	<u>F</u>	<u>S</u>	<u>P</u>	<u>L</u>	<u>R</u>	<u>I</u>	<u>A</u>	<u>P</u>
451	AGAGCAGGGA	TCAGACGCC	ACCTGGGTTT	AGCCCACTTC	GTATTGCACC												
	<u>A</u>	<u>S</u>	<u>S</u>	<u>R</u>	<u>M</u>	<u>I</u>	<u>R</u>	<u>R</u>	<u>P</u>	<u>P</u>	<u>G</u>	<u>F</u>	<u>S</u>	<u>P</u>	<u>F</u>	<u>R</u>	
501	AGCGTCTTCG	AGAATGATCA	GACGCCCACT	TGGGTTTAGC	CCATTTTCGTA												
	<u>I</u>	<u>A</u>	<u>P</u>	<u>A</u>	<u>I</u>	<u>V</u>	*										
551	TTGCCCCAGC	AATCGTTTAG															

Fig. 2 - Nucleotide sequences and amino acid translations of the open-reading frames encoding *Rana palustris* skin kininogen 2 (RPSK-2) (A) and skin kininogen 3 (RPSK-3) (B) deduced from cloned skin secretion library cDNAs. Putative signal peptides are double-underlined, sequences encoding previously identified peptides are single-underlined and stop codons are indicated by asterisks.

RPSK-2 contained two domains encoding canonical bradykinin, one domain encoding A-19-A, one domain encoding R-12-FR, one domain encoding A-17-R and one domain encoding all [Leu⁸]-bradykinin related peptides contained within A-15-A (Fig. 3). RPSK-2 thus differed from RPSK-1 in the absence of a second A-17-R encoding domain and that domain encoding

RE-12-R. RPSK-3, the smallest translated precursor protein, contained two canonical bradykinin-encoding domains and three additional domains encoding A-19-A, R-12-FR and A-15-A, respectively. RPSK-3 did not contain any domains encoding A-17-R. The nucleotide sequences and translated open-reading frames of *R. palustris* skin kininogens 1 through 3 (RPSK 1-3)

(A) *Rana palustris* skin kininogen 1.Signal peptide domain ***MFTLKKSLLLLFFLGTISLSLCEQE*****BLP-encoding domains**

1.	R	DADEDEYAGDAKAEDV	KR	AGYS	RVISLPAGLSPLR	IAPASS
2.				R	MIR RPPGFSPFR	IAPASTL
3.	KR	DADEDEYAGEAKAEDV	KR	AGYS	RVISLPAGLSPFR	IAPASTL
4.	KR	DADEDEYAGEAKAEDV	KR	AGYA	RVISLPAGLSPLR	IAPASTL
5.	KR	DADEDEYAGEAKAEDV	KR	AGYA	RVISLPAGLSPLR	IAPASTL
6.	KR	DADEDEYAGEAKAEDV	KR	ARYS	RENSLPAGLSPLR	IAPASTL
7.				KR	AGIRRPFGFSPLR	IAPASS
8.				R	MIR RPPGFSPFR	IAPAIV*

(B) *Rana palustris* skin kininogen 2.Signal peptide domain ***MFTLKKSLLLLFFLGTISLSLCEQE*****BLP-encoding domains**

1.	R	DADEDEYAGDAKAEDV	KR	AGYS	RVISLPAGLSPLR	IAPASS
2.				R	MIR RPPGFSPFR	IAPASTL
3.	KR	DADEDEYAGEAKAEDV	KR	AGYS	RVISLPAGLSPFR	IAPASTL
4.	KR	DADEDEYAGEAKAEDV	KR	AGYA	RVISLPAGLSPLR	IAPASTL
5.	KR	DADEDEYAGEAKAEDV	KR		AGIRRPFGFSPLR	IAPASS
6.				R	MIR RPPGFSPFR	IAPAIV*

(C) *Rana palustris* skin kininogen 3.Signal peptide domain ***MFTLKKSLLLLFFLGTISLSLCEQE*****BLP-encoding domains**

1.	R	DADEDEYAGDAKAEDV	KR	AGYS	RVISLPAGLSPLR	IAPASS
2.				R	MIR RPPGFSPFR	IAPASTL
3.	KR	DADEDEYAGEAKAEDV	KR	AGYS	RVISLPAGLSPFR	IAPASTL
4.	KR	DADEDEYAGEAKAEDV	KR		AGIRRPFGFSPLR	IAPASS
5.				R	MIR RPPGFSPFR	IAPAIV*

Fig. 3 – Structural organization and domain topography of *Rana palustris* skin kininogens 1 through 3, respectively (A–C).**Table 1 – Bradykinin-related peptides identified by peptidomic analysis of the skin secretion of the North American pickerel frog, *Rana palustris***

Bradykinin	RPPGFSPFR
(Leu ⁸)-bradykinin-related peptides	IRRPPGFSPPLR IRRPPGFSPPLRIA AGIRRPFGFSPLR AGIRRPFGFSPLRIA
Family E peptides	AGYSRVISLPAGLSPLRIA AGYARVISLPAGLSPLR RVISLPAGLSPLRIA RVISLPAGLSPLRI RVISLPAGLSPFR RTISLPAGLSPLR ^a RVISLPAGLSPLR SENSLPAGLSPLR ^b RVISLPAGLSP

(Leu⁸)-bradykinin-related peptides from [1]. Family E peptides from [2].^a Not encoded by common precursor identified in this study.^b Not identified in present study but similar peptide (RE-NSLPAGLSPLR) encoded by domain #6 in skin kininogen 1.

have been deposited in the EMBL Nucleotide Sequence Database under accession numbers AM412048, AM412049 and AM412050, respectively.

4. Discussion

Bradykinin is among the most potent endogenous inflammatory mediators known [4,5] and this, coupled with its algescic properties, renders it an ideal component of a biomolecular cocktail whose function is the deterrence or disablement of predators. The molecular evolution of peptide components of amphibian defensive skin secretions has led to the genesis of bradykinin-related peptides that are sometimes subtly and other times radically different in primary structure from the canonical mammalian nonapeptide, a factor that is probably highly influenced by the spectrum of predators that prey on each species [13].

Although a large array of BRPs have been identified in and structurally characterized from amphibian defensive skin secretions, representing a broad spectrum of anuran taxa, the elucidation of encoding precursor or skin kininogen structure has only occurred relatively recently, using molecular cloning

technology, with the reports of that encoding maximakinin from the skin of the Chinese discoglossid toad, *B. maxima* [8]. Since this first description, the structures of skin kininogens, or preprobradykinins, have been reported from *B. orientalis* [9], *B. variegata* [10], *Rana (Odorrana) schmackeri* [17], *P. sauvagei* [7], *P. hypochondrialis* [11], *P. hypochondrialis azurea* [24], *Amolops loloensis* [18] and *Rana sakuraii* [23]. The most striking feature when these data are reviewed is the high degree of heterogeneity of precursor structure and organization both between but often even within a species. *B. orientalis* and *B. variegata* skin each contain two different and unique skin kininogens encoding a different BRP [9,10]. In *B. orientalis*, several coding domains for each BRP are found whereas in *B. variegata*, only single BRP-encoding domains are present. In *R. (Odorrana) schmackeri* skin, a single kininogen was found containing seven tandem repeat domains encoding canonical BK [17]. *P. sauvagei* and *P. hypochondrialis* skin contain a single kininogen encoding a BRP located at the C-terminus or in *P. hypochondrialis azurea* skin, multiple kininogens containing single different BRPs at the C-terminals [24]. Torrent frog (*A. loloensis*) skin contains a single BRP encoded as a single copy within its precursor [18]. It is thus not possible to predict the organization of the skin kininogens from amphibians even when data exists for closely related or indeed, con-generic species.

To add to this complexity of precursor organization, is a large degree of species-specific propeptide processing by endogenous proteases/peptidases that can generate an array of both N- and/or C-terminally extended molecular forms and also those attenuated at both terminals. If this were not enough to generate molecular complexity from a single template, processed BRPs can also be post-translationally modified most commonly by hydroxylation of the prolyl residue, analogous to position 3 in canonical bradykinin, or by sulfation of the terminal tyrosyl residue present in the phyllokinins of phyllomedusid frogs. In *P. sauvagei* skin, both of these post-translational modifications can be found on some phyllokinins [7]. The trend therefore in most species is to generate BRP structural diversity within the peptidome using a combination of both of these strategies.

In *R. palustris* skin, however, the data presented in this study would imply that the strategy adopted here appears unique among amphibian skin kininogens reported to date in that a high degree of structural diversity of BRP encoding domains within a single precursor occurs before differential peptidase processing generates a remarkable diversity of BRPs. With the exception of two peptides, 12 of the 14 [Leu⁸]-BK-related and Family E peptides identified in peptidomic studies of *R. palustris* skin secretion as reported by Basir et al. (Table 1) [1,2], were found to be encoded by skin kininogen 1 (RPSK-1)—a precursor whose open-reading frame deduced from cloned cDNA, contained 321 amino acid residues (Fig. 1). As described in the results section and illustrated in Fig. 2, two additional splice variants of RPSK-1, named RPSK-2 and -3 (skin kininogens 2 and 3), encoding precursor open-reading frames of 233 and 189 amino acid residues, respectively, were also consistently cloned from the *R. palustris* skin secretion-derived cDNA library. The BRP-encoding domain organizations of each precursor are shown in Fig. 3. The purpose of alternative splicing, known to alter function in the case of many proteins,

is in this case unknown, although it could indicate a hierarchal order of functionality of the peptides encoded by the precursors as BK, as well as A-19-A, R-12-FR and A-15-A are present in all kininogen splice variants. This proposal would have to be substantiated by both real-time PCR, to accurately determine the relative amounts of each transcript, and by quantitative peptidomics, to accurately determine the relative amounts of each processed peptide. However, as a cautionary note, quantity may actually be inversely proportional to biological potency.

While the biological relevance of this plethora of BRPs in *R. palustris* skin secretion remains obscure, this family of peptides almost certainly contributes to the toxic nature, including the amphibicidal effects, of the secretions, although these authors failed to demonstrate any bioactivity for these skin BRPs (excepting canonical bradykinin) in classical mammalian smooth muscle bioassays [1,2]. It may be that the peptides are directed against targets in non-mammalian predators that have quite different and as yet unexplored structure/activity requirements for activating ligands.

Only 2 of the 14 peptides identified in previous peptidomic studies of secretion from this species were not present within the BRP-coding domains of the skin kininogens identified here. R-13-R, exhibiting a threoninyl for valyl residue substitution at position 2, as identified in a previous peptidomic study, could arise as an allelic variant as a different sample of *R. palustris* was employed in both studies. S-13-R, with an N-terminal sequence SENS-, identified in the previous peptidomic study, was not encoded by any of the skin kininogens but a peptide with an N-terminal arginyl (R) residue and with an N-terminal sequence, RENS-, was encoded by domain #6 in kininogen 1. It is difficult to envisage how confusion could have arisen over the designation of a seryl and arginyl residue as they differ considerably in molecular mass (87 amu versus 156 amu). The peptide exhibiting an N-terminal seryl residue identified in the peptidomic study was subjected to automated Edman degradation and mass spectrometric analysis—both of which are consistent with this residue. However, the peptide encoded by kininogen 1, and whose primary structure was deduced from cloned cDNA, was represented in at least 10 different clones and hence is not a sequencing artefact. In addition, the peptides arising from processing of domains 1 and 3 through 6 of kininogen 1 are all cleaved between a seryl/alanyl and arginyl bond at the N-terminus. However, leaving this minor inexplicable discrepancy aside, it is obvious that in the skin of *R. palustris*, the high degree of diversity exhibited by BRPs is achieved by peptidase processing of the products encoded by a single, multi-domain precursor and by two related but truncated domain precursors that arise from differential mRNA editing.

Thus, diversity of BRPs in the skin secretion of *R. palustris* arise from a transcriptional economy that relies upon expression of a precursor (kininogen) with multiple heterogeneous BRP-encoding domains, the products of which are acted upon by a battery of site-specific peptidases. A formidable array of putative defensive peptides can thus be generated from a single template produced by a single translation event.

The robustness of nucleotide sequence data obtained by molecular cloning of defensive amphibian skin peptide

encoding cDNAs from lyophilized skin secretion-derived cDNA libraries has been demonstrated and exonerated once again through the data generated and fundamental question addressed in the present study. These data were obtained through non-invasive, non-lethal procedures on the amphibian specimens employed and it is the desire of the authors, having validated said procedure in many recent peer-reviewed publications, that it would be adopted by other workers in the field to spare the lives of the providers of such secretions.

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