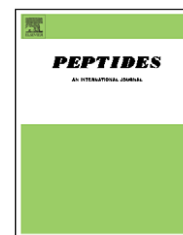


available at www.sciencedirect.comjournal homepage: www.elsevier.com/locate/peptides

Rapid identification of precursor cDNAs encoding five structural classes of antimicrobial peptides from pickerel frog (*Rana palustris*) skin secretion by single step “shotgun” cloning[☆]

Mei Zhou, Lei Wang, Damian E. Owens, Tianbao Chen, Brian Walker, Chris Shaw^{*}

Molecular Therapeutics Research, School of Pharmacy, Queen’s University, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland, UK

ARTICLE INFO

Article history:

Received 12 June 2007

Received in revised form

16 July 2007

Accepted 16 July 2007

Published on line 21 July 2007

Keywords:

Amphibian

Venom

Mass spectrometry

Peptide

Cloning

ABSTRACT

The skin secretion of the North American pickerel frog (*Rana palustris*) has long been known to have pronounced noxious/toxic properties and to be highly effective in defence against predators and against other sympatric amphibians. As it consists largely of a complex mixture of peptides, it has been subjected to systematic peptidomic study but there has been little focus on molecular cloning of peptide-encoding cDNAs and by deduction, the biosynthetic precursors that they encode. Here, we demonstrate that the cDNAs encoding the five major structural families of antimicrobial peptides can be elucidated by a single step “shotgun” cloning approach using a cDNA library constructed from the source material of the peptidomic studies—the defensive skin secretion itself. Using a degenerate primer pool designed to a highly conserved nucleic acid sequence 5′ to the initiation codon of known antimicrobial peptide precursor transcripts, we amplified cDNA sequences representing five major classes of antimicrobial peptides, such as esculentins, brevinins, ranatuerins, palustrins and temporins. Bioinformatic comparisons of precursor open-reading frames and nucleic acid sequences revealed high degrees of structural similarities between analogous peptides of *R. palustris* and the Chinese bamboo odorous frog, *Rana versabilis*. This approach thus constitutes a robust technique that can be used either alone or ideally, in parallel with peptidomic analysis of skin secretion, to rapidly extract primary structural information on amphibian skin secretion peptides and their biosynthetic precursors.

© 2007 Elsevier Inc. All rights reserved.

1. Introduction

Among anuran amphibians, the genus *Rana* is arguably the most diverse taxon and consequently the subject of numerous revisions by systematists. With a wide global distribution on all continents, excepting Antarctica, and with an estimated 250 species worldwide, including at least 36 species in North

America, this taxon represents a logical focus for bioactive peptide discovery and structural/functional characterization with subsequent assessment of such data sets for a range of biological/biomedical applications [5,7–9].

Many studies have focused on their skin secretion peptides, which possess antimicrobial activity, in the quest for novel agents with broad-spectrum activity against human or animal

[☆] The nucleotide sequences of clones encoding brevinin-1PLb, brevinin-1PLc, esculentin-2PLa, ranatuerin-2PLa, temporin-1PLa and palustrin-1c from the skin secretion of the North American pickerel frog, *Rana palustris*, have been deposited in the EMBL Nucleotide Sequence Database under the accession codes AM745087 through AM45092.

^{*} Corresponding author. Tel.: +44 2890 972129; fax: +44 2890 247794.

E-mail address: chris.shaw@qub.ac.uk (C. Shaw).

0196-9781/\$ – see front matter © 2007 Elsevier Inc. All rights reserved.

doi:10.1016/j.peptides.2007.07.019

pathogens that have either been resistant to conventional antibiotics or have developed resistance to such [4,6,10,13].

Many antimicrobial peptide families, defined by their exhibition of common primary structural features, are named after the species of origin of prototypes. These include the brevinins (*Rana brevipoda porsa*), esculentins (*Rana esculenta* complex), temporins (*Rana temporaria*) and palustrins (*Rana palustris*) [5]. There is no prescribed terminology to describe these peptides, and indeed peptides from different species that are clearly structurally related have been given different names. The system proposed by Simmaco et al. [13] and utilized extensively by Conlon et al. [5], uses the peptide family name followed by a single or double letter code derived from the species name in capitals and a letter in normal case indicating the isoform—so brevinin-1PLa would represent isoform a of brevinin-1 from *Rana palustris*. This system appears to provide much information on the peptide with little inherent confusion.

Rapid frog antimicrobial peptides may be grouped into at least eight major and discrete structurally related families that comprises: (1) brevinin-1 first isolated from *R. brevipoda porsa*, (2) brevinin-2 likewise originally from *R. brevipoda porsa*, (3) ranalexin from *Rana catesbeiana* tadpoles, (4/5) ranaturins 1 and 2 from adult *R. catesbeiana*, (6/7) esculentins 1 and 2 from *R. esculenta* and (8) temporins from *R. temporaria* [5]. In addition to these, other structural variants with apparently more restricted distribution within the taxon and that do not logically fit into major families, have been described—nigrocins from *Rana nigromaculata* [11], tigerinins from *Rana tigerina* [12], palustrins from *R. palustris* [1] and amurins from *Rana amurensis* [15], for example.

Generally, representative members of several families occur in the secretion of a single species leading researchers to speculate that there may either be a high degree of interaction between peptides in effecting bactericidal effects or that each may possess a different spectrum of activity against a broad range of specific microorganisms, in effect a type of natural combination therapy in a single secretion [4-6,8-10,15].

The North American pickerel frog, *R. palustris*, is found over a wide range within the eastern part of the continent and has long been known to possess a particularly noxious if not toxic skin secretion such that it is an excellent deterrent against predator attack and indeed has potent amphibicidal properties [1]. Several peptidomic studies that have been performed on the skin secretion of this species have revealed a high degree of component complexity with multiple antimicrobial peptides, bradykinin-related peptides and others that are of novel primary structures, many without known pharmacological functions [1,2]. Although a considerable body of information exists on peptide primary structures in the skin secretion of this species, there is little available information of peptide precursor structures derived from cloned cDNAs. Of interest was the recent finding of canonical *R. palustris* palustrins in the defensive skin secretion of the Chinese Bamboo Odorous frog (*Rana (Odorrana) versabilis*). These peptides were identified in skin secretions using a peptidomic approach and their primary structures confirmed by cloning cDNAs for their biosynthetic precursors [3]. In the present study, molecular cloning of the cDNAs encoding the

biosynthetic precursors of the major classes of antimicrobial peptides in *R. palustris* skin secretion has permitted additional evaluation of the degree of conservation of nucleic acids when compared to *R. versabilis* structural homologs, over entire open-reading frames, for the first time.

2. Materials and methods

2.1. Collection of skin secretion

Four young, wild *R. palustris* frogs were maintained in terraria at 24 °C with 12 h light:12 h darkness cycle and were fed on crickets. The skin secretions were obtained from the frogs by gentle electrical stimulation (4 ms pulse width, 50 Hz, 5 V), using platinum electrodes rubbed over the moistened dorsal skin surface for 10 s. Secretions were then washed off into a glass beaker, using deionised water. The resultant secretions were lyophilized in a Hetosicc 2.5 freeze-dryer (Heto, UK). Approximately 50 mg dry weight of skin secretion was obtained by this method.

2.2. "Shotgun" cloning of skin secretion peptide cDNAs

Polyadenylated mRNA was isolated from 5 mg of lyophilized skin secretion dissolved in stabilization buffer, using magnetic oligo-dT beads as described by the manufacturer (DynaL Biotech, UK), and was subsequently reverse-transcribed. The cDNA was subjected to 3'-RACE procedures to obtain full-length prepropeptide nucleic acid sequence data using a SMART-RACE kit (Clontech UK) essentially as described by the manufacturer. Briefly, the 3'-RACE reactions employed a NUP primer (supplied with the kit) and a degenerate sense primer (S1; 5'-GAWYYAYYHRAGCCYAAADATGTTCA-3') that was designed to a highly conserved domain of the 5'-untranslated region of previously characterized antimicrobial peptide cDNAs from *Rana* species (Fig. 1) [14-16]. The PCR cycling procedure was as follows—initial denaturation step: 60 s at 94 °C; 35 cycles: denaturation 30 s at 94 °C, primer annealing for 30 s at 61 °C; extension for 180 s at 72 °C. PCR products were gel-purified, cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated sequencer.

2.3. Identification and structural analysis of putative cDNA-encoded antimicrobial peptides

Five milligrams of lyophilized skin secretion were dissolved in 1 ml of 0.05% aqueous trifluoroacetic acid that was clarified of microparticulates by centrifugation. The clear supernatant was decanted and directly pumped onto a reverse phase HPLC column and subjected to LC/MS using a 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 240 min at a flow rate of 1 ml/min. A Thermoquest gradient reversed phase HPLC system, fitted with an analytical column (C-5), and interfaced with a Thermoquest LCQ™ DECA electrospray ion-trap mass spectrometer, was employed. The effluent from the chromatographic column was flow-split with approximately 10% entering the mass spectrometer source and 90% directed towards a fraction

GAWYYAYYHRAGCCYAAADATGTTCA (S1)

GAATCATTGAGCCTAAAGATGTTCA (AM233683)
 ----CATTGAGCCTAAAGATGTTCA (AM233687)
GATTCACCTGAGCCTAAAGATGTTCA (AM233685)
 -----GGTATGTTCA (AJ427746)
GAACTACCTGAGCCCAAAGATGTTCA (AJ427747)
GAACTACCCGAGCCCAAAGATGTTCA (AJ427748)
GAACCATCTGAGTCCAAAGATGTTCA (X77831)
GAACTACCAGAACCCAAAGATGTTCA (U22393)
 -----TGGAAAGGAGAATGTTGC (M62770)
 -----GAATTCGATTGATGTTCA (AJ968402)

W=A or T
 Y=C or T
 H=A, C, or T
 R=A or G
 D=A, G, or T

Fig. 1 – Design of degenerate sense primer S1. Nucleic acid sequences of appropriate regions (5' to signal peptide) of antimicrobial skin peptide precursor cDNAs are aligned. Accession numbers for complete transcripts are given in parentheses.

collector. Dead volume between column and fraction collector was minimal (20 µl). The molecular masses of polypeptides in each chromatographic fraction were further analysed using matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) on a linear time-of-flight Voyager DE mass spectrometer (Perseptive Biosystems, MA, USA) in positive detection mode using alpha-cyano-4-hydroxycinnamic acid as the matrix. Internal mass calibration of the instrument with known standards established the accuracy of mass determination as ±0.1%. The peptides with masses coincident with those predicted from cloned cDNAs were each subjected to primary structural analysis by MS/MS fragmentation sequencing using an LCQ™DECA system (Thermoquest Inc., San Jose, CA, USA).

3. Results

Six different prepropeptide cDNAs were cloned from the skin library and each encoded a single copy of a known *Rana* antimicrobial peptide homolog (sequencing of 250 clones, each sequence represented at least 10 times) (Figs. 2 and 3A). Open-reading frames consisted of between 62 through 78 amino acid residues. A BLAST search in the EMBL Nucleotide Sequence Database revealed that all cloned antimicrobial peptide precursors exhibited high degrees of structural similarity to other reported antimicrobial peptide precursors from other ranid frogs. In Fig. 4, the top four hits for each are given. Apart from the ranatuerin, other antimicrobial peptide precursors from *R. palustris* have high degrees of amino acid sequence similarity (95–99%, see Fig. 3B) to the peptides from the Chinese bamboo odorous frog, *R. versabilis*, reported previously [3]. Following the prediction of the molecular masses of the six antimicrobial peptides from the cloned

(A) Brevinin-1PLb
 M F T T K K S M L L L F F L G T I
 1 ATGTTACCA CAAAGAAATC CATGTTACTC CTTTCTTCC TTGGGACCAT
 N L S L C E E E R N A E E E R R
 51 CAACCTATCT CTCTGTGAGG AAGAGAGAAA TGCAGAGGAA GAAAGAAGAG
 D E P D E M N V E V E K R F L P L
 101 ATGAGCCAGA TGAATGAAT GTTGAAGTGG AAAACCGATT TTTACCACTA
 I A G L A A N F L P K I F C A I T
 151 ATTGCAGGCT TGGCCGCTAA TTTCTTGCCG AAAATATTTT GTGCAATAAC
 K K C *
 CAAAAAATGT TGA

(B) Brevinin-1PLc
 M F T L K K S M L L L F F L G T I
 1 ATGTTACCT TAAAGAAATC CATGTTACTC CTTTCTTCC TTGGGACCAT
 N L S L C E E E R N A E E E R R
 51 CAACCTATCT CTCTGTGAGG AAGAGAGAAA TGCAGAGGAA GAAAGAAGAG
 D E P D E M D V E V E K R F L P V
 101 ATGAGCCAGA TGAATGAAT GTTGAAGTGG AAAACCGATT TTTACCGATT
 I A G V A A K F L P K I F C A I T
 151 ATTGCAGGCG TGGCCGCTAA GTTCTTGCCG AAAATATTTT GTGCAATAAC
 K K C *
 CAAAAAATGT TGA

(C) Esculentin-1PLa
 M F T T K K S M L L L F F L G T I
 1 ATGTTACCA CGAAGAAATC CATGTTACTC CTTTCTTCC TTGGGACCAT
 S L S L C E E E R N A D E E E G
 51 CTCCTTATCT CTCTGCGAGG AAGAGAGAGG TGCCGATGAA GAAGAAGGAG
 D G E K L M K R G L F S I L K G V
 101 ATGGAGAGAA ATTGATGAAA AGAGGCTTTT TCTCGATCT CAAGGTTGTA
 G K I A L K G L A K N M G K M G L
 151 GGCAAAATG CACTCAAAGG TTTGGCCGAG AACATGGGCA AGATGGGGCT
 D L V S C K I S K E C *
 201 GGACCTGTG AGTTGCAAAA TTTCCAAGA ATGTTAA

(D) Ranatuerin-2Pla
 M F T T K K S M L L L F F F L G T I
 1 ATGTTACCA CAAAGAAATC CATGTTACTC TTTTCTTCC TTGGGACCAT
 S L S L C E Q E R G A D E D D G
 51 CTCCTTATCT CTCTGTGAAC AAGAGAGAGG TGCAGATGAA GACGATGGTG
 V E M T E E E V K R G I M D T V K
 101 TGGAAATGAC AGAGGAAGAA GTAAAAAGAG GTATCATGGA TACGGTAAAG
 N V A K N L A G Q L L D K L K C K
 151 AATGTAGCAA AGAATTGGC CGGACAGTTG CTGGATAAGT TAAATGTAA
 I T A C *
 201 AATTACTGCA TGTAA

(E) Temporin-1PLa
 M F T S K K S L L L L F F L G T I
 1 ATGTTACCT CAAAGAAATC CCTGTTACTC CTTTCTTCC TTGGGACCAT
 N L S L C E E E R D A D E E E R
 51 CAACCTATCT CTTTGTGAGG AAGAGAGAGA TGCCGATGAG GAAGAAGAA
 R D D P D E M N V E V E K R F L P
 101 GAGATGATCC AGATGAAATG AATGTGTAAG TAGAAAACCG ATTTTACCA
 L V G K I L S G L I G K *
 151 CTGTGTGAA AGATTCTCTC TGGTTAATT GGAAATAA

(F) Palustrin-1c
 M F T T K K S L L L L F F L G T I
 1 ATGTTACCA CGAAGAAATC CCTGTTACTC CTTTCTTCC TTGGGACCAT
 S L S L C E E E R G A D E E E G
 51 CTCCTTATCT CTCTGCGAGG AAGAGAGAGG TGCCGATGAA GAAGAAGGAG
 D G E K L T K R A L S I L R G L E
 101 ATGGAGAGAA ATTGACGAAA AGAGCTCTTT CGATCTCAG AGGTTAGAA
 K L A K M G I A L T N C K A T K K
 151 AAATTGGCCA AGATGGGGAT TGCCCTTACG AATTGCAAG CTACCAAAA
 C *
 201 ATGTTAA

Fig. 2 – Translated open-reading frames and nucleic acid sequences of cloned cDNAs encoding biosynthetic precursors of skin antimicrobial peptides from the pickerel frog, *R. palustris*. Putative signal peptides are double-underlined, mature peptides are single underlined and stop codons are indicated by asterisks.

precursors and compensation for post-translational modification (single disulfide bridge formation in the C-terminal loop = -2 amu, or a C-terminal amide rather than carboxyl group = -1 amu), each mature peptide was identified in respective skin secretion HPLC fractions and primary structures were confirmed. Most striking is the 100% primary structural identity between temporin-1, brevinin-1 and palustrin-1c mature peptides in each species. In fact, within the primary structure of the entire respective precursor open-reading frames, there are just single amino acid substitutions within the signal peptides of temporins and palustrins and just two, again within the signal peptides, of respective brevinins. The esculentin-2 precursors from both species exhibit five amino acid differences—two within the signal peptide and three within the mature peptide. All three substitutions within the mature peptide region - L/I position

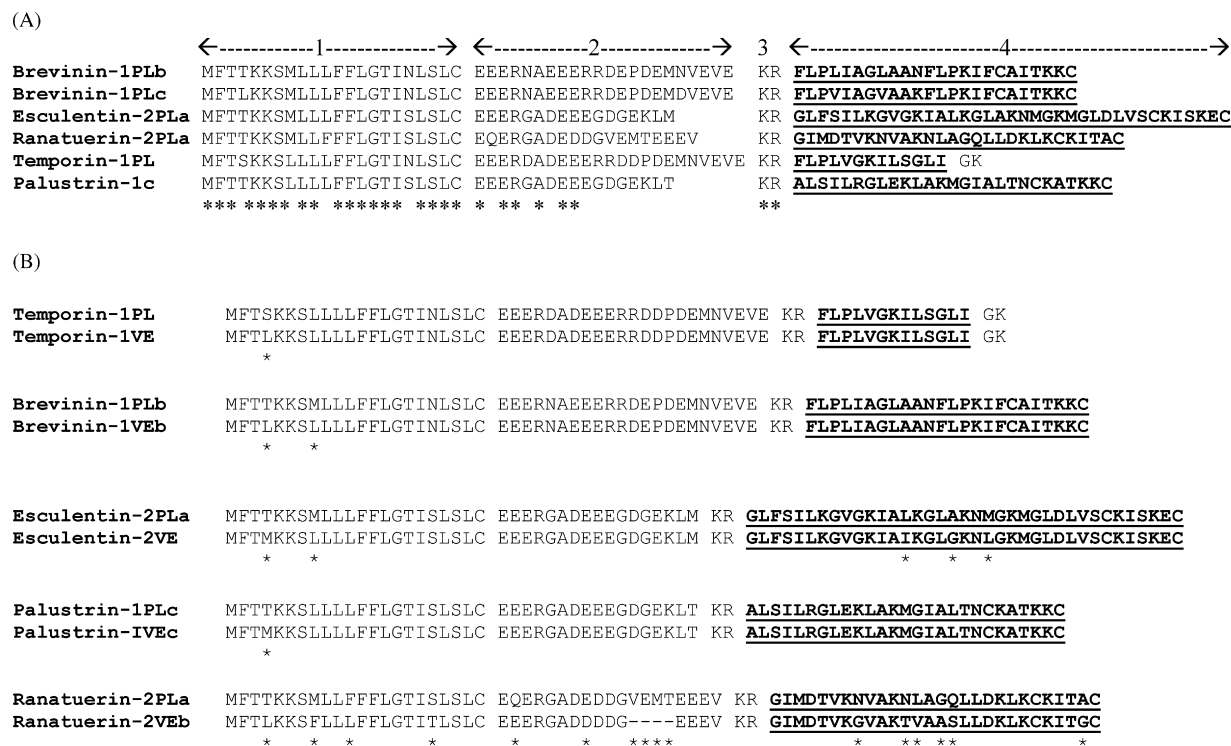


Fig. 3 – (A) Alignment and domain topology of biosynthetic precursors encoding *R. palustris* skin antimicrobial peptides. (1) Putative signal peptide. (2) Acidic residue-rich spacer peptide. (3) Propeptide convertase processing site. (4) Mature antimicrobial peptide domain. Asterisks indicate fully conserved amino acid residues that are located predominantly within the putative signal peptide domain. (B) Alignments of biosynthetic precursors of pickerel frog (*R. palustris*) skin antimicrobial peptides (PL) with homologs from the skin secretion of the Chinese bamboo odorous frog, *R. versabilis* (VE). Note the complete identity between mature temporin, brevinin-1 and palustrin-1c in each species and the high degree of conservation within the full precursors.

| | |
|-------------------------------|---|
| Preprotemporin 1PL | 98% <i>Rana versabilis</i> preprotemporin 1V. 92% <i>Rana ornativentris</i> preprotemporin 10b1. 93% <i>Amolops loloensis</i> preproamolopin 1b. 93% <i>Rana versabilis</i> preprobrevinin 1Vb. |
| Preprobrevinin 1PLb | 97% <i>Rana versabilis</i> preprobrevinin 1Vb. 89% <i>Rana pipiens</i> preprobrevinin 1Pb. 89% <i>Amolops loloensis</i> preproamolopin 1a. 88% <i>Rana esculenta</i> preprobrevinin 1E. |
| Preprobrevinin 1PLc | 95% <i>Rana versabilis</i> preprobrevinin 1Vb. 91% <i>Rana pipiens</i> preprobrevinin 1Pb. 89% <i>Odorrana versabilis</i> preprobrevinin 1V. 89% <i>Amolops loloensis</i> preproamolopin 1a. |
| Preproesculentin 2PLa | 97% <i>Rana versabilis</i> preproesculentin 2Vb. 91% <i>Rana versabilis</i> prepropalustrin 1c. 80% <i>Rana livida</i> preprolividin 4. 79% <i>Odorrana schmackeri</i> preproesculentin 2S. |
| Prepropalustrin 1c | 99% <i>Rana versabilis</i> prepropalustrin 1c. 93% <i>Rana versabilis</i> preproesculentin 2Vb. 92% <i>Rana livida</i> preprolividin 3. 92% <i>Rana plancyi fukienensis</i> preproesculentin 2P. |
| Preproranatuering 2PLa | 95% <i>Rana pipiens</i> preproranatuering 2P. 87% <i>Rana versabilis</i> preproranatuering 2Va. 82% <i>Rana versabilis</i> preproranatuering 2Vb. 87% <i>Rana temporaria</i> preprobrevinin 2Ta. |

Fig. 4 – Percentage identities of *R. palustris* skin antimicrobial peptide preproprotein nucleic acid sequences with those present in the NCBI database. The top four hits are given in each case.

14, A/G position 18 and M/L position 21 – are conservative. The precursors encoding the ranatuering-2 homologs from both species exhibited the greatest number of substituted sites – 12 in all – four within the signal peptide, two within the acidic spacer peptide and six within the mature peptide. The acidic spacer regions of each antimicrobial peptide precursor are compared in Fig. 5. Although not as highly conserved as the signal peptides, it is interesting to note the high degree of amino acid sequence similarity between these regions of brevinin/temporin precursors and esculentin/palustrin precursors, respectively. The corresponding region of the ranatuering precursor is quite different to these.

4. Discussion

Amphibian skin gland secretions are a rich source of antimicrobial peptides and a sufficient database of structures from many species now exists on-line, permitting several groups of researchers to perform bioinformatic analyses on the use of such structures as taxonomic clues [5,14]. Inferences made from these datasets are often compelling in that they produce phylogenetic trees that are, for the most part, quite consistent with those inferred from other measures of species relatedness that would be considered of classical usage such as digit and skeletal structure, tadpole morphometrics and

| | |
|------------------------|----------------------------|
| Brevinin-1PLb | -EEERNAEEEE-RRDEFDEMNVVE- |
| Brevinin-1PLc | -EEERNAEEEE-RRDEFDEMNVVE- |
| Temporin-1PL | -EEERDADEEEERRDDPFDEMNVVE- |
| | **** * ** ** * ** * ** * |
| Esculentin-2PLa | -EEERGADEEEEGDGEKLM- |
| Palustrin-1PLc | -EEERGADEEEEGDGEKLT- |
| | ***** |

Fig. 5 – Comparison of acidic spacer peptide domains of brevinins/temporin and esculentin/palustrin, respectively. Conserved residues are indicated by asterisks.

selected DNA sequences of conserved “housekeeping” proteins [5]. However, these results are often dependent on many factors such as population sampling (especially important for species with large geographical distributions), the range of species employed and the exact segments of skin peptide precursors that are subjected to analysis. For instance, the primary structures of antimicrobial peptide precursor signal peptides and indeed some of those of non-antimicrobial skin peptides, are highly conserved between many anuran families causing researchers to speculate that they have had a common ancestry [14]. These data might also reflect a common and fundamental function of various regions in precursor processing pathways within granular gland cells. Thus the degrees of conservation of the various regions of the biosynthetic precursors may be reflective of the mediation of essential intracellular processing/transport/transit events rather than function of the mature bioactive peptide.

Here, we describe the primary structures of biosynthetic precursors of five different classes of skin antimicrobial peptides from the pickerel frog, *R. palustris*, deduced from cDNAs cloned from a skin secretion-derived library. The open-reading frames of these cDNAs are compared with those that produced the highest similarity scores in on-line database interrogations. Interestingly, the closest homologs were all, with the exception of the ranatuerin, from the Chinese Bamboo Odorous frog, *Rana (Odorrana) versabilis* (Fig. 4) [3]. This high degree of primary structural similarity was also reflected at the level of nucleic acid sequence. Thus, the North American pickerel frog and the Chinese bamboo odorous frog, at least with respect to their skin antimicrobial peptides, appear to be more closely related to one another than to other ranid species that occupy similar geographical distributions.

As has been alluded to previously, the high degree of primary structural conservation of the skin antimicrobial peptide precursors cloned in the present study, is most evident within the signal peptide domains, moderate within the acidic spacer peptides and least within the matured antimicrobial peptide coding domains. The conservation of signal peptides has been noted and discussed previously with respect to functional relevance [5,14], but reference to Fig. 5, in which we have compared acidic spacer peptides, reveals an interesting finding. Brevinin/temporin acidic spacer peptides are highly conserved and esculentin/palustrin peptides even more so. The analogous ranatuerin peptide is different from both of these groups. These data may imply a close relationship between these two sets of precursors and perhaps that one has been derived from the other within each respective group.

The application of the simple robust molecular cloning technique described here has rapidly elucidated the nucleic acid sequences and derived primary structures of five classes of antimicrobial peptides from the skin secretion of the North American pickerel frog, *R. palustris*. Comparison of the primary structures of acidic spacer peptides within biosynthetic precursors, has revealed a close relationship between brevinins/temporins and esculentins/palustrins, respectively. The homologous domain of the ranatuerin biosynthetic precursor was quite different to that found in both of these groups and indeed, ranatuerins have been shown to be among the least conserved skin antimicrobial peptides of ranid frogs [5,14]. Thus, much data of fundamental interest can be generated from amphibian skin secretory peptidomes without sacrifice of or injury to the donor specimens.

Acknowledgement

Mei Zhou is in receipt of an Overseas PhD studentship from the Queen’s University of Belfast, Northern Ireland.

REFERENCES

- [1] Basir YJ, Knoop FC, Dulka J, Conlon JM. Multiple antimicrobial peptides and peptides related to bradykinin and neuromedin N isolated from skin secretions of the pickerel frog, *Rana palustris*. *Biochim Biophys Acta* 2000;1543:95–105.
- [2] Basir YJ, Conlon JM. Peptidomic analysis of the skin secretions of the pickerel frog *Rana palustris* identifies six novel families of structurally related peptides. *Peptides* 2003;24:379–83.
- [3] Chen TB, Zhou M, Rao P, Walker B, Shaw C. The Chinese bamboo leaf odorous frog (*Rana (Odorrana) versabilis*) and North American *Rana* frogs share the same families of skin antimicrobial peptides. *Peptides* 2006;27:1738–44.
- [4] Clarke BT. The natural history of amphibian skin secretions, their normal functioning and potential medical applications. *Biol Rev Camb Philos Soc* 1997;72:365–79.
- [5] Conlon JM, Kolodziejek J, Nowotny N. Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. *Biochim Biophys Acta* 2004;1696:1–14.
- [6] Conlon JM. The therapeutic potential of antimicrobial peptides from frog skin. *Rev Med Microbiol* 2004;15:1–9.
- [7] Duellman WE, Trueb L. *Biology of amphibians*. Baltimore, London: The Johns Hopkins University Press; 1994.
- [8] Erspamer V. Bioactive secretions of the integument. In: Heatwole H, Barthalmus GT, editors. *Amphibian biology*, vol. 1: the integument. Chipping Norton: Surrey Beatty and Sons; 1994 [Chapter 9].
- [9] Lazarus LH, Atilla M. The toad, ugly and venomous, wears yet a precious jewel in his skin. *Prog Neurobiol* 1993;41:473–507.
- [10] Nicolas P, Mor A. Peptides as weapons against microorganisms in the chemical defense system of vertebrates. *Ann Rev Microbiol* 1995;49:277–304.
- [11] Park S, Park SH, Ahn HC, Kim S, Kim SS, Lee BJ, et al. Structural study of novel antimicrobial peptides, nigrocins, isolated from *Rana nigromaculata*. *FEBS Lett* 2001;507:95–100.
- [12] Sai KP, Jagannadham MV, Vairamani M, Raju NP, Devi AS, Nagaraj R, et al. Tigerinins: novel antimicrobial peptides

- from the Indian frog *Rana tigerina*. *J Biol Chem* 2001;276:2701-7.
- [13] Simmaco M, Mignogna G, Barra D. Antimicrobial peptides from amphibian skin: what do they tell us? *Biopolymers* 1998;47:435-50.
- [14] Vanhoye D, Bruston F, Nicolas P, Amiche M. Antimicrobial peptides from hylid and ranin frogs originated from a 150-million-year-old ancestral precursor with a conserved signal peptide but a hypermutable antimicrobial domain. *Eur J Biochem* 2003;270:2068-81.
- [15] Zhou M, Liu Y, Chen TB, Fang X, Walker B, Shaw C. Components of the peptidome and transcriptome persist in lin wa pi: the dried skin of the Helongjiang brown frog (*Rana amurensis*) as used in traditional Chinese medicine. *Peptides* 2006;27:2688-94.
- [16] Zhou M, Chen TB, Walker B, Shaw C. Lividins: Novel antimicrobial peptide homologs from the skin secretion of the Chinese Large Odorous frog, *Rana (Oderana) livida*: identification by "shotgun" cDNA cloning and sequence analysis. *Peptides* 2006;27:2118-23.