Partial structure of the phylloxin gene from the giant monkey frog, *Phyllomedusa bicolor*: Parallel cloning of precursor cDNA and genomic DNA from lyophilized skin secretion

Tianbao Chen a, Ron Gagliardo b, Brian Walker a, Mei Zhou a, Chris Shaw a,∗

a School of Pharmacy, Queen’s University, Lisburn Road, Belfast BT9 7BL Northern Ireland, UK
b The Fuqua Orchid Center, Atlanta Botanical Garden, 1345 Piedmont Avenue NE, Atlanta, GA 30309, USA

Received 16 March 2005; accepted 27 April 2005
Available online 3 June 2005

Abstract

Phylloxin is a novel prototype antimicrobial peptide from the skin of *Phyllomedusa bicolor*. Here, we describe parallel identification and sequencing of phylloxin precursor transcript (mRNA) and partial gene structure (genomic DNA) from the same sample of lyophilized skin secretion using our recently-described cloning technique. The open-reading frame of the phylloxin precursor was identical in nucleotide sequence to that previously reported and alignment with the nucleotide sequence derived from genomic DNA indicated the presence of a 175 bp intron located in a near identical position to that found in the dermaseptins. The highly-conserved structural organization of skin secretion peptide genes in *P. bicolor* can thus be extended to include that encoding phylloxin (*plx*). These data further reinforce our assertion that application of the described methodology can provide robust genomic/transcriptomic/peptidomic data without the need for specimen sacrifice. © 2005 Elsevier Inc. All rights reserved.

Keywords: Phyllomedusinae; Venom; Peptide; Mass spectroscopy; Molecular cloning

1. Introduction

Antimicrobial peptides represent an abundant class of bioactive peptides present in amphibian defensive skin secretions [3,7]. Such peptides are often taxon specific in primary structure and are named accordingly after the first species from which the prototype was isolated, for example, bombinins (*Bombina bombina*), brevinins (*Rana brevipes*), esculentins (*Rana esculenta*), caerin (*Litoria caerulea*) and temporins (*Rana temporaria*). The nomenclature of others however, does not follow this general taxonomic rule, for example, *Xenopus laevis* magainins (from the Hebrew magen (shield) or *Phyllomedusa bicolor* dermaseptins (skin antiseptics). Most of the hitherto identified amphibian skin antimicrobial peptide families have one major attribute in common, they possess broad-spectrum activity against Gram-positive and Gram-negative bacteria and pathogenic yeasts, such as *Candida albicans* [11]. For this reason, their study has generated much interest, especially due to the emergence of multiple drug-resistant strains of many pathogenic microorganisms in recent years [9]. However, their exciting in vitro activities remain to be translated into clinical applications and efficacy [4]. Anti-microbial peptides frequently occur as multiple structurally-related isoforms even within the secretion of a single individual amphibian and as each isoform potentially possesses a slightly different spectrum of activity, this situation is regarded by many as the means whereby the amphibian retains and develops efficacy for its secretion for long periods of time and over a wide range of pathogenic microorganisms [3,7,11]. The means by which this can be achieved apparently resides in the dynamics of the genetics of the granular gland cells whose function has been compared in the past to a primitive, cell-free immune system. While this may be a scientifically-intriguing hypothesis, there is little information available as to the gene organizations of most skin...
secretion peptides and what is known is restricted to certain discrete species, such as the giant or two-colored monkey frog, P. bicolor. This species, as mentioned previously, was the origin of the prototypical antimicrobial peptide family, the dermaseptins. The primary structures of the dermaseptins are highly-variable, but all contain significant numbers of lysyl residues imparting a cationic and amphipathic helical character [1]. However, while dermaseptins are variable in primary structure, cloning of encoding skin cDNAs has revealed that the putative signal peptide and acidic spacer peptide domains of their precursors are highly-conserved. In fact, this observation is extended to nucleotide sequences of cDNAs within the 3'- and 5'-non-coding regions. The dermaseptin genes in this species appear to be likewise highly-conserved in terms of structural organization with a small intron of variable nucleotide length (but less than 200 bp) located between exon 1 (encoding signal peptide and first three residues of acidic spacer peptide) and exon 2 (remainder of acidic spacer peptide) and dermaseptin coding sequence through into 3'-non-translated region [1].

Phylloxin is a prototype narrow-spectrum antimicrobial peptide identified in P. bicolor skin [10]. Despite significant differences in primary structure to dermaseptins, the organization, nucleotide sequence of cloned cDNA, and primary structures of signal peptide and acidic spacer domains of the phylloxin precursor are remarkably highly-conserved [2,10]. Here, we report the use of our recently-described molecular cloning technique to acquire nucleotide sequences of both phylloxin precursor cDNA (from polyadenylated mRNA) and of the phylloxin gene (from genomic DNA) using cDNA libraries generated from a single sample of lyophilized skin secretion (a "surrogate" tissue) obtained by non-invasive, non-lethal means. This report extends the repertoire of non-invasive skin secretion sampling to determination of gene organization from genomic DNA contained within a single sample that can already demonstrably facilitate parallel primary structural characterization of secreted peptides and cloning of their respective precursor-encoding cDNAs [5,6].

2. Materials and methods

2.1. Specimen biodata and secretion acquisition

Adult five-year old P. bicolor (n = 5, 3 males, 2 females), were obtained from a source within the United States and had been raised from tadpoles imported from South America. The frogs had been adapted to their new surroundings in the Atlanta Botanical Gardens for 4 months prior to secretion harvesting. They were maintained in a purpose-designed amphibian facility at 20–25°C under a 12 h/12 h light/dark cycle and fed multivitamin-loaded crickets three times per week. Skin secretions were obtained from the paratoid glands and dorso-lateral skin folds by application of gentle pressure from snout to vent for a few minutes duration after which the white viscous secretion was pronounced. This method was employed as mild transdermal electrical stimulation was found to be ineffective. The secretion was washed from the skin using deionized water, snap-frozen in liquid nitrogen and lyophilized. Lyophilizate was stored at -20°C prior to analysis.

2.2. Cloning of P. bicolor phylloxin cDNA transcribed and amplified from trapped polyadenylated mRNA

A 5 mg sample of lyophilized P. bicolor skin secretion was dissolved in 1 ml of cell lysis/mRNA protection buffer supplied by Dynal Biotec, UK. Polyadenylated mRNA was isolated by the use of magnetic oligo-dT beads as described by the manufacturer (Dynal Biotec, UK). mRNA was eluted in 15 µl of 10 mM Tris-HCl and first strand cDNA synthesis, for subsequent RACE reactions, was performed using a SMART-RACE kit (Clontech UK) essentially as described by the manufacturer. Briefly, the RACE reactions were amplified using a sense primer (S1-PLX, 5'-TCTGAAATTGTAAGCCCAAACATG-3') and an antisense primer (AS-PLX, 5'-CAGTTAAACAGCTTTAATATAT-3'), designed to regions flanking the open-reading frame of the published sequence of P. bicolor phylloxin cDNA [10], by thermostable polymerase (Invitrogen). The PCR cycling procedure was as follows: an initial denaturation step for 1 min at 94°C followed by 35 cycles consisting of denaturation for 30 s at 94°C, primer annealing for 30 s at 52°C and extension for 3 min at 72°C. Gel electrophoresis of the PCR products generated from the library was followed by further purification, cloning using a pGEM-T vector system (Promega Corporation) and subsequent sequencing using an ABI 3100 automated capillary sequencer.

2.3. Cloning of P. bicolor phylloxin cDNA transcribed and amplified from genomic DNA

Total genomic DNA for PCR was extracted from 15 mg of lyophilized P. bicolor skin secretion using a Wizard® genomic DNA purification kit (Promega, UK). The partial phylloxin gene sequence, encoding the open reading frame, was amplified using the same sense (S-PLX) and antisense (AS-PLX) primers described in the previous section using thermostable polymerase (Invitrogen). The PCR cycling procedure was as follows: an initial denaturation step for 5 min at 94°C followed by 35 cycles consisting of denaturation for 30 s at 94°C, primer annealing for 30 s at 52°C and finally extension for 5 min at 72°C. The resulting PCR products were purified by a gel extraction system (Life and Technologies, UK), cloned using a pGEM-T vector system (Promega Corporation) and sequenced using a dye terminator cycle sequencing kit and an ABI 3100 automated DNA sequencer (Applied Biosystems).
Fig. 1. Nucleotide sequences of precursor cDNAs encoding Phyllomedusa bicolor phylloxin cloned from a skin secretion-derived library. The putative signal peptide (double-underlined), mature processed peptide (single-underlined) and stop codon (asterisks) are indicated.

Fig. 2. Partial sequence of the phylloxin (plx) gene from Phyllomedusa bicolor. PLX-B (R) represents the nucleotide sequence of phylloxin mRNA and PLX-B (D) the nucleotide sequence of genomic DNA. Both were cloned from libraries derived from pooled lyophilized skin secretions obtained from three individual specimens. Alignment indicates the position of intron 1 (176 bp—lower case) between exon 1 (encoding signal peptide and first three residues of acidic spacer peptide) and exon 2 (encoding remainder of acidic spacer peptide and phylloxin domain). The polyadenylation signal (AATAAA) is indicated. The translated open-reading frame is indicated in bold italic and the phylloxin sequence is underlined. The stop codon is indicated by an asterisk. Identical nucleotides are shaded in black.
3. Results

3.1. Cloning of P. bicolor phylloxin cDNA

Using the same primers, a single PCR product was consistently obtained from each cDNA library generated from polyadenylated mRNA and genomic DNA, respectively. However, the size of each product was different following gel electrophoresis with the product from the genomic library routinely running approximately 200 bp greater than that generated from the library constructed from trapped polyadenylated mRNA. Following cloning and sequencing (25 clones from each product) (Figs. 1 and 2), that obtained from the polyadenylated mRNA library was found to be identical in nucleotide sequence to that previously reported as encoding the P. bicolor phylloxin precursor [10] (Fig. 1).

4. Discussion

The defensive skin secretions of frogs are known to contain a plethora of biologically-active peptides, some of which share common primary structural features with endogenous vertebrate regulatory peptides but others, often indeed the most abundant, are taxon-specific broad-spectrum antimicrobials [3,7]. This area of research could thus generate lead compounds for the development of novel antimicrobial agents or act as a model for combined therapy for future treatment regimes that are less likely to produce resistance. However, the species that produce these naturally-selected libraries of antimicrobial peptides are in global decline due to a range of biotic and environmental factors [12]. It is thus essential that non-invasive, non-lethal methods of gaining both protein sequence and genetic data are developed, evaluated and widely-used to aid in the conservation of species and the preservation of individual specimens. These technologies and practices may in fact prove to have broader applications within the molecular biosciences. Typical frog skin peptide studies in the past relied upon killing and skinning specimens (often several hundreds or thousands) followed by chemical extraction of skins for peptide analysis or molecular studies. Tyler et al. [13] developed a non-invasive, non-destructive transdermal electrical stimulation technique for procuring frog skin secretions and this is extremely well- tolerated and now widely-used by several groups. In 2002, our group discovered that cDNA libraries could be constructed from lyophilized skin secretions obtained by this method as a consequence of interaction between endogenous polyadenylated mRNA and endogenous stabilization/protective peptides [5,6]. Although this technique has not been widely employed by other groups, it has been used to produce robust data in many studies from our group. Here we have extended the technique to isolate and amplify endogenous genomic DNA within the lyophilized secretion to obtain a partial gene structure for the novel antimicrobial peptide, phylloxin. The full-length precursor cDNA (derived from trapped polyadenylated mRNA) was also obtained from the same secretion sample. Alignment of both nucleotide sequences revealed the presence of an intron within the genomic DNA sequence that was not present within the nucleotide sequence derived from polyadenylated mRNA. This was 175 bases in length and hence was frame shifted from the coding exons. Of interest was that this intron was in exactly the same position as that previously reported for the partial gene sequence of dermaseptin [8], a broad-spectrum antimicrobial peptide found in the same species as phylloxin reported here. The intron/exon boundaries, and hence the gene structures of amphibian skin defensive skin secretion peptides, appear to be highly-conserved. The original identification of phylloxin in P. bicolor, and subsequent determination of its precursor structure from cloned cDNA, required the sacrifice of two specimens [10]. Our current data have now unequivocally shown that such data can be generated without this requirement and without compromise of robustness and quality.

Acknowledgement

Mei Zhou is currently in receipt of an Overseas Research Studentship from Queen’s University, Belfast, Northern Ireland.

Appendix A. Data deposition footnote

The nucleotide sequence of partial structure of the phylloxin gene from the giant monkey frog, Phyllobates bicolor, has been deposited in the EMBL Nucleotide Sequence Database under the accession code AJ867213.

References


