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Expression of genes encoding antimicrobial peptides in the Harderian gland of the bullfrog *Lithobates catesbeianus*

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ABSTRACT

The Harderian gland is an orbital gland found in many tetrapod species that possess a nictitating membrane. While the main role of the Harderian gland is lubrication of the eyeballs, numerous other functions are attributed to this gland. In amphibians, mast cells have been detected in the Harderian gland, suggesting that the gland is involved in the host's system of innate immunity defending against microbial invasions. Using reverse-transcription polymerase chain reaction, we cloned from the bullfrog Harderian gland total RNA preparations, cDNAs encoding biosynthetic precursors for the antimicrobial peptides temporin-CBa (FLPIASLLGKYL-NH_2), previously isolated from an extract of bullfrog skin, and chensirin-2CBa (IIPLPLGY-FAKKP) that contained the amino acid substitution $Thr^{13} \rightarrow Pro$ compared with chensirin-2 from the Chinese brown frog, *Rana chensinensis*. By means of *in situ* hybridization using digoxigenin-labeled cRNA probes for preprotemporin-CBa and preprochensirin-2CBa, we have demonstrated for the first time in an amphibian the presence of mRNAs encoding these two precursors in the cytoplasm of the glandular cells in the bullfrog Harderian gland.

1. Introduction

The vertebrate eye is situated at the interface between the organism and its environment and as so requires defensive systems to protect against invasion by pathogenic microorganisms in the environment. Amphibians have a worldwide distribution, occupying aquatic, semi-aquatic, and terrestrial environments and thus are exposed to a wide range of microorganisms. The Harderian gland (HG) is an orbital gland found in many tetrapod species that possess a nictitating membrane (Harder, 1694). Although the function of HG is still not completely clear, its size and widespread occurrence among vertebrates suggest potentially important roles. In fact, numerous functions are attributed to this gland such as lubrication of the eye and nictitating membrane, a source of pheromones and growth factors, as well as osmoregulatory, thermoregulatory and immunoregulatory functions (Payne, 1994; Chieffi et al., 1996). In amphibians, the HG is a seromucoid acinar gland located at the medial corner of the orbit and consists of a single type of epithelial cells (Chieffi Baccari et al., 1991; Payne 1994). It is known to show seasonal changes in secretory activity in a temperature-dependent manner (Minucci et al., 1990). Because of these seasonal changes and its spatial localization in the orbit, it is speculated that the amphibian HG may be involved in the innate immune system of the host's eyes, defending against pathogenic microorganisms encountered in the environment.

Antimicrobial peptides (AMPs) are gene-encoded polypeptides of various lengths and structures found in all organisms including vertebrates, invertebrates, plants, and bacteria. In vertebrates, these molecules are important components of the innate immune system of the organisms and protect against microbial infections before adaptive immunity is activated (Auvynet and Rosenstein, 2009). AMPs can target and neutralize a broad range of microorganisms by mechanisms that involve non-specific interactions with the cell membrane. Although there are no obvious conserved amino acid sequences among these peptides, most AMPs are hydrophobic and cationic, and have a propensity to form an amphipathic helical conformation in a membrane-mimetic environment (Brogden et al., 2005).

Amphibians are a promising source of AMPs. Based on limited amino acid sequence similarities, AMPs in ranid frogs are classified into several well-established groups that include the temporin, brevinin, ranatuerin, palustrin, esculentin, nigrocin, and japonicin families (Conlon et al., 2004). Although extensive amino acid sequence variations are observed

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among these AMPs, their precursor proteins show a high degree of sequence similarity suggesting that they have arisen from a common ancestral gene (Nicolas et al., 2003). Molecular cloning studies have demonstrated that the biosynthetic precursors of these peptides comprise three domains: a signal peptide region, an intervening sequence region, and an AMP region (Simmaco et al., 1996). While nucleotide sequences of the AMP-coding regions of ranid AMP precursors are hypervariable, those of the signal peptide and the intervening sequence regions are moderately to strongly conserved among different AMP families. In addition, nucleotide sequences of the 3′-untranslated region (UTR) of amphibian AMP precursor mRNAs are also well conserved among each AMP family (Ohnuma et al., 2010). Using these nucleotide sequence similarities, we have successfully amplified several cDNAs encoding biosynthetic precursors of AMPs from total RNA samples prepared, not only from skin, but also from several extradermal tissues of ranid frogs by a one-step reverse-transcription polymerase chain reaction (RT-PCR) procedure (Iwamuro et al. 2006; Suzuki et al., 2007b; Ohnuma et al., 2010). In the present study, we have employed this protocol using a set of preprotemporin genes-specific primers to clone cDNAs encoding AMPs from the bullfrog HG and then performed *in situ* hybridization analyses in order to clarify whether the AMP genes are expressed in the gland. Nomenclature adopted for AMPs from frogs of the Ranidae family follows recent guidelines (Conlon, 2008), and species nomenclature follows the taxonomic recommendations of Frost (2010).

2. Materials and methods

2.1. Tissue collection and total RNA extraction

Adult male and female bullfrogs, *Lithobates catesbeianus* (formerly *Rana catesbeiana*) were captured in Ibaraki Prefecture, Japan, in July for molecular cloning studies, and in November for morphological investigations and *in situ* hybridization experiments. Specimens were purchased from Ouchi Aquatic Animal Supply (Saitama, Japan). All experiments were approved by Saitama and Waseda Universities Bioethics and Animal Ethics Committees and carried out by authorized investigators. Animals ($n=45$) were anesthetized by immersion in ice water and sacrificed by decapitation. Since the gland was pinkish in color, similar to the color of *musculi bulbi*, care was taken during sample collection to avoid contamination. HGs were dissected from the orbits under a microscope and immediately frozen on dry ice. Total RNA was extracted by the acid phenol–guanidinium–isothiocyanate procedure (Chomczynski and Sacchi, 1987). RNA concentrations were estimated by measurement of the absorbance at 260 and 280 nm, and quality was checked by the ratio OD_{260}:OD_{280}. Samples were stored at −80 °C.

2.2. Amplification of open reading frame (ORF) of AMP cDNAs from HG total RNA by RT-PCR

ORFs of AMP precursor cDNAs were amplified by RT-PCR in a volume of 50 μL using a One-Step RT-PCR kit (Qiagen, Chatsworth, CA, USA) according to the method described in a previous report (Iwamuro and Kobayashi, 2010). Briefly, aliquots of 100 ng of total RNA along with the set of preprotemporin gene-specific primers were incubated at 50 °C for 30 min for reverse transcription, and then at 95 °C for 15 min for denaturation of reverse transcriptase. Subsequently, PCR was performed under the following conditions: 5 min at 94 °C for DNA denaturation followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C, and 30 s at 72 °C with a final extension step of 7 min at 72 °C. The forward primer (5′-ATGTTCACCTTGAAGAAATC-3′) and reverse primer (5′-AGATGATTTC-CAATTCCAT-3′) were designed according to the nucleotide sequence of temporin precursor cDNAs obtained from several Japanese ranid frogs (Ohnuma et al., 2007; Suzuki et al., 2007a; Tazato et al., 2010). Synthetic oligonucleotides were purchased from Sigma Genosys (Ishikari, Japan). RT-PCR products (10 μL) were separated by electrophoresis on a 1.2% agarose gel, stained with ethidium bromide, and visualized on an UV transilluminator. The DNA bands were excised and purified using a Wizard SV gel and a PCR clean-up system (Promega, Madison, WI, USA) and subcloned into pSTBlue-1 vector using the Acceptor Vector Kit (Novagen, Darmstadt, Germany). Nucleotide sequence analysis was performed by the dideoxy-chain termination method using a Big-Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) by Biomatrix Company (Chiba, Japan). Nucleotide and amino acid sequence identities and prediction of the secondary structures were analyzed using Genetyx Mac version 15.0.1 (Software Development Corporation, Osaka, Japan).

2.3. In situ hybridization analyses

Aliquots of the RT-PCR products of preprotemporin-CBa and preprochensirin-2CBa (Section 2.2) were subcloned into pBluescript II plasmid vector (Stratagene, La Jolla, CA, USA) and subjected to PCR in order to obtain cDNA templates for *in vitro* transcription. The reaction conditions were as follows: 5 min at 94 °C for DNA denaturation, followed by 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 30 s at 72 °C, with a final extension step of 7 min at 72 °C. Sets of primers for amplification of cDNA templates for antisense (forward: M13 forward primer; reverse; 5′-GTAAAACGACGGCCAGT-3′) and sense (forward: 5′-GGAAACAGCTATGACCATG-3′; reverse: M13 reverse primer) cRNA probes were used. Digoxigenin (DIG)-labeled antisense and sense probes were synthesized using a DIG RNA labeling kit (Roche Diagnostics, Basel, Switzerland). Three fresh HG specimens from adult male bullfrogs were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetechnical, Tokyo, Japan) by immersion in liquid nitrogen-cooled isopentane. Frozen sections (10 μm) were cut and thaw-mounted onto MAS-coated slides (Matsunami, Osaka, Japan). Sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, rinsed in PBS, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0), and washed in 2× saline sodium citrate (SSC; 1× SSC = 150 mM NaCl and 15 mM sodium citrate, pH 7.0). The sections were prehybridized for 2 h at 50 °C in prehybridization buffer (50% formamide, 2× SSC, 1× Denhardt's solution, 500 μg/mL yeast tRNA, 500 μg/mL heparin sodium, 0.1% sodium pyrophosphate). Hybridization was performed at 50 °C for 16 h with DIG-labeled preprotemporin-CBa and/or preprochensirin-2CBa cRNA probes diluted with hybridization buffer (prehybridization buffer supplemented with 10% dextran sulfate). After hybridization, the sections were washed in 2× SSC at room temperature, 2× SSC-50% formamide at 50 °C for 1 h, 1× SSC-50% formamide at 50 °C for 1 h, and 2× SSC at room temperature. Slides were rinsed in buffer 1 (100 mM Tris–HCl, 150 mM NaCl, pH7.5), followed by incubation in buffer 1 containing 2% blocking reagent (Roche Diagnostics). Subsequently, sections were incubated with anti-DIG antibody conjugated with alkaline phosphatase (Roche Diagnostics), and then washed with buffer 1 followed by immersion in buffer 2 (100 mM Tris–HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH9.5). The bound antibody-conjugate was visualized with nitro-blue tetrazolium chloride (Sigma-Aldrich, St. Louis, MO, USA) and 5-bromo-4-chloro-3'-indolylphosphatase *p*-toluidine salt (Sigma-Aldrich) in buffer 2 at room temperature for 18 h in the dark. These sections were viewed using a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan), and digital images were captured using an Olympus DP70 CCD camera (Olympus, Tokyo, Japan).

3. Results

3.1. Cloning of AMP precursor cDNAs from the bullfrog HG samples

Two cDNA clones (clones 1 and 2) were amplified by RT-PCR using the set of preprotemporin-specific primers. Nucleotide sequence analysis revealed that clone 1 consisted of 209 bp and included an ORF of 186 bp (including a stop codon) and a 3′-UTR of 23 bp (Fig. 1). The deduced