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Identification and expression of a new member of the *pyrokinin/pban* gene family in the sand fly *Phlebotomus papatasi*

Man-Yeon Choi [a,1,*], Neil D. Sanscrainte [a,1], Alden S. Estep [a,b], Robert K. Vander Meer [a], James J. Becnel [a,*]

[a] United States Department of Agriculture, Agriculture Research Service, Center for Medical, Agricultural and Veterinary Entomology (CMAVE), 1600 SW 23rd Drive, Gainesville, FL 32608, USA

[b] Navy Entomology Center of Excellence, Box 43, Naval Air Station, Jacksonville, FL 32212-0043, USA

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ABSTRACT

The major family of neuropeptides (NPs) derived from the *pk* (pyrokinin)/*pban* (pheromone biosynthesis activating neuropeptide) gene are defined by a common FXPRL-NH_2 or similar sequence at the C-termini. This family of peptides has been found in all insect groups investigated to date and is implicated in regulating various physiological functions, including pheromone biosynthesis and diapause, but other functions are still largely unknown in specific life stages. Here we identify two isoforms of *pk/pban* cDNA encoding the PBAN domain from the sand fly *Phlebotomus papatasi*.

The two *pk/pban* isoforms have the same sequence except for a 63 nucleotide difference between the long and short forms, and contain no alternative mRNA splicing site. Two NP homologues, DASGDNGSDSQRTRPPFAPRLamide and SLPFSPRLamide are expected, however, sequence corresponding to the diapause hormone was not found in the *P. papatasi pk/pban* gene. The PBAN-like amino acid sequence homologue SNKYMTPRL is conserved in the gene, but there is no cleavage site for processing a functional peptide. Characterizing the expression of the isoforms in developmental stages and adults indicates that the short form is differentially transcribed depending on the life stage. The *P. papatasi pk/pban* gene is the only known *pk/pban* gene with two transcriptional isoforms and from examination of endoproteolytic cleavage sites is expected to produce fewer peptides than most of the *pk/pban* genes elucidated to date; only *Drosophila melanogaster* is simpler with a single NP detected by mass spectroscopy. A phylogenetic analysis showed *P. papatasi pk/pban* grouped more closely with other nematoceran flies rather than higher flies.

1. Introduction

Insect peptide neurohormones regulate a variety of physiological functions such as fat body homeostasis, feeding, digestion, excretion, circulation, reproduction, metamorphosis, and behavioral events during development and reproduction (Gäde and Goldsworthy, 2003). The pyrokinin (PK)/pheromone biosynthesis activating neuropeptide (PBAN) (=*pk/pban*) family, a major neuropeptide group found in insects, has been shown to have varying functions across orders (reviewed in Rafaeli, 2009). These include (among many others) stimulation of sex pheromone production and induction of melanization in lepidopterans (Raina et al., 1989; Matsumoto et al., 1990), hindgut muscle contraction in blattodeans (Holman et al., 1987), and most recently, stimulation of trail pheromone biosynthesis in hymenopterans (Choi and Vander Meer, 2012). This family of peptides shares a C-terminal FXPRL-NH_2 (or similar sequence), which is required for physiological activity, and are produced in the neurosecretory cells of the central nervous system (CNS) of insects (Raina and Kempe, 1990). In Lepidoptera, PBAN is synthesized in the subesophageal ganglion (SEG) and is released into the hemolymph via the corpora cardiaca, a neurohemal organ. To date, about 200 PK/PBAN family peptides have been reported from over 40 species (Choi et al., 2012).

Three genes can potentially produce FXPRL peptides: (1) *pk/pban* gene usually codes for four or five FXPRL-NH_2 (or similar sequence) peptides (diapause hormone (DH), PBAN-like peptides); (2) *capa* gene usually codes for two periviscerokinin (PVK) peptides (with a C-terminal PRV-NH_2) and one DH-like PK (different from the DH found in the PBAN/PK gene); and (3) *hugin*, found in *Drosophila*, is the equivalent to *pk/pban* which codes for two

* Corresponding authors at: USDA-ARS Horticultural Crops Research Laboratory, 3420 NW Orchard Avenue, Corvallis, OR 97330, USA (M.-Y. Choi). United States Department of Agriculture, Agriculture Research Service, Center for Medical, Agricultural and Veterinary Entomology (CMAVE), 1600 SW 23rd Drive, Gainesville, FL 32608, USA (J.J. Becnel).

E-mail addresses: mychoi@ars.usda.gov (M.-Y. Choi), James.Becnel@ars.usda.gov (J.J. Becnel).

[1] N.S. and M.C. contributed equally to this work.

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PRL-NH_2 peptides (Jurenka and Nusawardani, 2011) although subsequent studies have shown only a single functional peptide detected by mass spectroscopy (Baggerman et al., 2002, 2005). While peptides similar to those derived from *capa* genes have been found in crustaceans (the whiteleg shrimp *Litopenaeus vannamei*), chelicerates (the tick *Ixodes scapularis*, *Metaseiulus occidentalis*), and *Caenorhabditis elegans*, to date *pk*/*pban* genes and peptides have only been found in insects (Torfs et al., 2001; Lindemans et al., 2009; Neupert et al., 2009).

The first PBAN, a 33 amino acid peptide, was discovered in *Helicoverpa zea* (Raina et al., 1989), and while the physiological function of most PBANs is largely unknown, the majority of functional work has focused on the regulation of sex pheromone biosynthesis in Lepidoptera. Recently, the fire ant PBAN has been demonstrated to stimulate a trail pheromone production in the Dufour's gland of ant workers (Choi and Vander Meer, 2012). Explorations of these neuropeptides in Diptera have also revealed much about the genes involved in synthesizing these peptides. Some indications of function have been demonstrated (Zdárek et al., 1997; Verleyen et al., 2004) by showing that the PK peptide accelerates pupation in the flesh fly, *Sarcophaga bullata.* In mosquitoes, expressions of the *pk*/*pban* gene, as well as the PBAN and DH receptor genes, have been determined for all life stages of the mosquito *Aedes aegypti* and both receptors were shown to be functional through expression binding assays (Choi et al., 2013). Hellmich et al. (2014) localized PK/PBAN-like peptides to specific neuronal groups in the nervous system of larval and adult *Ae. aegypti*. These two studies are the first to characterize the *pk*/*pban* gene and expressed peptides in a species that vectors disease, potentially opening a new pathway by which pests can be targeted for control (Choi et al., 2012).

The sand fly *Phlebotomus papatasi* is an Old World vector of several species of *Leishmania* protozoan parasites. Leishmaniasis, a disease whose main reservoir is rodents, is responsible for over 70,000 human deaths annually (Kedzierski, 2010). *P. papatasi* vectors *L. major* and *L. arabica* in North Africa and the Middle East, and *L. major* and *L. turanica* in Iran and neighboring countries (Ready, 2013). These species of *Leishmania* cause cutaneous leishmaniasis, a chronic and slow healing disease that causes sores on the skin or mucous membranes. Other species of sand fly vector the more dangerous visceral leishmaniasis, which spreads to the internal organs and is responsible for the majority of fatalities from this disease (Kaye and Scott, 2011). Current methods for protecting people from sand fly bites, such as treating soldiers' uniforms or bednets with insecticides, have not been proven effective in reducing leishmaniasis cases (Alten et al., 2003; Courtenay et al., 2007). Studies in Israel have shown that *P. papatasi* populations could be significantly reduced by the application of attractive toxic sugar baits (ATSB), a mixture of juices, sugars, and boric acid as the toxicant (Müller and Schlein, 2011). Strategies such as this, combined with a better understanding of the physiology and molecular biology of phlebotomines, may produce methods more tailored to preventing sand fly vectored diseases.

In this study, we identified and characterized the *pk*/*pban* gene from *P. papatasi* (Phlpa-*pk*/*pban*), the first from a sand fly. This is the only known *pk*/*pban* gene with two transcriptional isoforms. Expression of the Phlpa-*pk*/*pban* gene transcription was examined in egg, larval, pupal, and adult life stages, and the phylogeny of all available transcripts that code for PK/PBAN-like peptides is discussed.

2. Materials and methods

2.1. Insects

The *P. papatasi* colony at USDA-ARS-CMAVE, Gainesville, FL was established from the Israeli strain colony at Walter Reed Army Institute of Research (Silver Spring, MA). Larvae were reared on a diet containing rabbit feces, and adults were membrane fed using bovine blood. Immature life stages of *P. papatasi* were collected as pools of approximately 0.1 mL of eggs, 1st instar larvae, 4th instar larvae, and pupae. Male and female adults were sampled at 1–2D post-emergence (PE), 4–6D PE, and 8–9D PE. Groups of 100 females of each adult age group were decapitated with heads and abdomens collected separately. Fifty to 100 whole females were collected in tandem. All dissections were performed on dry ice, and samples were held at −80 °C until processing.

2.2. Total RNA isolation and cDNA synthesis

Total RNA was extracted from all *P. papatasi* life stage samples using the RNAqueous-4PCR Total RNA Isolation Kit (Ambion/Life Technologies) as per the manufacturer's instructions, including a DNAse I step to remove any contaminating DNA. RNA was quantified on a Nanodrop 2000 (Thermo Scientific). RACE-ready cDNA was generated with the GeneRacer Kit with SuperScript III RT (Invitrogen/Life Technologies) using a mixture of 1000 ng of total RNA isolated from whole females from each age group. The manufacturer provided GeneRacer 5′ RNA oligo was ligated to full length capped RNA and then reverse transcription was carried out using GeneRacer oligo dT primer, resulting in 5′ and 3′ RACE-ready cDNA. First-strand cDNA libraries for expression studies were generated from 300 ng of total RNA from each life stage using the Cloned AMV First-Strand cDNA Synthesis Kit (Ambion/Life Technologies) and stored at −20 °C.

2.3. Molecular cloning

To identify putative sand fly homologues, the *Ae. aegypti* PK/PBAN protein sequence, AAEL012060-PA, and PK1 protein sequence, AAEL012796-PA, were collected from Vectorbase (www.vectorbase.org) and used as a query against the WGS database sequences of *P. papatasi* and *L. longipalpis* on the genome server of NCBI using the tBLASTn option. Genomic sequences were examined using ORFinder to determine putative open reading frames in the area of the initial match and for initial primer design. The 5′ end of the *pk*/*pban* transcript was amplified using the sense GeneRacer 5′ primer 5′-CGACTGGAGCACGAGGACACTGA-3′ and the gene specific antisense primer 5′-CCTTCCGAGTCTGGGTGAGAAGG GTAA-3′. The 3′ end of the *pk*/*pban* transcript was amplified using the gene specific sense primer 5′-CCGCGTCTTGGACGAGATGCATC TGGA-3′ and the antisense GeneRacer 3′ primer 5′-GCTGTCAACG ATACGCTACGTAACG-3′. Touchdown PCR was performed as per the GeneRacer protocol with the following modifications: 5 cycles of 94 °C for 30 s and 72 °C for 45 s; 5 cycles of 94 °C for 30 s and 70 °C for 45 s; and 25 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 45 s. Bands of interest were gel purified and cloned using the pGEM T-Easy Vector System (Promega) per the manufacturer's instructions, and then sequenced by Macrogen USA.

2.4. Reverse transcriptase (RT)-PCR for pk/pban expression

Total RNA from each *P. papatasi* life stage (20 ng per sample) was used to amplify the full *pk*/*pban* gene sequence with the primer set, 5′-GAGTATTGACTTGGATATCGAAACG-3′ and 5′-AACCAAT TTATTTCAAAATTTGC-3′. A 101-bp fragment of the sand fly ribosomal protein S7 (RPS7) gene was also amplified for a positive control using primers 5′-GCAGTGAACCGGATGAGTTT-3′ and 5′-CGTTATGT GCAAATCCCTCA-3′. The one-step RT-PCR was performed as follows: 1 cycle of 50 °C for 30 min and 40 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min using RT/Taq mix polymerase (Invitrogen/Life Technologies). Then PCR products were visualized using GelRed (Biotium) under a UV light after 1.5% agarose gel