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Structure and expression of a shrimp prohormone convertase 2

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ABSTRACT

Although many crustacean neuroendocrine hormones have been reported, the enzymes responsible for post-translational modification of neuroendocrine hormones have rarely been characterized. A prohormone convertase 2 (PC2)-like enzyme has been isolated from the optic lobe of the giant tiger shrimp, *Penaeus monodon* and referred as PmPC2. The full length cDNA sequence of PmPC2 has been identified and found to resemble evolutionarily conserved PC2 enzymes of vertebrates and invertebrates. PmPC2 was expressed in all larval developmental stages and in neuroendrocrine cells in the adult optic lobe. Its expression was found to be negatively related with shrimp body weight by qPCR ($P < 0.05$). Immunohistochemistry results using an anti-rPmPC2 antibody with adult shrimp revealed high staining intensity in specific neurosecretory cells including the sinus gland, the organ of Hanström (also referred to as the medullar terminalis X-organ) and the organ of Bellonci (also referred to as the sensory or X-organ). By using the yeast two hybrid technique, PmPC2 was found to bind with *P. monodon* hyperglycemic hormone (Pem-CHH1) that plays an important role in glucose metabolism. Since PmPC2 is a subtilisin-like serine proteinase, it is expected to cleave the synthetic substrate, pyr-RTKR-MCA, but the expressed recombinant catalytic domain of PmPC2 (rPmPC2-cat) showed no enzymatic activity as expected. *In vivo* injection of dsRNA-PmPC2 resulted in reduced transcripts for both PmPC2 and Pem-CHH1 on day 3 post injection, but there was no accompanying reduction of glucose level in the hemolymph. Taken together, PmPC2 localization, expression and activity suggest that it has a function(s) in the shrimp neuroendrocrine system and that it may not only activate Pem-CHH1 but also affect its expression. However, there is no obvious explanation for the negative correlation between PmPC2 expression level and shrimp body weight.

1. Introduction

Peptide hormones and neuropeptides are frequently synthesized as precursors that are biologically inactive until they are cleaved and modified through multiple, post-translational processing steps. One critical modification is a proteolytic cleavage to release one or more biologically active products. PC2 prohormone convertases (PCs) are enzymes involved in the proteolytic maturation of neuropeptide precursors [16]. Homologues of PC2 have also been characterized in a few invertebrates including the nematode *Caenorhabditis elegans* [7], three mollusk species (*Lymnaea stagnalis* [18], *Aplysia californica* [13], *Haliotis asinine* [5]) and two arthropod species (*Lucilia cuprina* [12] and *Drosophila melanogaster* [8]). A vertebrate PC2 has been reported to cleave protein precursors including proinsulin, proglucagon, pro-opiomelanocortin (POMC), proglucagon and prosomatostatin [23,14]. When PC2 knockout (KO) mouse models became available, numerous neuropeptide and peptide hormone precursor processing defects were revealed using quantitative peptidomic approaches [24].

Crustacean hormones, principally peptides, released from the XO-SG system are known to control many physiological processes, including molting, somatic growth and sexual maturation, as well as metabolic adaptation to changing environmental conditions. The crustacean hyperglycemic hormone (CHH) is a member of the eyestalk CHH/MIH/GIH neuropeptide family and originates from a larger preprohormone in the crustacean medulla terminalis X-organ (MTXO) [4]. The role that CHH plays in the regulation of glucose metabolism has been studied in a number of crustaceans [6]. The mechanism of preproCHH processing is still unknown,

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but co-expression of PC2 and CHH-I was reported in the cells of the X-organ of the crayfish *Orconectes limosus*, suggesting that PC2 might be involved in the maturation of CHH [20]. Thus, it is possible that CHH maturation, as in vertebrates, involves removal of a signal peptide from the preprohormone to yield an excreted prohormone that, in turn, yields the mature hormone by additional proteolytic cleavage by PC2.

In this study, PmPC2-cDNA was identified and characterized, and its expression was examined in larval and juvenile shrimp. It was also subjected to yeast-two-hybrid screening for interacting partners, and its activities were studied using dsRNA knockdown and an expressed recombinant protein for its purported catalytic domain (rPMPC2-cat). In addition, an anti-rPmPC2-cat antibody was used by immunohistochemistry to study PmPC2 distribution in the optic lobes of juvenile shrimp.

2. Materials and methods

2.1. Experimental shrimp

Juvenile giant tiger shrimp *Penaeus monodon* were purchased from a commercial farm in Thailand, and kept in a recirculated seawater system. Shrimp were anesthetized in an ice-water mixture for 3 min before dissection of optic lobes, thoracic ganglia, abdominal ganglia, hematopoietic tissue, hemocytes, lymphoid organs, stomach tissue, hepatopancreatic tissue, intestines, hearts, gills, ovaries, pleopods and muscle tissue for transfer to Trizol reagent (Invitrogen, USA). Larvae at the stages of zoea 3, mysis 3 and post-larva 5 were sampled and kept in RNAlater solution (Ambion) at 4 °C overnight before being stored at −20 °C until used. For analysis of relative PmPC2-mRNA expression versus body weight, large and small sizes of male shrimp were selected from the >90 and <10 percentile regions of the growth distribution curve, respectively. Large male (LM) and small male (SM) sub-populations had average body weights of 42.96 ± 1.85 and 11.29 ± 3.24 g, respectively. The optic lobes of small and large males were collected in RNA later solution. Total RNA was extracted from shrimp tissues using Trizol reagent according the manufacturer's manual.

2.2. Isolation and characterization of PmPC2-like cDNA from P. monodon

A partial EST sequence of PmPC2 (240 bp) was screened from an optic lobe-SSH library of *P. monodon* [19]. Specific primers were designed to perform 5′RACE and 3′RACE according SMART™ RACE cDNA amplification kit instructions (Clontech Laboratories, Inc.). Briefly, the cDNAs were synthesized by using 3 μg of total RNA before specific amplification with PC2-specific primers (5′RACE-PC2-3 or 3′RACE-PC2-1) and the kit universal primer mix (UMP, Clontech). All primer sequences used in this study are shown in Table 1. The PCR products were analyzed by agarose gel electrophoresis and bands of expected sizes were excised and purified using a gel/PCR DNA Fragment Extraction kit (Geneaid, Taiwan). The purified DNA fragments were cloned into the pGEM-T Easy vector (Promega, USA) and sequenced. The amplification of the full-length cDNA was carried out by PCR using the primer pair PC2-Met and PC2-stop (Table 1) with Pfu DNA polymerase (Promega, USA), before cloning and sequencing to obtain complete PmPC2 sequences.

2.3. RT-PCR and quantitative PCR

Temporal expression of PmPC2 in *P. monodon* whole larval stages and tissue distribution in juvenile shrimp were assessed by RT-PCR. Equivalent amounts of total RNA (100 ng) were used as the template for amplification of a PmPC2-fragment using a Superscript III one-step RT-PCR kit with Platinum®*Taq* (Invitrogen) with specific primers of PmPC2 (PC2F and PC2R). Amplification of PmEF-1α by primers EF-1αF and EF-1αR was also performed to normalize the intensity of the PmPC2 product. The primers for *P. monodon* crustacean hyperglycemic hormone I precursor were designed from Pem-CHH1 (GenBank Accession No. AF233295) [21]. To determine the quantity of Pem-CHH1 transcript in a dsRNA-PmPC2 knockdown experiment, primers CHH-1-F and CHH-1-R were used. The protocol consisted of reverse transcription at 50 °C for 30 min followed by an initial denaturing step at 94 °C for 2 min followed by 28 cycles for PmPC2 but 20 cycles for EF-1α at 94 °C for 20 s, 62 °C for 20 s and 68 °C for 20 s with a final extension at 68 °C for 5 min. The amplicon products (232 bp for PmPC2, 156 bp for PmEF-1α and 305 bp for Pem-CHH1) were resolved by 1.5% agarose gel electrophoresis.

The expression of PmPC2 in the optic lobes of small and large males was determined by qPCR using specific primers (PC2F and PC2R). The first-strand cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) with 1 μg total RNA template and oligo $(dT)_{18}$ according the manufacturer's manual. Gene expression was assessed by SYBR Green PCR Master Mix (KAPA BIOSYSTEMS) in a LightCycler® 480 (Biorad) with a program consisting of 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 62 °C for 10 s and 72 °C for 10 s. Sample reactions were performed in triplicate and included negative (without target DNA) and positive plasmid (efficiency determination) controls. Relative quantification was performed according to the "ΔΔCt method" [10] by employing PmEF-1α for normalization. Student's test was used to compare the relative expression of PmPC2 in small and large males, and means were considered significantly different at $p < 0.05$.

2.4. Expression and purification of recombinant PmPC2

The putative catalytic domain of PmPC2 (Nucleotide Nos. 331–1260) was PCR-amplified and cloned into a pET17b expression vector (Novagen). The specific primers (HindIII-CatPC2-331 and SacI-CatPC2-1260) used are shown in Table 1. The constructs were subsequently sequenced to confirm in-frame reading before transformation into the expression host *E. coli* strain BL21 (Novagen). Transformants were cultured in LB medium supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenical overnight at 37 °C. Isopropyl-b-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM to induce expression for 4 h at 20 °C and production of recombinant PmPC2 (rPmPC2-cat) was examined by SDS–PAGE. The gels were stained with Coomassie brilliant blue G250. Western blot analysis was carried out to confirm the identity of rPmPC2 using anti-His antibody (GE Healthcare), a horseradish peroxidase-tagged secondary antibody (Zymed) and immunochemical staining using TMB Membrane peroxidase substrate (KPL, USA).

The inclusions of rPmPC2 were solubilized in 8 M urea in PBS at pH 8.0. The solubilized fractions were purified by Ni-NTA agarose supplemented with imidazole, according to the manufacturer's protocol (QIAGEN). The fusion protein was eluted with 300 mM imidazole buffer containing 8 M urea before buffer replacement with PBS pH 7.4 and concentration by ultrafiltration (Vivaspin 6; cut off 10 kDa, GE Healthcare). Total protein concentration was determined according to Bradford using bovine serum albumin as the standard. The purified rPmPC2 was stored at −20 °C until used for amino acid sequencing and enzymatic activity assays. For amino acid sequence determination, purified rPmPC2 from Coomassie stained bands was excised and digested with trypsin to prepare for liquid chromatography–electrospray ionization tandem mass spectrometry (LC–MS/MS). MASCOT program was used to analyze the results.