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Characterization of *Cherax quadricarinatus* prohibitin and its potential role in spermatogenesis

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ABSTRACT

Prohibitin (PHB) proteins have diverse functions, such as cellular signaling, transcriptional control and mitochondrial biogenesis. In this study, we characterized PHB gene and its protein expression in *Cherax quadricarinatus*. *PHB* cDNA comprises 1472 nucleotides with an open reading frame of 828 bp, which encodes 275 amino acid residues. The highest transcript levels were found during the spermatogonial developmental phase, with the lowest levels detected during the resting phase in the reproductive cycle. Western blot analysis revealed that PHB is an approximately 30 kDa protein, and occurs in a number of unexpected isoforms, ranging from 30 kDa to greater than 180 kDa in the testes of different developmental phases, which may be the ubiquitinated substrates. The strongest immunolabeling signal was found in spermatogonia, with lower levels of staining in secondary spermatocytes, and weak or absent expression in mature sperm. Immunogold electron microscopy results confirmed the localization of PHB in the inner mitochondrial membranes. The results showed that PHB is a substrate protein for spermatogenesis, with a potential reproductive function involving sperm ubiquitination in invertebrates.

1. Introduction

Prohibitin (PHB) is an evolutionally conserved protein belonging to a Band-7 or PHB domain family (Merkwirth and Langer, 2009; Mishra et al., 2006) and mainly localized in mitochondria (Artal-Sanz and Tavernarakis, 2009; Ikonen et al., 1995; Terashima et al., 1994; Thompson et al., 1999). Two highly homologous forms of this protein exist termed PHB1 and PHB2 (Mishra et al., 2006). Besides the initially proposed role in cell cycle progression (Cummins, 1998; Mishra et al., 2006; Nijtmans et al., 2000; Nuell et al., 1991; Piper et al., 2002; Sharma and Qadri, 2004; Theiss et al., 2007), PHBs have also been implicated in transcriptional regulation (Sun et al., 2004), sister chromatid cohesion regulation (Schmittgen and Livak, 2008), cellular signaling (Mishra et al., 2006; Rajalingam et al., 2005), apoptosis (Fusaro et al., 2003) and mitochondrial biogenesis (Artal-Sanz and Tavernarakis, 2009; Berger and Yaffe, 1998; Nijtmans et al., 2000). PHB1 mRNA and protein were reported to be expressed differentially during germ cell development. Subsequently, it was suggested that PHB1, a conserved 30 kDa component of the inner mitochondrial membrane, is also expressed as an unusual, high-molecular-mass isoform in mammalian spermatozoa (Sutovsky, 2003). However, little is known regarding the functional significance of PHB1 in the male germ cell in invertebrate animals.

The mitochondrion is a complex sub-cellular organ present in the cytoplasm of all animal and plant cells (Sanz et al., 2003). Several studies have indicated an important role for mitochondria in spermatogenesis and fertility (Sharma and Qadri, 2004; Shivaji et al., 2009). Mitochondria are a double-membrane structure consisting of outer and inner membranes (IM) separated by an inter-membrane space. The IM forms numerous folds inside the mitochondrial matrix known as "cristae" and is unusually high in protein content. PHB1 and PHB2 associate to form a macromolecular structure of approximately 1 MDa at the mitochondrial IM (Nijtmans et al., 2002).The PHB complex has been implicated in regulation of membrane protein degradation by the mitochondrial m-AAA protease (Langer, 2000). Ubiquitination is a versatile and universal mechanism for protein recycling, through which misfolded or aged proteins are tagged for degradation by the covalent attachment of one or more molecules of ubiquitin (Sutovsky, 2003). Mitochondrial DNA mutations have been reported in mitochondrial

Abbreviations: PHB, prohibitin; IM, inner membranes; *Cq*-PHB, *Cherax quadricarinatus* prohibitin; RT-qPCR, real-time quantitative RT-PCR; ML, maximum likelihood; JTT, Jones–Taylor–Thornton; PVDF, polyvinylidene difluoride; IF, immunofluorescence; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; IEM, immunogold electron microscopy; TBS, Tris buffered saline; H&E, hematoxylin and eosin; S SPFH, tomatin/prohibitin/flotillin/HflK/C; mtDNA, mitochondrial DNA.

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respiration defects giving rise to meiotic arrest and abnormalities in sperm morphology (Ankel-Simons and Cummins, 1996; Cummins, 1998; Merkwirth et al., 2008; Spiropoulos et al., 2002).

Cherax quadricarinatus is a native species in the north of Queensland (Australia) and the southeast of Papua New Guinea (Jones, 1997). It is an important cultured species in aquaculture that has been reared for human consumption since 1985. Knowledge of the reproductive biology of male *C. quadricarinatus* is mainly limited to the anatomy of the reproductive system and the effects of nutrition or neuroendocrine regulation on fecundity (López Greco and Lo Nostro, 2008). Hence, a better understanding of the molecular mechanisms of spermatogenesis has become an immediate research priority. PHB functions as a reproduction-related and mitochondrial membrane protein, *PHB* cDNA and protein had been characterized from yeast (Tatsuta et al., 2005), *Pneumocystis carinii* (Narasimhan et al., 1997), *Drosophila* (Eveleth and Marsh, 1986), mouse (Terashima et al., 1994), rat (Altus et al., 1995; Choongkittaworn et al., 1993) and human (Sato et al., 1992), but no studies have been conducted in the Decapoda, Parastacidae.

The main objectives of this present study were: (1) to clone the PHB gene from *C. quadricarinatus* (designated *Cq-PHB*); (2) to investigate the tissue distribution and temporal mRNA expression of *Cq-PHB* in testes during spermatogenesis, and (3) to locate Cq-PHB in testes and sperm mitochondria. Insight into the PHB gene and its expression pattern in spermatogenesis is important for understanding the molecular mechanisms of crayfish which may be conducive to the development of reproduction biology. In combination with the identification of its localization in testes and sperm mitochondria, the information reported here may facilitate further investigations of inducible PHB in the freshwater crayfish and inform further investigation of its mitochondrial inheritance in invertebrates.

2. Materials and methods

2.1. Animals

Fifteen male *C. quadricarinatus* (70–110 g) were purchased from Shanghai Jinshan aquaculture farm during May (spring), July (summer) September (autumn) and December (winter) in 2010, and then placed in an ice bath for 3 to 5 min until lightly anesthetized prior to sacrifice. Various tissues were dissected, immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Isolation of total RNA and cDNA synthesis

Total RNA was extracted using Trizol reagent (RNA Extraction Kit, CA, USA) according to the protocol provided by the manufacturer. The concentration and quality of total RNA were estimated by spectrophotometry (absorbance at 260 nm) and agarose gel electrophoresis, respectively. Total RNA (2 μg) isolated from testis was reverse transcribed using the SMART™ cDNA kit (Clonetech, CA, USA) for cDNA cloning and using the Prime Script™ RT-PCR Kit (TaKaRa, Japan) for semi-quantitative RT-PCR (RT-PCR) analysis or the PrimeScript Real-time PCR Kit (TaKaRa, Japan) for real-time quantitative RT-PCR (RT-qPCR) analysis.

2.3. PHB gene isolation

Target fragments of cDNAs encoding PHB were amplified with the degenerate primers DP1 and DP2. All primers were synthesized at Shanghai Invitrogen Biotech Co., Ltd. (Shanghai, China) and are listed in Table 1. The obtained target sequences were verified and analyzed for similarity with other known PHB sequences using BLAST programs (Altschul et al., 1997). The cDNA for 3′ or 5′ RACE was amplified using the kit recommended reaction system and two pairs of gene-specific primers (Gp5-1, Gp5-2, Gp3-1, and Gp3-2; Table 1), which were designed based on the obtained cDNA partial sequence of *Cq-PHB*. The full-length *Cq-PHB* cDNA was obtained using the RACE cDNA amplification kit according to the protocol provided by the manufacturer (Bioscience Clontech, CA, USA).

Table 1
Sequences of primers.

Primers	Sequence	Code
Degenerate primers for target PHB		
Forward primer	5′-ACCATGNCYTCHGGGTRCATMGA-3′	DP-R
Reverse primer	5′-GGBGATGAGYTNTTNACYGACA-3′	DP-F
Gene-specific primers for PHB cloning		
Gene-specific primer pairs for RACE	5′-GAGTTCTCCAGCATCATACTGAGCCA-3′	Gp5-1
	5′-GGAATGTCCCTGTAAACACTGGCAGT-3′	Gp3-1
	5′-AGGGTGGTCTGGTTGCTTGGTAG-3′	Gp5-2
	5′-TATCTATTACTCATCTGACCTTCGGC-3′	Gp3-2
RT-PCR primers		
PHB 5′ primer	5′-TTGCCGAAGGTCAGATGAGTAATA-3′	RT-R
PHB 3′ primer	5′-GGAATGTCCCTGTAAACACTGGC-3′	RT-F
qRT-PCR primers		
PHB 5′ primer	5′-TGAAGCATCACCATCAGCACTAAT-3′	Real-R
PHB 3′ primer	5′-TATCTATTACTCATCTGACCTTCGGC-3′	Real-F
18S rRNA primers for RT-PCR and qRT-PCR		
18S rRNA reverse	5′-GGAGGTAGTGACGAAAAATAACG -3′	18S-R
18S rRNA forward	5′-GGAGCGTGACAGTAAGCACCATCGG -3′	18S-F

2.4. Alignment of multiple sequences and phylogenetic analysis

Multiple alignments of the full-length PHB sequence were compared with PHB sequences from other species. Amino acid sequences of various crustacean species were retrieved from the NCBI GenBank and analyzed using the ClustalW 2.0 Multiple Alignment program. Phylogenetic analyses were conducted using maximum likelihood (ML) approach (Holmes, 2003; Huelsenbeck and Rannala, 1997). It is known to be robust to a number of systematic biases of phylogenetic reconstruction. A ML phylogenetic tree was constructed using MEGA software version 5.0 (Holmes, 2003; Tamura et al., 2011). Jones–Taylor–Thornton (JTT) model was used as substitution model for phylogenetic tree construction (Jones et al., 1992). Reliability of nodes was estimated by ML bootstrap percentages (BPML) (Sullivan, 2005) obtained after 1000 pseudo replications, using the previously estimated ML parameters.

2.5. PHB mRNA transcript expression patterns

Quantitative analysis of tissue- and testes developmental cycle-dependent mRNA expression was conducted via Real-time RT-qPCR. First-strand cDNA was prepared as described in Section 2.2. RT-qPCR primers (Q-R and Q-F, Table 1) were designed based on the cloned Cq-PHB cDNA to produce a 310 bp amplicon. PCR reactions were performed according to the PrimeScript Real-time PCR Kit protocol. The primers 18S-F and 18S-R were designed based on the sequence of *C. quadricarinatus* 18S rRNA (AF235966) to amplify a 232 bp fragment. Samples were run in triplicate and normalized to the selected control gene, 18S rRNA. All PCR reactions were performed in triplicate using extracted RNA (pooled) of the same concentration. *Cq-PHB* expression levels were calculated by the $2^{-\Delta\Delta Ct}$ comparative CT method (Colebatch et al., 2002). Mean and standard deviation values were calculated from triplicate experiments, and presented as fold differences in expression relative to 18S rRNA. Data were analyzed using the CFX Manager™ software version 1.0.