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Molecular characterization and function of the Prohibitin2 gene in *Litopenaeus vannamei* responses to *Vibrio alginolyticus*

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ABSTRACT

Prohibitin2 (PHB2), a potential tumor suppressor protein, plays important roles in inhibition of cell cycle progression, transcriptional regulation, apoptosis and the mitochondrial respiratory chain. To explore its potential roles in crustaceans' immune responses we have identified and characterized *Lv*PHB2, a 891 bp gene encoding a 297 amino acids protein in the shrimp *Litopenaeus vannamei*. Expression analyses showed that *Lv*PHB2 is expressed in all examined tissues, and largely present in cytoplasm, correlating with its known anti-oxidation function in mitochondria. Luciferase reporter assays showed that over-expression of *Lv*PHB2 could activate the p53 pathway, indicating that it might participate in apoptosis regulation. Quantitative real-time PCR revealed that infection with *Vibrio alginolyticus* induces its up-regulation in hepatopancreas. Moreover, RNAi knock-down of *Lv*PHB2 in vivo raises mortality rates of *L. vannamei* infected by *V. alginolyticus*, and affects expression of STAT3, Caspase3 and p53 genes. We found significantly higher reactive oxygen species production, DNA damage and apoptosis rates in *Lv*PHB2-silenced shrimp challenged with *V. alginolyticus* than in controls injected with a Green Fluorescent Protein-silencing construct. Our results suggest that *Lv*PHB2 plays a vital role in shrimp responses to *V. alginolyticus* infection through its participation in regulation of oxidants and apoptosis.

1. Introduction

The pacific white shrimp, *Litopenaeus vannamei*, is one of the most important aquacultured prawn species globally, especially in China. In recent years the prawn breeding industry has faced severe challenges and losses due to the rapid development of intensive culture, environmental pollution and outbreaks of pathogens (Briggs et al., 2005), notably the bacterium *Vibrio alginolyticus* (Cheng et al., 2004; Su and Chen, 2008). The main shrimp defenses against such pathogens include phagocytosis and respiratory bursts initiated by their innate immune systems (Rajesh et al., 2014). These systems require tight regulation because respiratory bursts involve rapid production of reactive oxygen species (ROS), which disrupt organisms' redox balances and induce apoptosis (Cha et al., 2015; Xian et al., 2010).

Known molecular elements of shrimps' immune responses include the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway (Okugawa et al., 2013). More specifically, STAT gene activation is known to play a defensive role during bacterial infection in shrimp (Peng et al., 2016; Wei et al., 2008). Another possibly important agent is prohibitin (PHB), a potential tumor suppressor protein first discovered in rat liver by McClung (Mcclung et al., 1989). PHB is an evolutionarily conserved protein found in diverse organisms, including bacteria, yeast, protozoans, plants, and mammals (Mcclung et al., 1995). It comprises two highly homologous subunits, Prohibitin1 (PHB1) and Prohibitin2 (PHB2), both of which participate in various physiological activities of cells. They play crucial roles in the inhibition of cell cycle progression, senescence, suppression of cell proliferation, transcriptional regulation, apoptosis and mitochondrial respiratory chain assembly (Peng et al., 2015).

Recent studies have shown that PHB2 protein has various cellular locations, which are closely linked to its functions. Therefore, the localization of PHB2 in cells might determine its function. For example, on a plasma membrane PHB2 may modulate

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membrane transport (Sharma and Qadri, 2005), while PHB2 located in the nucleus may participate in regulation of transcription, cell cycle and apoptosis (Gamble et al., 2004). Moreover PHB2 in mitochondria appears to be crucial for the stability of mitochondrial morphology, and regulation of both mitochondrial dynamics (Nijtmans et al., 2000; Rossi et al., 2014) and the mitochondrial apoptotic pathway (Kirchman et al., 2003). Thus, PHB2 is clearly a key regulator in mitochondria. Accordingly PHB2 deletion impairs the function and form of mitochondria, and increases both ROS levels and cells' susceptibility to ROS in *Caenorhabditis elegans* (Artalsanz et al., 2003). Conversely, PHB up-regulation can protect cells against injury and apoptosis induced by oxidative stress (Ye et al., 2015).

Clear links between oxidative stress, PHB2 and apoptosis regulation have also been found. For example, expression of PHB2 and Caspase3, a major apoptotic executioner protease, is significantly and positively correlated in rat brain (Xu et al., 2014). Furthermore, PHB2 over-expression can prevent activation of apoptosis pathways by dampening ROS generation, reducing mitochondrial cytochrome *c* release and decreasing caspase-3 activation in a mouse model of transient forebrain ischemia (Kurinami et al., 2014). Over-expression of PHB also inhibits apoptosis by affecting activity of Caspase3, while PHB-silencing enhances sensitivity to apoptosis induction in rat granulosa cells (Chowdhury et al., 2013). Up-regulation of prohibitin expression can also promote apoptosis in retinoic acid-resistant acute promyelocytic leukemia cells (NB4-R1) (Liu et al., 2014).

Findings that PHB co-localizes and directly interacts with p53 in human epidermal HaCaT cells (Yang et al., 2014) provide further evidence that expression and distribution of PHB are intimately connected to apoptosis and regulation of cell proliferation through complex mechanisms. The tumor-suppressor gene p53 plays a key role as a specific transcription factor in inhibition of the cell cycle, promotion of cell apoptosis and aging (Jian and Lin, 2005). PHB appears to modulate this activity by binding to p53 and inducing p53-mediated transcription in regulation of apoptosis pathways, according to chromatin immunoprecipitation assays (Fusaro et al., 2003). In addition, changes in PHB expression reportedly induce corresponding changes in levels of caspase 7 (another apoptosis executioner protein), and PHB might affect apoptosis by activating the caspase 7 promoter, which has p53 binding sites (Joshi et al., 2007).

Given its importance in anti-oxidative processes and apoptosis there are clear practical reasons for elucidating interactions and roles of PHB2, in organisms generally and crustaceans particularly. Thus, in recent years several findings on PHBs in crustaceans have been published. Notably, PHB1 transcripts are reportedly down-regulated in *L. vannamei* 1 and 3 h after infection by white spot syndrome virus (WSSV), and up-regulated 24 h after infection (Clavero-Salas et al., 2007). PHB2 gene sequences have also been obtained from a testis cDNA library of the shrimp *Penaeus monodon*, and used in an analysis of differences in expression profiles between the organism's testes and ovaries (Leelatanawit et al., 2009). However, there is very little knowledge of the role of PHB2 in crustaceans' immune responses. Thus, in efforts to address this knowledge gap we have investigated its role in responses of *L. vannamei* to challenge by *V. alginolyticus*. For this purpose, we cloned full-length PHB2 cDNA of *L. vannamei*, examined its tissue expression profile and subcellular localization, and examined effects of *V. alginolyticus* injections with and without silencing the PHB2 gene. Finally, our present results support that *Lv*PHB2 participated in regulating the ROS production and apoptosis signal pathway, and plays an important role in the innate immune response of *L. vannamei* to *V. alginolyticus* infection.

2. Material and methods

2.1. Animals

Healthy L. *vannamei*, averaging 10–13 g in weight and 6–7 cm in length, were obtained from a shrimp farm in Panyu, Guangdong Province, China. These shrimps were kept for 2 weeks in circulating water tanks with filtered seawater at 10‰, pH 7.8–8.0 and 24±2 °C. The shrimp were fed three times daily with commercial feed.

2.2. Cloning full-length cDNA of PHB2 and sequence analyses

To clone a cDNA fragment of *Lv*PHB2, primers *Lv*PHB2 F1 and *Lv*PHB2 R1 (Table 1) were designed, based on conserved regions of

Table 1
The primer sequences used in this paper.

Names	Sequence (5′-3′)
LvPHB2-5′	GAACCATCGCCCCGGCGCCG
LvPHB2-3′	CTTTCCCTATACATTTATTTGC
LvPHB2 -pEGFP-N1-F	CCCTCGAGCTATGGGCGACAAACTGAACGA
LvPHB2 -pEGFP-N1-R	GCGAATTCGACTTCTTTGTCACACGGGTAG
LvPHB2-pEGFP-N3-F	GGGGTACCGATGGGCGACAAACTGAACGA
LvPHB2-pEGFP-N3-R	CGGGATCCCGCTTCTTTGTCACACGGGTAG
LvPHB2 F1	AAGGACTGGTGGATGTTGGG
LvPHB2 R1	TCTTCAGCCGTATTGGAGGA
Lv18SRNA- F	TATACGCTAGTGGAGCTGGAA
Lv18SRNA- R	GGGGAGGTAGTGACGAAAAAT
LvSTAT F	ATAGTTTGTGGTGTGTTGGG
LvSTAT R	TATATCCGAATGTGCCTAAG
LvP53 F	CGAATCCCCACATCCACG
LvP53 R	GGCGGCTGATACACCACC
LvCaspase3 F	ACGAGAAGTCGCCAGGAGGT
LvLvCaspase3R	CGGTCGCATTGTGATGATAAAA
LvPHB2i-F (with T7)	TAATACGACTCACTATAGGGCTTATGGCATCTCGCAGTC
LvPHB2i-F	GCTTATGGCATCTCGCAGTC
LvPHB2i-R (with T7)	TAATACGACTCACTATAGGCAAGTTGAGCATTAGTGTGTC
LvPHB2i-R	CAAGTTGAGCATTAGTGTGTC
GFPi-F (with T7)	TAATACGACTCACTATAGGGAGAGTGCCCATCCTGGTCGAGCT
GFPi-F	GTGCCCATCCTGGTCGAGCT
GFPi-R (with T7)	TAATACGACTCACTATAGGGAGATGCACGCTGCCGTCCTCGAT
GFPi-R	TGCACGCTGCCGTCCTCGAT

*T7 RNA polymerase binding site is underlined.

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