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p53 is involved in shrimp survival via its regulation roles on MnSOD and GPx in response to acute environmental stresses

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ABSTRACT

The tumor suppressor gene p53 plays a critical role in safeguarding the integrity of genome in mammalian cells. It acts as a sequence-specific transcription factor. Once activated by a variety of cellular stresses, p53 transactivates downstream target genes, through which it regulates cell cycle and apoptosis. However, little is known about p53 as well as its downstream target genes in invertebrates. A full length cDNA that encodes a 453-amino-acid p53 protein (Lvp53) was characterized in the Pacific white shrimp (*Litopenaeus vannamei*) to explore the potential relationships between p53 and two antioxidant enzyme genes: Mn-superoxide dismutase (MnSOD) and glutathione peroxidase (GPx) in eliminating cell stresses in *L. vannamei*. Sequence analysis revealed a close phylogenetic relationship between Lvp53 and that of *Marsupenaeus japonicus*, and a high degree of conservation in critical amino acids residues is involved in DNA and zinc binding among species. Quantitative real-time PCR showed that Lvp53 was expressed with varied levels in all the 11 tissues under investigation. In response to acute pH challenge, the relative expression of Lvp53 was induced in a pH- and time-dependent manner, with the peak observed at pH 6.1 and after 24 h of treatment, in which condition, both the relative mRNA expressions and the enzymatic activities of LvMnSOD and LvGPx were increased correspondingly. In response to acute cadmium (Cd) exposure, the relative expression of Lvp53 was upregulated in a time- and concentration-dependent manner, with the maximum detected at Cd 6.6 μM and after 48 h of exposure, in which case, both the transcripts and the enzymatic activities of LvMnSOD and LvGPx were also induced. After Lvp53 transcripts were declined by double-strand RNA injection, the relative mRNA expressions of LvMnSOD and LvGPx were decreased correspondingly. Meanwhile, pH 6.1 or 6.6 μM Cd could not induce the transcripts or the enzymatic activities of LvMnSOD or LvGPx any more in Lvp53-silenced shrimp, but increased shrimp mortalities. These results indicated the involvement of Lvp53, LvMnSOD and LvGPx in mediating cell stress caused by suboptimal pH and elevated levels of Cd in *L. vannamei*, and that the expressions of LvMnSOD and LvGPx were positively regulated by Lvp53, which is a potential mechanism for shrimp to survive the oxidative stress that occurs during short-term exposure to Cd or challenge with acidic pH. This finding will contribute to better understanding of p53 signaling pathways and redox regulation in invertebrate organisms.

1. Introduction

Environmental toxicants can cause a variety of physiological and behavioral responses in aquatic animals, even if the toxicity is not at a lethal level (Ikuta and Kitamura, 1995; Ikuta et al., 2003; Vuorinen et al., 2003). Generally, when organisms are exposed to a specific environmental stressor or simultaneously to multiple stressors, a range of defense mechanisms in cells such as regulation of oxidative stress, detoxification of xenobiotics, management of denatured molecules, regulations of the cell cycle, DNA damage, if the DNA repair fails, may be activated (Farcy et al., 2008). Usually, the initiation of these response mechanisms needs the upregulation of the involved biomolecules in cells.

The tumor suppressor gene p53 has been well characterized in various species such as *Homo sapiens* (Levine, 1997), *Oncorhynchus mykiss* (Caron de Fromentel et al., 1992), *Drosophila melanogaster* (Jin et al., 2000), *Bombyx mori* (Huang et al., 2011), *Spodoptera frugiperda* (Huang et al., 2011), and *Tigriopus japonicus* (Hwang et al., 2010); is a member of the cell cycle regulation machinery, playing pivotal roles in apoptosis (programmed cell death). In response to cellular stresses including DNA damage, hypoxia, and nucleotide deprivation, both the levels and the activities of p53 are induced (Lakin and Jackson, 1999). The most important biological function of the phosphorylation-activated p53 under stressful conditions is the induction of cell cycle arrest and apoptotic cell death (Ohtsubo et al., 2000; Koumenis et al., 2001; Hong et al., 2006; Rani et al., 2008). Specifically, if DNA is

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damaged in cells, the p53 protein puts a brake on the cell cycle; cellular repair systems are then activated, to remove the damaged cells by apoptosis (Bitomsky and Hofmann, 2009). Previous studies have indicated that p53 was closely related to the impacts of the endocrine disruption chemicals (EDCs), the genotoxic chemicals, and the environmental chemicals on ecosystem biota and human health (Bhaskaran et al., 1999; Abu-Qare and Abou-Donia, 2001; Yang et al., 2006; Hwang et al., 2010). However, limited information exists about the responses of p53 under acute environmental stresses.

In aquatic ecosystem, both pH variations and Cd pollutions are common phenomena in recent years. There is a growing awareness that acid or Cd exposure can induce oxidative stress in aquatic organisms. Reactive oxygen species (ROS) are continuously generated by aerobic metabolism or oxidative stress and can damage important biomolecules, such as DNA, proteins, and lipids (Stohs and Bagchi, 1995; Halliwell and Gutteridge, 1999; Liu et al., 2007; Chang et al., 2009; Zhang et al., 2009). In cells, oxidative stress is controlled by multiple, interacting, low and high molecular weight components, such as ferritin, catalase (CAT; EC 1.11.1.6), cytosolic manganese superoxide dismutase (cMnSOD; EC 1.15.1.1), glutathione peroxidase (GPx; EC 1.11.1.9), glutathione *S*-transferase (Mu-GST; EC 2.5.1.18), thioredoxin (Trx; EC 1.11.1.15), heat shock proteins (HSPs) (Zhou et al., 2008; Wang et al., 2009; Zhou et al., 2009; Qian et al., 2012); among them, SOD, GPx, and CAT play a central role (Spector, 2000); SOD first catalyzes the dismutation of superoxide radicals to H_2O_2, which is further metabolized to H_2O and O_2 by CAT and GPx. Past research has shown that expression levels of MnSOD and GPx were increased after expression of p53 was induced (Hussain et al., 2004). In addition, ROS can induce p53 to cross-talk with MnSOD to regulate oxidative stress and mitochondrial DNA repair in organelles (Bakthavatchalu et al., 2009; Pani et al., 2009). The above observations indicate that p53 may extend its protective functions by participating in antioxidant defense. The mRNA expression of MnSOD and GPx is transactivated by p53 in several vertebrate species including human, mouse, and tilapia (Tan et al., 1999; Hussain et al., 2004; Sablina et al., 2005; Mai et al., 2010). In other species, the antioxidant enzyme levels or activities have been shown to be useful as biomarkers of exposure to pollutants in aquatic environment. However, there is a paucity of data regarding the mechanism on how the enzymes were increased or initiated in response to acute environmental stresses.

The Pacific white shrimp, *Litopenaeus vannamei*, has become one of the important species in Chinese aquaculture in recent years and was chosen as the subject species to investigate the molecular mechanisms of cell stress response in the present study. The main purposes of this study were: (1) to obtain the full length cDNA sequence of Lvp53; (2) to clarify the mRNA expression profiles of Lvp53 in different tissues of *L. vannamei*; and (3) to investigate the potential relationships of Lvp53, LvMnSOD, and LvGPx in relieving cell stresses. Results obtained from this study would provide a potential molecular mechanism for shrimp's survival from oxidative stress caused by either suboptimal pH or elevated levels of Cd.

2. Materials and methods

2.1. Shrimp sampling and maintenance

Twenty shrimp (*L. vannamei*) obtained from a local commercial farm (20–26 g and 13–16 cm), were used for cDNA cloning and to obtain the tissues used for RNA extraction, and *L. vannamei* juveniles (4.5–6.4 cm) obtained from the same farm were selected for stress treatment and dsRNA injection experiments. Before the experiments, shrimp were acclimated to the laboratory conditions for 7 days in tanks with aerated seawater under the same conditions specified in Qian et al. (2012). During acclimation, shrimp were fed twice daily with commercial feed, and water was changed every day (Buikema et al., 1982). After screening for viral contamination and molting status, 6 White Spot Syndrome Virus (WSSV) and Taura Syndrome Virus (TSV)-free shrimp (3 females and 3 males) in intermolt stage were finally selected for cDNA cloning and the 11 target tissues (hepatopancreas, heart, gill, stomach, intestine, muscle, testis, ovary, epidermis, eyestalk, and swimming leg) were collected and used immediately for RNA extraction.

2.2. Acute pH challenge

After acclimation, 100 *L. vannamei* juveniles were placed into each of three plastic aquaria (water volume, 100 L) which contained test solutions of pH 6.1, pH 8.1, and pH 10.1, respectively. pH values were set with 1.0 M HCl or 1.0 M NaOH and were monitored during the whole experiment with an Orion 320 pH meter. Hepatopancreas of 6 shrimp from seawater (pH 8.1) was collected and used as the control before challenge, and hepatopancreas of 6 shrimp from pH 6.1, pH 8.1, and pH 10.1 was collected at 3, 6, 12, 24 and 48 h post challenge separately. All collected hepatopancreas samples were used immediately for RNA isolation. The cumulative mortality was recorded each 12 h.

2.3. Acute Cd exposure

After acclimation, 100 *L. vannamei* juveniles were placed in each of the 5 plastic containers (water volume, 200 L) which contained 5 test solutions of Cd 3.9 nM (seawater), 1.1 μM Cd, 2.2 μM Cd, 4.4 μM Cd, and 6.6 μM Cd, which were chosen from the results of previous studies (Frias-Espericueta et al., 2001; Castillo, 2004). The concentration of Cd in the seawater was measured with the conventional methods (APHA-AWWA-WPCF, 1999), and the remaining concentrations of Cd were nominal in the present study. The test solutions were prepared by regulating seawater with proper volume of the Cd stock solution which was 100 times of the 96-h LC50 obtained with water-soluble salts $CdCl_2 \cdot 2.5H_2O$ dissolved in Milli-Q deionized water. All values reported here stand for the total concentrations of Cd. Each test solution was renewed daily according to a static renewal method for toxicity tests (Buikema et al., 1982). Hepatopancreas of 6 shrimp from each container was collected at 3, 6, 12, 24, 48, 72, and 96 h post exposure separately. Before exposure, hepatopancreas of 6 shrimp from seawater (Cd 3.9 nm) was collected as 0 h samples and served as the control. All the samples were used immediately for RNA isolation. The cumulative mortality was recorded each 12 h.

2.4. RNA isolation and reverse transcription

Total RNA was extracted from all the samples using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's protocol. RNA integrity was assessed by electrophoresis on 1.2% agarose gel and its purity was verified by measuring the absorbance at 260 and 280 nm using ND-2000 spectrophotometer (NanoDrop Technologies, USA). Prior to reverse transcription, residual genomic DNA was removed by DNase I (TaKaRa, Japan). For sequence cloning, cDNA template was prepared from 3 μg of total RNA with M-MLV reverse transcriptase (Invitrogen) and oligo$(dT)_{18}$ primer, while for qRT-PCR, cDNA for each sample was synthesized from equal amount of total RNA (500 ng) with PrimeScript™ RT reagent kit (TaKaRa, Japan). All the cDNAs obtained as described above were stored at −20 °C until used as templates for PCR reactions.

2.5. Cloning of Lvp53 full length cDNA

The full-length cDNA sequence of Lvp53 was obtained in three consecutive steps by reverse transcriptase-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) strategies from the gill tissue of *L. vannamei*. A cDNA fragment of Lvp53 was firstly obtained by PCR using a pair of primers (Table 1) designed based on the *Marsupenaeus japonicus* p53 cDNA sequence (GenBank accession No.: AB559569.1). The purified products were cloned into the pMD18-T vector (TaKaRa, Japan) and sequenced by GenScript Corporation (Nanjing, China) using ABI 3730 automated DNA sequencer (BigDye Terminator