Comparative Biochemistry and Physiology, Part B 159 (2011) 244–251

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Expression analysis and response of *Penaeus monodon* 14-3-3 genes to salinity stress

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ARTICLE INFO

Article history:
Received 22 February 2011
Received in revised form 12 May 2011
Accepted 12 May 2011
Available online 23 May 2011

Keywords:
14-3-3
Osmoregulation
ATPase
Salinity stress
Shrimp
Penaeus monodon

ABSTRACT

Two isoforms of 14-3-3 protein, namely 14-3-3A and 14-3-3B, from the shrimp *Penaeus monodon* were investigated for their potential role in adaptation to salinity stress. Transcripts of 14-3-3A were found in various shrimp tissues whilst expression of 14-3-3B transcripts was more specific being observed in the osmoregulatory tissues, that is in the gills and epipodites. In shrimp gills, the 14-3-3A transcript levels slightly changed after transfer of the shrimp from 3 to 25 or to 40 ppt. In contrast, significant change in the mRNA levels in response to salinity stress was detected for 14-3-3B, where a significant decrease of 14-3-3B transcript was observed in gills of shrimp transferred from hypo-osmotic (3 ppt) salinity to iso-osmotic or hyper-osmotic (25 and 40 ppt, respectively) salinity. On the other hand, shrimp transferred from 40 ppt to 3 ppt showed a strong induction of 14-3-3B mRNA expression in the gills. These transcript expression analyses suggest that 14-3-3B is likely to be involved in the hyper-osmotic regulation in *P. monodon*. In addition, 14-3-3B appeared to regulate ATPase function since suppression of the gene by RNA interference resulted in a significant decrease in the total ATPase activity.

1. Introduction

The black tiger prawn, *Penaeus monodon*, is a euryhaline species that is capable of adaptation to a wide range of environmental salinity levels. It exhibits hypo- and hyper-osmotic regulation to seawater at salinities below and above the iso-osmotic concentrations (24–26 ppt salinity), respectively (Castille and Lawrence, 1981). The ability to resist dramatic changes in environmental salinities is believed to depend on the osmoregulatory mechanisms. These mechanisms are important for the natural life cycle of shrimps and also shrimp farming since salinity stress affects the growth, survival and development of shrimps (Chanratchakool, 2003; Pan et al., 2005; Silva et al., 2010). Penaeids begin their life cycle as planktonic larvae in coastal waters, enter estuarine nursery grounds as postlarvae before going back to oceanic conditions as adults (Wickins, 1976).

Osmoregulation mechanisms include the regulation of the shrimp body fluid composition with regard to the ambient medium by modifying the surface permeability, urine production and ion transport (Ferraris et al., 1987; Spanings-Pierrot et al., 2000; Buranajitpirom et al., 2010). In euryhaline crustaceans, many organs are involved in osmoregulation, including the gills, epipodites, antennal gland, part of the intestine and hepatopancreas. The majority of experimental evidence suggests that the gills serve as the primary site of osmoregulatory ion transport (Mantel and Farmer, 1983; Péqueux et al., 1989; Taylor and Taylor, 1992; Postel et al., 2000; Lucu and Towle, 2003; Freire et al., 2008).

Adaptations to varying salinity by crustaceans are regulated through transport mechanisms including Na^+/K^+-ATPase, $V(H^+)$-ATPase, HCO_3^--ATPase, K^+ and Cl^- channels, Na^+ channels, Cl^-/HCO_3^- exchangers, $Na^+/K^+/2Cl^-$ co-transporter, Ca^{2+}-pumps, Na^+/Ca^{2+} exchangers and carbonic anhydrase (Mantel and Farmer, 1983; Henry 1984; Péqueux et al., 1989; Böttcher et al., 1990; Péqueux, 1995; Henry and Watts, 2001; Freire et al., 2008). However, although 14-3-3 proteins, which participate in a wide variety of signal transduction processes, have been reported to play an important role in salinity adaptation this has mostly been studied in plants (Chen et al., 2006; Xu and Shi, 2006; Xu and Shi, 2007; Wang et al., 2008). The 14-3-3 proteins are abundant acidic proteins with a molecular mass of around 30 kDa and are expressed in a wide range of organisms and tissues (Aitken, 2006). In humans there are several isoforms of the 14-3-3 gene, denoted as the β, γ, ε, η, σ, τ (θ) and ζ isoforms, while yeast and plants contain 2 to 15 genes. 14-3-3 proteins sequester other proteins that are phosphorylated on serine (and sometimes threonine) residues and can interact with many different proteins and play a role in diverse functions, such as signaling, cell growth, division, adhesion, differentiation, apoptosis, and ion-channel regulation (Aitken, 2006; Gardino et al., 2006; Fiol and Kultz, 2007).

Several studies have reported the involvement of 14-3-3 proteins in the responses of higher plants to salt stress. 14-3-3 proteins are regulators of the plant plasma membrane H^+-ATPase

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doi:10.1016/j.cbpb.2011.05.004

and K^+ channels that play a role in salt stress (Baunsgaard et al., 1998; Morsomme and Boutry, 2000; Ve´ry and Sentenac, 2003), and also interact with ascorbate peroxidase (Zhang et al., 1997; Badawi et al., 2004) and the phytohormone abscisic acid (ABA) signaling pathways (Schultz et al., 1998; Jia et al., 2002; Schoonheim et al., 2007), which play an important role in plant adaptation to salt stress. Additionally, heterologous overexpression of a tomato 14-3-3 protein in *Arabidopsis thaliana* improved the plant's salt tolerance (Xu and Shi, 2007). In mammals, the 14-3-3 proteins are known to regulate several ion channels (Kagan et al., 2002; Allouis et al., 2006; Czirjak et al., 2008), and are also an essential part of the signaling network regulating Na^+/K^+-ATPase activity and endocytosis in response to G protein-coupled receptor ligands at the plasma membrane. The functional relevance of this interaction was observed in opossum kidney cells stably expressing the rodent Na^+/K^+-ATPase α1-subunit and subsequently transfected with plasmids encoding for either the wild type 14-3-3 or a deletion mutant (Efendiev et al., 2005).

However, the role(s) of 14-3-3 proteins in ion homeostasis in aquatic animals has not been widely studied. There is some evidence to suggest the involvement of the 14-3-3 proteins in salinity adaptation of aquatic animals, for example, the observation of a potential binding site of 14-3-3 protein in the N terminus of Na^+/K^+-ATPase α -subunit of the shore crab, *Pachygrapsus marmoratus*, the sea urchin, *Strongylocentrotus purpuratus*, and the zebra fish, *Danio rerio*, (Jayasundara et al., 2007) as well as the increased 14-3-3 mRNA and protein levels in the gills of the euryhaline teleost fish, *Fundulus heteroclitus*, upon transfer from sea water to fresh water (Kültz et al., 2001).

In the present study, we identified two isoforms of 14-3-3 proteins from the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th) (Tassanakajon et al., 2006) and investigated their potential role in salinity adaptation by examining the mRNA expression in various shrimp tissues and in response to hypo-osmotic (3 ppt salinity) or hyper-osmotic (40 ppt salinity) stress. Furthermore, RNA interference mediated gene silencing was performed to examine the effect of 14-3-3 gene knockdown on the ATPase activity. The results revealed the likely function of 14-3-3 protein in controlling osmoregulation in *P. monodon* shrimp.

2. Materials and methods

2.1. Animal and sample preparation

Shrimp samples were purchased from local farms and were randomly chosen for use in the study without determining their sex. Sub-adult intermolt (20 g body mass) *Penaeus monodon* were used for mRNA expression analysis while juvenile shrimp (5 g body mass) were used for *in vivo* gene silencing experiments. Low (hypo-osmotic) and high (hyper-osmotic) salinity stressed shrimp were reared in 3 or 40 ppt salinity, respectively, compared to the iso-osmotic (25 ppt salinity) conditions that all shrimp were initially reared in for two weeks in laboratory tanks. Shrimp were acclimated at 28 ± 1 °C. They were fed with commercial shrimp pellets (CP, Bangkok, Thailand). Not all groups of shrimp that were subjected to the low salinity stress showed detectable levels of 14-3-3B mRNA by RT-PCR, which is in contrast to that for 14-3-3A mRNA levels, which were detected in all individual shrimp tested. Therefore, 10 randomly selected shrimp from each group were acclimatized at 3 ppt salinity for 1 day and then examined for the presence or absence of 14-3-3B mRNA in shrimp gills by RT-PCR as described in 2.3.

2.2. Sample collection and total RNA preparation

Gills, epipodites, antennal gland, heart, hemocytes, hepatopancreas, intestine, lymphoid organ and eyestalk were each isolated separately from an individual shrimp and immediately frozen in liquid nitrogen (−176 °C) in order to preserve the intact tissue. Total RNA was isolated from the collected tissues using TRI REAGENT (Molecular Research Center, Cincinnati, Ohio, USA) and then treated with DNase I (Promega, Sunnyvale, CA, USA) following the manufacturer's protocol. Genomic DNA contamination in total RNA preparations was determined by subjecting RNA samples to 35 cycles of PCR amplification with β-actin primers (Table 1). There was no PCR product from the DNase I treated RNA samples.

2.3. Semi-quantification of 14-3-3 mRNA expression by RT-PCR

Semi-quantitative RT-PCR was used to examine mRNA expression levels of the 14-3-3A and 14-3-3B isoforms in the different tissues and in response to salinity stress of *P. monodon*. Portions of the total RNA extract served as the template for cDNA synthesis using the ImPromp-II Reverse Transcription System (Promega, Sunnyvale, CA, USA). Single-strand cDNA was synthesized from 1 μg DNase-treated total RNA in a final volume of 20 μl. The cDNA then served as the template for the second stage PCR assay using β-actin as the positive control or as the internal standard. Beta-actin was chosen as the internal standard for semi-quantitative analysis of the study, since β-actin was the most accurate housekeeping gene under salinity stress as compared to elongation factor 1α and 18S ribosomal protein. Based on microarray data, the expression ratio between salinity stressed (3 ppt or 40 ppt) and control (25 ppt) shrimp of β-actin gene was about 1.2 or 1.02, respectively (Tassanakajon et al., unpublished data). The specific primers for 14-3-3A and 14-3-3B (Table 1) were designed from the cDNA sequences. Amplification reactions were performed in a 25 μl total volume containing 1 μl cDNA sample, 1X PCR buffer, 0.2 μM each dNTP, 0.8 μM each primer and 0.25 units *Taq* DNA polymerase (Fermentas, Burlington, Ontario, CA). The cycle profile was 95 °C for 2 min, followed by 25 or 35 cycles of 95 °C for 30 s,

Table 1
Primer sequences, annealing temperatures and cycle numbers for semi-quantitative RT-PCR and dsRNA interference.

Gene locus	Primer name[a]	Primer sequence (shown as 5' to 3')[b]	T_A (°C)[c]	Cycle[d]	Size (bp)[e]
14-3-3A	14-3-3AF	CCACAAGGGCGGGATTTA	57	25	267
	14-3-3AR	CTGGGTGCTCTGCCTATT			
14-3-3B	14-3-3BF	CGAAGAACTGCCTGGAGAGCCCTCAGC	62	35	402
	14-3-3BR	GCAACTCCGGGGTCCTCATTGACT			
β-actin	β-actinF	GCTTGCTGATCCACATCTGCT	55	25	317
	β-actinR	ACTACCATCGGCAACGAGA			
14-3-3B	14-3-3BFT7	[T7]CGAAGAACTGCCTGGAGAG	–	–	–
	14-3-3BRT7	[T7]AACTCCGGGGTCCTCATTGACT	–	–	–

[a] F = forward primer, R = reverse primer.
[b] T7 = T7 promoter sequence (5'-GGATCCTAATACGACTCACTATAGG-3') at the 5' end of the shown primer sequence.
[c] TA = annealing temperature used in the PCR amplification.
[d] Cycle = the number of thermocycles used in the PCR amplification.
[e] Size = the expected amplicon size in base pairs (bp).