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Ontogeny and expression analysis of tube (interleukin-1 receptor-associated kinase-4 homolog) from *Penaeus monodon* in response to white spot syndrome virus infection and on exposure to ligands

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Tube, an IRAK- 4 (Interleukin-1 receptor-associated kinase-4) homolog, is a key component in the Toll signalling pathway that has diverse role in the innate immunity of organisms including the ontogenic development. In the present study, Tube from *Penaeus monodon* (*Pm*IRAK-4) was studied in response to infection with white spot syndrome virus (WSSV) and on exposure to various ligands. The ontogenic expression pattern of *Pm*IRAK-4 in different developmental stages of *P. monodon* showed that the gene was constitutively expressed in all the stages tested with the maximum expression detected in egg. Immune-modulation of *Pm*IRAK-4 in response to WSSV was studied in post-larvae, juveniles and adult *P. monodon in vivo*, and in primary haemocyte cultures at different time-points post-infection *in vitro*. *Pm*IRAK-4 displayed significant up-regulation in haemocytes, gill, lymphoid organ and stomach at all time-points *in vivo* as well as in primary haemocyte cultures *in vitro*. To understand the post-injection stress on the immune-modulation, we have compared the expression level of *Pm*IRAK-4 using zero hour (un-injected) and phosphate buffered saline (PBS)-injected controls, wherein the trend in expression was found to be similar. Following *in vitro* stimulation with ligands such as lipopolysaccharide and peptidoglycan, significant up-regulation of the gene could be observed at all time-points. However, poly I:C induction resulted in down-regulation of the same at early time-points. The ubiquitous expression of *Pm*IRAK-4 in different larval and post-larval stages implies the involvement of the gene in defense mechanism during early developmental stages of *P. monodon*. Further, the modulation of expression of *Pm*IRAK-4 in response to WSSV and different ligands indicates its possible role in immune responses in shrimp.

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1. Introduction

In recent years, extensive studies have been focused on the innate immune system of economically important aquatic invertebrates due to the occurrence of severe disease problems in aquaculture (Li et al., 2010). Although invertebrates lack antibodies and complements, they can initiate a rapid and effective response against intruding pathogens through an innate immune response (Uematsu et al., 2008). Toll-like receptors (TLRs), the extensively-studied group of pattern recognition receptors (PRRs), play a critical role in the initiation of innate immunity through the recognition of pathogen and damage-associated molecular patterns (PAMPs and DAMPs, respectively) (Moossavi, 2014).

Interleukin-1 receptor-associated kinases (IRAKs) are important mediators in the signal transduction of TLR family members (Janssens and Beyaert, 2003), modulating the functions of both innate and adaptive immunity (Lye et al., 2008; Suzuki et al., 2003). They are members of the serine/threonine protein kinase family and can be recruited to the TLR complex to further mediate downstream signalling (Ge et al., 2011). Among the four members of IRAK family (IRAK-1, 2, M, and 4), IRAK-4 is the last IRAK family member identified (Gottipati et al., 2008; Li et al., 2002). Signal transduction by TLRs, which signal *via* TIR (Toll-IL-1R) domains, is entirely or in part dependent upon IRAK-4 (Takeda and Akira, 2004). In the TLR signalling pathway, all TLR except TLR3 can bind with their ligands and recruit the adaptor molecule MyD88 through the TIR domain, mediating the MyD88-dependent pathway. MyD88 then recruits IRAK-4, which phosphorylates IRAK-1. The phosphorylated

Abbreviation: ANOVA, analysis of variance; BLAST, basic local alignment search tool; DAMPS, damage-associated molecular patterns; DD, death domain; Dpi, days post infection; EFα, elongation factor α; *Es*Tube, *Eriocheir sinensis* tube; hpi, hour post-infection; IRAK-4, Interleukin-1 receptor-associated kinase-4; L-15, Leibovitz 15 medium; LPS, lipopolysaccharide; MEGA, Molecular Evolutionary Genetics Analysis; NCBI, National Center for Biotechnology Information; PAMPs, pathogen associated molecular patterns; PBS, phosphate buffered saline; PGN, peptidoglycan; PL, post-larvae; Poly I:C, polyinosinic:polycytidylic acid; PRRs, pathogen recognition receptors; SEM, standard error of the mean; SPSS, statistical package for social sciences; TLRs, Toll-like receptors; WSSV, white spot syndrome virus; YHV, yellow head virus.

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IRAK-1 gets associated with tumor necrosis factor receptor-associated factor 6 (TRAF6) and subsequently results in the activation of transcription factors, such as NF-kB, that regulates immune-modulatory genes, including pro-inflammatory cytokines (Phelan et al., 2005). Based on the mutagenesis studies, it was demonstrated that the active kinase IRAK-4 is indispensable for the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Li et al., 2002). Studies on mice and clinical observations of IRAK-4 deficient patients showed that the absence/deficiency of IRAK-4 results in severe immune deficiency and susceptibility to infection (Maglione et al., 2014; Bela et al., 2012; Medvedev et al., 2003; Suzuki et al., 2002a).

Tube is an IRAK-4 homolog in Toll-pathway that plays a key role in the development and immunity in Drosophila (Towb et al., 2009). It has been described in different shellfish such as, *Litopenaeus vannamei* (Li et al., 2014a, 2014b; Y.W. Li et al., 2014), *Scylla paramamosain* (Li et al., 2013a), *Haliotis diversicolor* (Ge et al., 2011), *H. rufescens* (Valenzuela-Munoz and Gallardo-Escarate, 2014) and *Eriocheir sinensis* (Yu et al., 2014) and immune-modulation of this gene was reported on exposure to different pathogens and ligands (Umasuthan et al., 2015; Li et al., 2014a, 2014b; Y.W. Li et al., 2014; Yu et al., 2014; Li et al., 2013a; Ge et al., 2011; Mateo et al., 2010; Wiens et al., 2007; Li et al., 2002). An IRAK-4 homolog from *Penaeus monodon* has been previously reported by Watthanasurorot et al. (2012), and its role in gut immunity of the animal after bacterial challenge was studied. Recently, we have demonstrated that Toll-pathway in *P. monodon* responds to WSSV infection (Deepika et al., 2014a), by studying the responses of the adaptor molecule MyD88 and the downstream signalling molecule TRAF6.

Penaeid shrimp is an economically important species for the aquaculture industry. However, production of shrimp larvae is often hindered by considerable mortality rates due to disease outbreaks (Jiravanichpaisal et al., 2007). It is assumed that, the early developmental stages of animals are more susceptible to pathogens than adults (Aguirre and Ascencio, 2000; Ausvet, 1997; Momoyama and Sano, 1989) and their susceptibility to infectious diseases could be related to immune-competence during the early larval stages (Jiravanichpaisal et al., 2007). Therefore, a better understanding of penaeid shrimp immunity during ontogeny would help in designing efficient strategies for disease control and ensure long-term viability of shrimp aquaculture (Bachere, 2000). The role played by IRAK-4 in the early developmental stages of many animals has been reported (Yu et al., 2012; Ge et al., 2011; Phelan et al., 2005; Muller-Holtkamp et al., 1985). However, nothing is known about its role in the ontogeny of any crustacean.

Against this background, we have analysed the ontogenic expression pattern of tube (*Pm*IRAK-4) in different developmental stages of *P. monodon* such as egg, nauplii 1–3, zoea 1–3, and mysis 1–3, and post-larval (PL) stages 1–5, 7, 10, 14 and 18. Further, we have attempted to determine the role of the *Pm*IRAK-4 in response to white spot syndrome virus infection in larvae, juveniles and adult *P. monodon in vivo* through different modes of infection (injection and immersion) and on exposure to different pathogen-mimicking ligands as well as WSSV *in vitro*.

2. Materials and methods

2.1. Animals

Live, healthy adult *P. monodon* weighing 25–33 g, were collected from Shakthi Aqua Farm Pvt. Ltd. Mumbai, Maharashtra, India and used for viral challenge studies. To study the effect of PBS injection, a separate experiment was conducted using healthy adults of similar size (weighing 25–30 g) procured from a local farm. In both the experiments, the animals were acclimatised in 15‰ seawater, and fed daily with a commercial diet at 5% of their body weight twice a day for 7 days before the experiments. Early developmental stages of shrimp (egg, nauplii, zoea, mysis and post-larvae) were acquired from Vasavi Hatchery, Kakinada (India), and transported to the laboratory in RNA*later*® (Qiagen, USA).The immersion-challenge experiment, using post-larvae at PL18 stage and juvenile weighing 3 g size, was carried out at Kakinada centre of Central Institute of Fisheries Education.

2.2. WSSV inoculum preparation

WSSV inoculum was prepared from gill and pleopod tissue samples of WSSV-infected *P. monodon* collected from field outbreaks and stored at −80 °C, using the procedure described by Rajendran et al. (1999). Briefly, the tissue samples were homogenised in sterile chilled phosphate buffered saline (PBS), the homogenate was clarified by centrifugation at 3000*g* for 20 min at 4 °C and the supernatant was then filtered through a 0.22 μm membrane syringe filter. For *in-vitro* experiments, WSSV inoculum was prepared in cold 2× L-15 cell culture medium instead of PBS (Jose et al., 2010). The homogenate was clarified by centrifugation at 3000*g* for 20 min at 4 °C and the supernatant was then filtered through a 0.22 μm membrane syringe filter. The virulence of the WSSV inoculum was confirmed by injecting 100 μL of the inoculum in healthy shrimp. For the immersion challenge, the tissue homogenate was directly used without filtration.

2.3. Challenge experiments

2.3.1. Immersion challenge of P. monodon post-larvae (PL) and juveniles with WSSV

For the larval challenge, 1 mL of WSSV inoculum (5.7×10^5 copies/mg tissue) was added to 1 L sterile seawater (15‰) containing 250 number of post-larvae (PL18 stage). After 3 h of exposure, they were transferred to fresh seawater having the same salinity. Three PL were sampled and pooled at 2, 6, 12, 24, 48, 72 h and 5 days post-immersion challenge. Three such pools were used as sample replicates. Post-larvae not exposed to WSSV inoculum were used as control. The experiment with *P. monodon* juveniles was carried out by exposing 30 animals (average weight-3 g) to 2.5 mL WSSV inoculum in 3 L 15‰ seawater with continuous aeration. Control animals in both the cases were treated with same amount of PBS. After 3 h exposure, the animals were transferred to separate tanks containing seawater with the same salinity. Tissues including gill, stomach, hepatopancreas, midgut and hindgut were collected at 2, 6, 12, 24, 48, 72 h and 5 days post-immersion challenge. Three replicates were used for each time-point.

2.3.2. In-vivo challenge with white spot syndrome virus by injection in adults

Healthy, adult *P. monodon* were injected intramuscularly in the third abdominal segment with 100 μL of WSSV inoculum (1.3×10^3 copies/μL). The animals in the control group were injected with 100 μL of PBS. Three animals were sampled at each time-point (2, 6, 12, 24, 48 and 72 h post-injection). Following the procedure described by Deepika et al. (2014a), haemolymph was drawn from heart using a 24 gauge needle containing equal volume of anticoagulant (10% tri-sodium citrate) and haemocytes were collected by centrifugation at 800*g* for 2 min at 4 °C. Different tissues such as gill, lymphoid organ, stomach, hepatopancreas, midgut and hindgut were collected from each challenged animal. Similarly, tissues were collected from control animals.

2.4. RNA extraction and cDNA synthesis

For the gene expression study, total RNA was extracted from eggs, different larval stages (nauplii, zoea, mysis and PL), haemocytes and other target tissues of *P. monodon* using Trizol® reagent (Invitrogen, USA) and treated with DNAse 1, RNAse-free (Thermo Scientific, USA). The RNA was quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). One microgram of total DNase-treated RNA was reverse transcribed into first-strand cDNA in a total reaction volume of 20 μL using RevertAid™ First Strand cDNA Synthesis kit (Thermo

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