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Identification of the thioredoxin-related protein of 14 kDa (TRP14) from *Litopenaeus vannamei* and its role in immunity

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ABSTRACT

The thioredoxin system plays essential roles in maintenance and regulation of the redox state of cysteine residues in cellular proteins. The thioredoxin-related protein of 14 kDa (TRP14) is an important member of the TRX superfamily which acts on various substrate proteins, some of which are not overlapped with those of thioredoxin. The knowledge on the function of TRP14 in invertebrates is limited to date. In this study, a TRP14 gene was identified from Pacific white shrimp *Litopenaeus vannamei* (LvTRP14) and its role in immune responses was investigated. We demonstrated that the expression level of LvTRP14 was high in hepatopancreas and intestine, low in eyestalk, and medium in other tissues of healthy shrimp. The transcription of LvTRP14 *in vivo* was significantly down-regulated in Relish-silencing shrimp but up-regulated in STAT-silencing shrimp, indicating a complex regulation of LvTRP14 expression. Although the LvTRP14 expression showed little change after immune stimulation with different type of pathogens, knockdown of LvTRP14 expression using RNAi strategy could significantly facilitate the infection of white spot syndrome virus (WSSV) and *Vibrio parahaemolyticus* in shrimp. Dual luciferase reporter assays demonstrated that LvTRP14 enhanced the transcription factor activity of Relish but attenuated that of Dorsal. Furthermore, silencing of LvTRP14 *in vivo* had opposite effects on expression of different type of antimicrobial peptides. These suggested that LvTRP14 could play a complex role in shrimp immunity.

1. Introduction

In cells, the structure and function of most proteins depend on the reversible reduction/oxidation (redox) state of cysteine residues, which can be modified by reactive oxygen-, nitrogen- and sulfur species (ROS, RNS, RSS) [1,2]. The thioredoxin (TRX) superfamily, a group of evolutionarily conserved small proteins characterized by abilities of their reduced forms to recognize and reduce proteins containing disulfides, plays essential roles in maintenance of the intracellular redox state [3,4]. The TRX system consists of TRX isoforms, NAPDH, TRX reductase (TRXR), and their cellular substrates [4,5]. With NAPDH as the electron donor, TRXR transfers electrons to TRXs, which further catalyze the reduction of disulfide bonds in a number of TRX-dependent enzymes and proteins [6]. At present, three Trx isoforms, TRX1, TRX2 and TRX3, have been characterized in human, all of which contain a conserved Cys-XX-Cys (CXXC) motif (also known as the WCGPC motif) in the redox-active site [7,8]. The thioredoxin-related protein of 14 kDa (TRP14), also called the thioredoxin domain containing 17 protein (TXNDC17), is an evolutionarily well-conserved member of the TRX superfamily [9]. Since first cloned from human Hela cells, TRP14 gene has been widely identified in bacteria, archaea, and eukaryotes. It possesses a conserved CPDC motif and shares similar disulfide reductase activities with TRX1 [10]. However, TRP14 exhibits a different crystal structure in the vicinity of the redox-active site from that of TRX and recognizes a distinctive group of substrate proteins [11].

In mammals, members of the TRX superfamily are essentially involved in various biological processes, including cell growth, apoptosis and signal transduction [12–15]. Accumulating evidence has suggested that TRX1 is ubiquitously expressed in immune-related cells and implicated in cytokine secretion, antigen presenting and lymphocyte differentiation [16–19]. In recent years, more and more TRXs have also been identified and functionally studied in invertebrates, including

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insects and crustaceans [20–22]. The Pacific white shrimp *Litopenaeus vannamei* is a representative species of crustaceans and is now the major aquaculture shrimp in the world [23]. The TRX protein from *L. vannamei* (LvTRX) has been well studied [24]. Previous studies have suggested that LvTRX possesses antioxidant activity and is involved in virus infection [25,26]. The crystallographic structure of LvTRX has also been established using X-ray crystallographic analysis [27,28]. However, the knowledge on other members of the TRX superfamily in invertebrates is still limited. In this study, the TRP14 from *L. vannamei* (LvTRP14) was identified and its role in immunity was investigated, which could enrich the knowledge on crustacean immunity and the function of invertebrate TRX superfamily.

2. Materials and methods

2.1. Animals and pathogens

Pacific white shrimp (~10 g) were collected from an aquaculture farm in Zhanjiang, Guangdong Province and were randomly sampled to ensure to be free of white spot syndrome virus (WSSV) and *Vibrio parahaemolyticus* by PCR as previously described [29,30]. Shrimp were acclimated at ~28 °C for over 7 days in air-pumped seawater (2.0% salinity) in a recirculating water tank system before experiments. The stocks of *V. parahaemolyticus* and WSSV were prepared as previously described [31].

2.2. Cloning of LvTRP14 gene

The expressed sequence tag (EST) homologous to TRP14 was retrieved from a previously reported *L. vannamei* transcriptome library [32]. The full open reading frame (ORF) of LvTRP14 mRNA was amplified by rapid amplification of cDNA ends (RACE) using a SMARTer RACE cDNA Amplification kit (Clontech, Japan) and sequenced.

2.3. Bioinformatics analysis

The amino acid sequences of many TRX superfamily members were obtained from the National Center for Biotechnology Information (NCBI) databases. Sequence alignments were made by Clustal W 1.8 with parameters as previously described [33]. Phylogenetic tree was generated by the neighbor-joining (NJ) method using MEGA 5.0 software with a Poisson model and other parameters as follows: substitution type (amino acid), rates among sites (uniform rates), gap missing deletion treatment (complete deletion), and number of bootstrap replication (1000 replicates).

2.4. Real-time PCR

To detect the distribution of LvTRP14 mRNA, many tissues were sampled from *L. vannamei* as previously described [34]. For challenge experiments, shrimp were intramuscularly injected at the second abdominal segment with *Staphylococcus aureus* (10^6 CFU), *V. parahaemolyticus* (10^6 CFU), WSSV (10^6 copies) and PBS buffer (as control). The gills were sampled at 0, 4, 12, 24, 48, 72 and 96 h post injection. RNA extraction, cDNA synthesis and real-time PCR were performed following the strategy and methods as previously described [32]. The EF-1α gene (Genbank accession No. GU136229) was used as internal control. Sequences of primers used in this study were listed in Table 1.

2.5. Dual luciferase reporter assays

The coding sequence of LvTRP14 was cloned into pAc5.1/V5-His A plasmid (Invitrogen, USA) to generate pAc-TRP14. The pAc-Dorsal and pAc-Relish vectors and the firefly luciferase plasmids containing the promoters of microRNA-1959 and WSSV *ie1* gene were prepared as previously reported [35,36]. For the dual luciferase reporter assays, S2 cells were transfected using 0.2 μg reporter Firefly luciferase plasmid, 0.3 μg pAc5.1-LvTRP14 plus pAc5.1-Relish/pAc5.1-Dorsal vectors, and 0.02 μg pRL-TK renilla luciferase plasmid (as internal control) (Promega, USA). Cells were harvested at 48 h post transfection and lysed for examination of firefly and renilla luciferase activities using a Dual-Luciferase Reporter Assay System (Promega, USA). Three independent experiments were performed and all assays were performed in four independent transfections.

2.6. RNA interference

Double stranded RNAs (dsRNAs) specific to LvTRP14 (dsLvTRP14) and green fluorescent protein (dsGFP, as control) were prepared by *in vitro* transcription using a T7 RiboMAX™ Express RNAi System (Promega, USA) as previously described [34]. Shrimp were injected with 50 μl PBS containing 5 μg LvTRP14 and GFP dsRNA (as control). The RNA interference (RNAi) efficiency was determined using real-time PCR. At 48 h post injection, the dsRNA treated shrimp were further injected with 10^6 copies of *V. parahaemolyticus* and WSSV. Experiments were done in triplicate and the cumulative mortality was recorded every 4 h. At 48 h and 72 h post infection, the muscle tissue near the tail was sampled and the copy number of WSSV in muscle were determined using absolute quantitative real-time PCR as previously described [37]. To primarily explore the regulatory mechanism of LvTRP14 expression, shrimp were also injected with the dsRNAs specific to Dorsal, Relish, STAT and interferon regulatory factor (IRF) and sampled at 48 h post injection. The expression of LvTRP14 and other immune related genes were detected using real-time PCR. The primers used were listed in Table 1.

2.7. Statistical analysis

The statistical procedures were carried out using SPSS statistical software version 16.0. The mean and standard deviation (SD) from three detections was calculated. Student's t-test was used to compare the two means. The Kaplan-Meier plot (log-rank $\chi 2$ test) was used to analyze the mortalities between different groups.

3. Results

3.1. Cloning and bioinformatics analysis of LvTRP14

The ORF of LvTRP14 is 381 bp encoding a protein of 126 amino acids with a calculated molecular weight of 13.69 kDa and a theoretical isoelectric point of 4.87 (Fig. 1A). LvTRP14 was predicted to possess a typical thioredoxin domain covering the 6–125 residue region, which contained a conserved CPDC motif. Multiple-sequence alignment showed that LvTRP14 shared identities of 54.0% with TRP14 from crustacean *Daphnia pulex*, 49.2% with insect *Drosophila melanogaster*, 47.9% with zebrafish *Danio rerio*, and 46.3% and 48.8% with mammals *Mus musculus* and *Homo sapiens*, respectively. In the constructed phylogenetic tree, the analyzed TRP14 genes could be categorized into the clades of Vertebrate, Arachnoidea, Crustacea and Insecta, and LvTRP14 was clustered into the Crustacea category.

3.2. Tissue distribution and expression profile of LvTRP14

The distribution of LvTRP14 mRNA was analyzed using real-time PCR, which demonstrated that all the detected tissues expressed LvTRP14 (Fig. 2A). The lowest level of LvTRP14 expression was observed in eyestalk. The hepatopancreas and intestine expressed the highest levels of LvTRP14, which were 14.7- and 20.0-fold higher than that in the eyestalk, respectively. The expression of LvTRP14 in other tissues was 2.0- to 6.3-fold higher than that in eyestalk. Next, the regulatory mechanism of LvTRP14 expression was initially investigated (Fig. 2B). Compared with the control, silencing of Relish *in vivo*