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Short communication

LvCdc42 is a potential negative regulator of Lvp53 in *Litopenaeus vannamei* exposed to *Vibrio alginolyticus* stress

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ABSTRACT

As a crucial molecular switch, Cdc42 is a signal regulation hub which is involved in a wide range of cellular processes, including cytokinesis, gene expression, cell cycle progression and apoptosis. It has been reported that this GTPase promotes host defense against fatal infection and plays a vital role in the innate immunity system of mammals. But whether and how Cdc42 participates in innate immunity in invertebrates, such as the shrimp *Litopenaeus vannamei*, is still unknown. In this study, confocal microscopy analysis showed that LvCdc42 located in both cytoplasm and nucleus of S2 cells depended on its structure. The silencing LvCdc42 induced an increase in the expression of Lvp53 and Lvcaspase-3. When LvCdc42-silenced shrimps were stressed with *Vibrio alginolyticus*, the expression of Lvp53 and Lvcaspase-3 was markedly up-regulated. Moreover, the increase in the apoptosis rate in hemocytes and in cumulative mortality were in line with Lvp53 mRNA expression. These data suggest that the molecular switch LvCdc42 acts as a negative regulator of Lvp53 and participates in the apoptosis of hemocytes when *L. vannamei* is challenged with *V. alginolyticus*.

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1. Introduction

Cdc42 is a small GTPase which is a member of the Rho family; it acts as a molecular switch by cycling from an inactive GDP-bound state to the active GTP-bound state (Phillips et al., 2008). The physiological functions of Cdc42 in the mammalian immune response have been widely reported. In response to in vitro stimuli, Cdc42 transduces signals to regulate cytoskeletal changes, promote DNA synthesis in quiescent fibroblasts, and induce apoptosis in immune cells (Giesler, 2000). A primary apoptosis signaling pathway which is controlled by p53 is activated by numerous stresses. Previous studies have found that the expression of p53 has a close relationship with the genes encoding components of the Cdc42 pathway in mammals. Inactivation of Cdc42 can induce apoptosis through the p53, ERK and Akt pathways (Zugasti et al., 2001). Moreover, endogenous p53 is activated by Cdc42 deficiency and induces apoptosis in normal fibroblasts, but inhibiting Cdc42 activity does not lead to apoptosis in p53$^{-/-}$ cells (Lassus et al., 2000). These observations suggest that Cdc42 is implicated in the regulation of p53. Consequently, we seek to explore the connection between LvCdc42 and Lvp53 in the regulation of apoptosis in the innate immunity system of shrimp.

The shrimp *Litopenaeus vannamei* is a major cultivated member of the Penaeidae which exhibits innate immunity. The immune response of the innate immunity system includes immune recognition, signal transduction and production of effectors (Hultmark, 2003). Nevertheless, it is susceptible to diseases caused by pathogenic bacteria and viral infections, especially when grown in shrimp farms. *Vibrio alginolyticus*, a gram-negative bacterium, is a common pathogen in aquaculture environments (Moriarty, 1997). It is currently highly infective in aquaculture systems, causing tremendous damage to shellfish and crustaceans, especially prawns, leading to infections of the carapace and hepatopancreas (Hörmansdorfer et al., 2000). Understanding the shrimp immune response to infectious pathogens is essential for disease control.

2. Materials and methods

2.1. Animal

Litopenaeus vannamei specimens were obtained from an

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aquaculture farm located in Panyu, a suburb of Guangzhou City in China. Shrimp (3–4 g in weight) were selected and acclimated in seawater (10% salinity, 24–26 °C) for 7 days prior to the experiment.

2.2. Cloning LvCdc42 gene and bioinformatics analysis

The complete ORF of LvCdc42 was cloned based on Litopenaeus vannamei Cdc42 sequence published on Gene Bank (Accession KM278989). Using gene-specific primers (Supplementary Table 1), the ORF of LvCdc42 was obtained and cloned into pMD-18 T vector (TaKaRa, Japan). The sequence was confirmed by BGI.

Nuclear localization sequence was predicted using cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi). Multiple sequences alignment of LvCdc42 amino acid sequence was conducted with ClustalW program. A neighbor-joining phylogenic tree based on amino acid sequences was constructed using MEGA 5.1 program and its bootstrap sampling was reiterated 1000 times.

2.3. Cell culture, transient transfection and subcellular localization assay

Drosophila Schneider S2 cells (Invitrogen, USA) were seeded into 24-well plates and cultured in Schneider's Drosophila media (Gibco, USA) containing with 10% fetal bovine serum (Gibco, USA). The culture condition of incubator was set as follow: 28 °C, CO_2 free.

Recombination plasmid pAc5.1-LvCdc42-EGFP was constructed with LvCdc42 ORF and pAc5.1-EGFP vector using gene-specific primers (Supplementary Table 1). Then the recombination plasmid was mixed with Lipofectamine 3000 reagent (Invitrogen, USA) for 20 min. Subsequently, S2 cells were incubated with the mixture for 5 h and cultured in SDM for another 24 h. To confirm the subcellular localization of LvCdc42 in cells, nuclei were stained with DAPI (1 mg/ml). Lastly, the light emitted from nuclei and EGFP could be observed and captured under a laser scanning confocal microscopy (Zeiss, Germany).

2.4. DsRNA preparation and LvCdc42 silencing

Gene-specific primers with the T7 promoter sequence (Supplementary Table 1) were used to amplify a coding fragment of LvCdc42. After being purified, the LvCdc42 fragment with the T7 promoter sequence was used for synthesizing LvCcd42 dsRNA (dsLvCdc42) following the manufacturer's instructions for the T7 RiboMAX Express RNAi System (Promega, USA). Similarly, GFP dsRNA (dsGFP) was prepared using a pEGFP plasmid as template and specific T7 primers as described.

Healthy shrimp pretreated as described in section 2.1 were injected intramuscularly with equal amounts of dsLvCdc42 or dsGFP (2.5 μg/g shrimp) to assess their effect (Peng et al., 2015). At 0, 24, 48 and 72 h post injection, hepatopancreas from three random shrimp per group was sampled for total RNA isolation and cDNA preparation. RT-qPCR assay of LvCdc42 expression was performed with the above cDNA and gene-specific primers (Supplementary Table 1). Lv18 S rRNA (AF186250), was used as an internal control to calibrate the RT-qPCR results.

2.5. RT-qPCR analysis of Lvp53 and Lvcaspase-3 in LvCdc42-silenced shrimp

After 24 h following dsLvCdc42 injection, the expression of Lvp53 (KC422442.1) and Lvcaspase-3 (EU421939.1) was detected using cDNA prepared from hepatopancreas samples from LvCdc42-silenced shrimp by RT-qPCR. Gene-specific primers are listed in Supplementary Table 1.

2.6. V. alginolyticus challenge in LvCdc42-silenced shrimp

Shrimp adapted to seawater were treated with dsLvCdc42 or dsGFP as described in section 2.4. After 24 h of treatment, 10 μl of *V. alginolyticus*, diluted to a final concentration of 10^7 CFU/ml with PBS, was intramuscularly injected into shrimp (Wang et al., 2012). Then hepatopancreas and hemolymph samples were collected from three random shrimp per group at 1.5, 3, 6 and 12 h post challenge. Hepatopancreas was stored in liquid nitrogen for RT-qPCR analysis and hemolymph was stored at 4 °C for apoptotic cell count.

2.7. RT-qPCR analysis of LvCdc42, Lvp53 and Lvcaspase-3 expression in shrimp following LvCdc42 silencing and V. alginolyticus challenge

Hepatopancreas samples of shrimp treated with dsRNA and *V. alginolyticus*, were collected for cDNA preparation. The expression levels of LvCdc42, Lvp53 and Lvcaspase-3 were detected by RT-qPCR.

2.8. Apoptosis rate in hemocytes after dsRNA and V. alginolyticus injection

Apoptosis was analyzed with an Annexin V-FITC/PI apoptosis detection kit (Invitrogen, USA) using a FACSCalibur Cell Analyzer (BD, USA). Hemolymph samples collected as described in section 2.6 were treated according to the manufacturer's protocol. Finally, 10,000 hemocytes for each sample were grouped into early apoptotic hemocytes, late apoptotic hemocytes, necrotic hemocytes and living hemocytes. Apoptosis rate was calculated as the ratio of the sum of the numbers in the first three groups to the total number of hemocytes.

2.9. Cumulative mortality rate

Sixty shrimp individuals were randomly divided into two groups and treated with dsLvCdc42 or dsGFP, following *V. alginolyticus* challenge. The number of dead animals in each of the two groups was recorded after 1.5, 3, 6, 12 and 24 h.

2.10. Statistical analyses

All experiments in this study were repeated at least three times and data were subjected to one-way analysis of variance (ANOVA) using the SPSS 18 analysis program. Differences between samples were considered significant when $p < .05$.

3. Results

3.1. Identification of LvCdc42 and phylogeny analysis

The ORF of LvCdc42 was 576 bp long, encoding a polypeptide of 191 amino acids (Supplementary Fig. 1a). A nuclear localization sequence was predicted at amino acid 162–189. Meanwhile, the amino acid sequence of Cdc42 from *L. vannamei* shared 90%–95% similarity with other known Cdc42 sequences (Supplementary Fig. 1b). Lastly, phylogenetic analysis showed that *L. vannamei* Cdc42 and the one from other arthropods were grouped into one cluster (Supplementary Fig. 1c).

3.2. Subcellular localization of LvCdc42

In Supplementary Figs. 1d and S2 cells harboring recombination plasmid pAc5.1-LvCdc42-EGFP showed green fluorescence in both

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