Short communication

Molecular cloning and expression analysis of a dorsal homologue from *Eriocheir sinensis*

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**ABSTRACT**

Dorsal as a crucial component of Toll signaling pathway, played important roles in induction and regulation of innate immune responses. In this study, we cloned a NF-κB-like transcription factor Dorsal from *Eriocheir sinensis* and designated it as *EsDorsal*. The full-length cDNA of *EsDorsal* was 2493 bp with a 2202 bp open reading frame (ORF) encoding a 673-amino acid protein. This protein contained a 171-residue保守的Rel homology domain (RHD) and a 102-residue Ig-like, plexins and transcription factors domain (IPT). By phylogenetic analysis, *EsDorsal* was clustered into one group together with other invertebrate Dorsals or NF-κB, and then clustered with vertebrate NF-κB. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis results showed that a) *EsDorsal* had higher expression level in immune organs; (b) *EsDorsal* differentially induced after injection of lipopolysaccharides (LPS), peptidoglycan (PG) and zymosan (GLU). Importantly, *EsDorsal* was more responsive to LPS than GLU and PG. Collectively, *EsDorsal* was differentially inducibility in response to various PAMPs, suggesting its involvement in a specific innate immune regulation in *E. sinensis*.

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1. Introduction

NF-κB/Rel transcription factors are central regulators of mammalian immunity and also play crucial roles in some important physiological and pathological processes such as apoptosis, proliferation and differentiation, as well as in the innate immune responses of invertebrates (Dixit and Mak, 2002; Ghosh et al., 1998; Hayden and Ghosh, 2008). In mammalian, five different NF-κB/Rel family members have been found: NF-κB/Rel1 (p50/p105), NF-κB/Rel2 (p52/p100), RelA (p65), RelB and c-Rel, in which p105 and p100 belong to class I NF-κB factors that have the Rel homology domain (RHD) and the IkB-like domain, while c-Rel, RelB and RelA belong to class II NF-κB factors that contain only the RHD domain (Baeuerle and Henkel, 1994). In *Drosophila*, up to now, three NF-κB/Rel family members have been identified: Dorsal and Dorsal-related immunity factor (Dif) and Relish. Lacking the IkB-like domain, Dorsal and Dorsal-related immunity factor (Dif) belong to the class II NF-κB family, while Relish belongs to the class I NF-κB factors with one IkB-like domain. These three members are involved in mediating the induction of some antibacterial peptide genes following injury and infection via Toll or IMD signal pathway (Dushay et al., 1996; Huang et al., 2010; Reichhart et al., 1993; Steward, 1987; Steward et al., 1984). Prior to Toll signaling pathway activation, Dorsal is present in the cytosol. When Toll signaling pathway was activated by Gram-positive bacteria or fungi, Dorsal translocate into the nucleus to regulate the transcription and expression of Toll-dependent genes (Imler and Hoffmann, 2000, 2001; Lemaitre and Hoffmann, 2007; Tauszig et al., 2000; Wang et al., 2009).

Recently, more and more papers about the Chinese mitten crab immunity mechanism have been increasing because of its importance in the control of disease. For example, *EsRelish* was proposed to be involved in the innate immune responses against fungus and bacterium in Chinese mitten crab (Li et al., 2010b). What was different from *EsDorsal*, *EsRelish* not only had the RHD and IPT domains, but also contained some ankyrin-related motifs in the C-terminal domain of it. Two antibacterial C-type lectins from *Eriocheir sinensis* possessed microbial-binding activities and microbial killing activities, meanwhile, they also could stimulate the cellular encapsulation in vitro (Jin et al., 2013a). Another novel C-type lectin from *E. sinensis* not only could bind to various PAMPs and microorganisms, but also enhance encapsulation by crab hemocytes (Wang et al., 2013b). In addition, two down syndrome cell adhesion molecules (Dscams) from *E. sinensis* also were identified and confirmed that they could produce different isoforms through alternative splicing and participate in pathogen recognition (Jin et al., 2013b; Wang et al., 2013a). Actually, these studies focused on the relation between immune genes and PAMPs largely basing on the hemocytes, which also confirmed the importance of...
hemocytes in crab innate immunity to some extent. As the effector immune cells of crab, hemocytes not only participated directly in the pathogen recognition and elimination by phagocytes, encapsulation, nodule formation and melanization, but also produced humoral defense components, including protease inhibitors, anti-LPS factor, antimicrobial peptide and lysosomal enzymes (Hong et al., 2013; Söderhäll and Cerenius, 1992; Wu et al., 2012).

The main objectives of this current study were (1) to clone the full-length cDNAs of EsDorsal from E. sinensis; (2) to investigate the mRNA expression patterns of EsDorsal in different tissues; and (3) to detect the temporal responses of EsDorsal in the hemocytes induced by LPS, PG and GLU challenge. Together, our results determined the potentially important roles of EsDorsal in the innate immune responses to exogenous pathogenic stimulation in the Chinese mitten crab.

2. Materials and methods

2.1. Animal immune challenge and sample collection

Healthy adult Chinese mitten crabs (n = 200; 80 ± 20 g wet weight) were collected from the Tong Chuan Aquatic Product Market in Shanghai, China. After acclimation for one week at 23 ± 2 °C in filtered, aerated freshwater, crabs were placed in an ice bath for 1–2 min until each was lightly anesthetized. Hemolymph was drawn from the hemocoel in the arthrodial membrane of the last pair of walking legs using a syringe (~2.0 mL per crab), added to an equal volume of anticoagulant solution (0.1 M glucose, 30 mM citrate, 26 mM citric acid, 0.14 M NaCl and 10 mM EDTA) (Söderhäll and Smith, 1983) and centrifuged at 500 g at 4 °C for 1–2 min until each was lightly anesthetized. Hemolymph was drawn from the hemocoel in the arthrodial membrane of the last pair of walking legs using a syringe (~2.0 mL per crab), added to an equal volume of anticoagulant solution (0.1 M glucose, 30 mM citrate, 26 mM citric acid, 0.14 M NaCl and 10 mM EDTA) (Söderhäll and Smith, 1983) and centrifuged at 500 g at 4 °C to isolate hemocytes. The other tissues (hepatopancreas, gills, muscle, stomach, intestine, testis, ovary, thoracic ganglia, brain and heart) were harvested, snap frozen in liquid nitrogen, and stored at −80 °C prior to nucleic acid analysis. For cloning and subsequent in-depth analysis, tissues from five crabs were pooled, and ground with a mortar and pestle prior to extraction.

For stimulation by PAMPs, 120 crabs were divided equally into four groups. The three experimental crab groups were injected into the arthrodial membrane of the last pair of walking legs with approximately 100 μL of LPS from Escherichia coli (Sigma–Aldrich, St. Louis, MO, USA), 100 μL of PG from Staphylococcus aureus (Sigma–Aldrich) and 100 μL of zymosan (GLU from Saccharomyces cerevisiae, Sigma–Aldrich) resuspended (500 μg/mL) in E. sinensis saline (ESS, 0.2 M NaCl, 5.4 mM KCl, 10.0 mM CaCl₂, 2.6 mM MgCl₂, 2.0 mM NaHCO₃; pH 7.4). Meanwhile, the control group crabs were each administered 100 μL ESS (pH 7.4) in the same manner. Five crabs were randomly selected at each time interval of 0 (as blank control), 2, 6, 12 and 24 h after injection of each type of PAMP. Hemocytes were harvested as described above and stored at −80 °C after the addition of 1 mL Trizol reagent (Invitrogen, Carlsbad, CA, USA) for subsequent RNA extraction.

2.2. Total RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from E. sinensis tissues sampled as described in Section 2.1 using Trizol® reagent (RNA Extraction Kit, Invitrogen) according to the manufacturer’s protocol. The total RNA concentration and quality were estimated using spectrophotometry at an absorbance of 260 nm and agarose-gel electrophoresis respectively.

Total RNA (5 μg) isolated from hemocytes was reverse transcribed using the SMARTer™ RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA) for cDNA cloning. For RT-PCR and quantitative real-time RT-PCR (qRT-PCR) expression analysis, total RNA (4 μg) was reverse transcribed using the PrimeScript™ Real-time PCR Kit (Takara, Shiga, Japan).

2.3. EST analysis and cloning of full-length EsDorsal cDNA

Partial cDNA sequences of EsDorsal were obtained from the transcriptome data of the hepatopancreas (Jiang et al., 2009; Zhang et al., 2011), testis (He et al., 2013) from E. sinensis. To obtain the full-length cDNA, 5' RACE and 3' RACE were performed with gene-specific primers and adaptor primers (UPM) (shown in Table 1 of supporting information) using a SMARTer™ RACE cDNA Amplification kit (Clontech, USA). PCR amplification conditions for both the 3’ and 5’ RACE were as follows: 5 cycles at 94 °C for 30 s, 72 °C for 3 min; 5 cycles at 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min; 20 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. The PCR amplicons were size separated and visualized on an ethidium bromide stained 1.2% agarose gel. Amplicons of expected sizes were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and inserted into the pMD19T vector (Takara, Japan). Positive clones containing inserts of an expected size were sequenced using T7 and SP6 primers (shown in Table 1 of supporting information).

2.4. Sequence analysis and phylogenetic analysis

Full-length cDNAs of EsDorsal and deduced amino acid sequences were compared against sequences from other representative vertebrates and invertebrates reported in the National Center for Biotechnology Information (NCBI) GenBank, using the BLAST program (BLAST: Basic Local Alignment Search Tool). The homologous conserved domains were identified by SMART (Simple Modular Architecture Research Tool, http://smart.embl-heidelberg.de). Calculated molecular masses and theoretical isoelectric points were predicted by the Protein Mol. Wt & AA Composition Calculator (http://www.proteomics.com.cn/proteomics/pi_tool.asp). Multiple sequence alignment was done by the ClustalX and ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). An unrooted neighbor-joining (NJ) phylogenetic tree of selected sequences was constructed with MEGAS5.0 software (http://www.megasoftware.net) based on amino-acid sequences and the reliability of the branching was tested using bootstrap resampling with 1000 pseudo-replicates.

2.5. Tissue distribution and expression profiles of EsDorsal in hemocytes after LPS, PG and GLU challenge

qRT-PCR was conducted using the CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA). Gene-specific primers (EsDorsal-RT-F, EsDorsal-RT-R) (shown in Table 1 of supporting information) were designed to analyze the tissue distribution of EsDorsal and the expression profiles in hemocytes upon LPS, PG, and GLU challenge. Samples were run in triplicate and normalized to the control gene β-actin, and EsDorsal expression levels were calculated by the 2-ΔΔCt comparative CT method (Livak and Schmittgen, 2001). PCR conditions were as follows, 95 °C for 30 s; followed by 40 cycles of 95 °C, and a 0.5 °C/s incremental increase from 60 °C to 95 °C that lasted 30 s per cycle. Data were analyzed using the CFX Manager™ software (ver. 1.0).

2.6. Statistical analysis

Statistical analysis was performed using SPSS software (ver. 20.0). Data are represented as the mean ± standard error (S.E.). Statistical significance was determined by one-way analysis of variance (ANOVA) (Snedecor and Cochran, 1971) and post hoc
Duncan multiple range tests. In this study, differences were considered to be significant at $P < 0.01$.

## Results and discussion

### 3.1. Identification and characterization of EsDorsal

The Dorsal sequence of *E. sinensis* was deposited in the GenBank database under accession number KC900086, and named as *EsDorsal*. The full-length cDNA of *EsDorsal* was 2493 bp with a 2022-bp ORF encoding a 673-amino acid protein, a 144-bp 5’ UTR and a 327-bp 3’ UTR containing a non-canonical polyadenylation signal site (AATAAA) (shown in Fig. 1 of supporting information). In addition, the theoretical pI/MW of *Es* Dorsal were 6.74/72.79 kDa.

Under the analysis of deduced amino acid sequence by the SMART program, *EsDorsal* contained 673 residues with a conserved Rel homology domain (RHD) containing 171 residues, an Ig-like, plexins and transcription factors domain (IPT) containing 102 residues and a nucleus localization signal (shown in Fig. 1 of supporting information). As the signature sequence of Dorsal protein, FRYICEG and a dorsal domain profile were also detected in the deduced amino acid sequence of *EsDorsal*. Lacking the IκB-like domains, *EsDorsal* belongs to the class II NF-κB family, which was consistent with most invertebrate Dorsals.

### 3.2. Multiple sequence alignments and phylogenetic analysis

Considering the conservation of the RHD and TIG domains among Dorsals, the multiple sequence alignments of the two domains of *EsDorsal* with those of other selected invertebrate Dorsals were shown in shown in Fig. 2 of supporting information. The results showed that the RHD domain of *EsDorsal* was most similar to *Litopenaeus vannamei* Dorsal (*Lv*Dorsal, 93% identity) and *Fenneropenaeus chinensis* Dorsal (*Fc*Dorsal, 93% identity), followed by other selected invertebrate Dorsals (71–77% identity). Meanwhile, the IPT domain of *EsDorsal* shared the highest identity (89%) to those of *Lv*Dorsal and *Fc*Dorsal, 84% identity to that of *Aedes aegypti* Dorsal and 61–68% identity to those of other selected invertebrate Dorsals (shown in Fig. 2 of supporting information).

NJ trees were constructed based on the phylogenetic analysis of *EsDorsal* (Fig. 1) with representative invertebrate and vertebrate sequences from Protein Blast results. The results revealed that...
EsDorsal was clustered together with invertebrate Dorsals firstly, and then with invertebrate NF-κB, finally with vertebrate NF-κB.

3.3. Tissue distribution and expression profiles of EsDorsal in hemocytes post LPS, PG and GLU immune challenged

Information on the tissue distribution can offer useful clues into putative gene functions. As determined by qRT-PCR, EsDorsal was widely expressed in all the detected tissues of healthy crabs, but with great differences in levels between diverse tissues (Fig. 2A), which suggested it may be involved in a wide variety of physiological processes. Importantly, the highest expression was observed in gill (Fig. 2A), which was similar to that LvDorsal transcripts observed in shrimp (Huang et al., 2010). Gills were mainly involved in the continuous water exchange with the outside environment, therefore, they were more susceptible to pathogen infection. Thus, the higher expression of EsDorsal in gill also showed its importance roles in crab innate immunity to some extent.

As the representative PAMPs of Gram-negative bacterium, Gram-positive bacterium and fungi, LPS, PG and GLU were widely used in the immune-related experiments (Chettri et al., 2011; Krävchenko and Kaufmann, 2013; Vollmer et al., 2008; Watthanasurot et al., 2011). Considering the importance of the hemocytes in crab innate immunity, we detected the transcriptional expression profiles of EsDorsal in hemocytes after PG, LPS and GLU challenge by qRT-PCR. The results revealed that the expression of EsDorsal was significantly up-regulated at 2–24 h after GLU challenge (P < 0.01) (Fig. 2D). After LPS challenge, the expression of EsDorsal increased to 6.3, 1.7, 3.7 and 3.5 times above the blank control at 2, 6, 12 and 24 h, respectively (Fig. 2C). EsDorsal expression was kept significantly up-regulated at 2–24 h post-injection with PG (~1.5-fold compared to control, P < 0.01), and up to the peak at 24 h after challenged by PG (~2.9-fold compared to control, P < 0.01) (Fig. 2B). Control reactions, in which ESS was used for induction, yielded no significant variation in expression levels of EsDorsal (white bars in Fig. 2).

In this study, the mRNA expression level of EsDorsal in hemocytes increased to the peak at 2 h post-LPS injection, at 6 h post-GLU injection and at 24 h post-PG injection comparing to the control, which suggested that the transcription of EsDorsal was responsive to both bacteria and fungi challenge. The similar results that Dorsal can be induced diverse expression level by different pathogen stimulus also were found in other invertebrate Dorsals. For example, in Drosophila, DmDorsal was also differentially activated depending on pathogens used for infections, i.e. Gram-positive versus Gram-negative bacteria (Hoffmann and Reichhart, 2002). In shrimp Dorsals, LvDorsal was also responsive to fungi and some Gram-positive bacteria challenge in diverse degree, while FcDorsal mainly induced effective immune responses against Gram-positive bacteria and Gram-negative bacteria (Huang et al., 2010; Li et al., 2010a).

In summary, EsDorsal was identified from E. sinensis for the first time and highly expressed in immune tissues. Moreover, EsDorsal was involved in the crab anti-bacterial and anti-fungal immune responses, which contributed to the crab mounting appropriate defense responses against different PAMPs. Further investigations are necessary to clarify the function and regulation mechanism of EsDorsal from the cell and protein levels.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dci.2013.08.013.

References


