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Identification and functional analysis of *immune deficiency* (IMD) from *Scylla paramamosain*: The first evidence of IMD signaling pathway involved in immune defense against bacterial infection in crab species

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ABSTRACT

Immune deficiency (IMD) pathway, one of the most essential pattern recognition receptor signaling pathways, plays vital roles in innate immune responses to eliminate pathogen infection in invertebrates. In the present study, an *immune deficiency* (*IMD*) gene and two NF-κB family members, *Relish* and *Dorsal*, were identified and characterized in mud crab *Scylla paramamosain* for the first time. The deduced *Sp*IMD, *Sp*Relish and *Sp*Dorsal protein contained conserved death domain and classical NF-κB domains, respectively. Phylogenetic analysis suggested that *Sp*IMD was classified into the invertebrate IMD branch, and *Sp*Relish could be classified into the type I NF-κB class while *Sp*Dorsal could be grouped into the type II NF-κB class. Tissue distribution results showed these three genes were ubiquitously expressed in all tested tissues. The expression patterns of IMD signaling pathway and NF-κB genes, including *SpIMD*, *SpIKKβ*, *SpIKKε*, *SpRelish* and *SpDorsal*, were distinct when crabs were stimulated with *Vibro alginolyticus*, indicating that they might be involved in responding to bacterial infection. When *SpIMD* was silenced by *in vivo* RNA interference assay, the expression levels of IMD pathway and antimicrobial peptides (AMPs) genes, including *SpIKKβ*, *SpRelish*, *SpALF1-6* and *SpCrustin*, were significantly down-regulated ($p < 0.05$). Correspondingly, the bacteria clearance ability of hemolymph was extremely impaired in IMD silenced crabs. Overall, the IMD played vital roles in innate immune response by regulating the expressions of its down-stream signaling genes and AMPs in *S. paramamosain*. These findings might pave the way for a better understanding of innate immune system and establish a fundamental network for the IMD signaling pathway in crustaceans.

1. Introduction

Invertebrates lack adaptive immunity and rely highly on an efficient innate immune system to defend themselves from pathogen invasions [1]. Innate immunity is activated when pattern-recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides, lipoproteins, peptidoglycans, mannans and zymosan [2,3]. Upon recognition, PRRs activate multiple and complex signaling cascades that manipulate the transcription of immune-related genes encoding effector molecules [4]. Notably, different types of pathogens would elicit specific transcription programs [5].

Invertebrates have two major pattern-recognition receptor pathways that control the activation of NF-κB/Rel homologues and the induction of the antimicrobial peptides (AMPs), which are known as the immune deficiency (IMD) and Toll pathways [4,6–8]. NF-κB is an essential nuclear transcription factor which participates in innate immune responses, and contains a typical structure called Rel homology domain (RHD), which is involved in DNA binding and interaction with inhibitor κB (IκB) [9]. Up to now, three NF-κB family members, including Relish, Dorsal and Dorsal-related immunity factor (Dif), have been identified in *Drosophila* [9–11]. The Toll pathway, which is stimulated by fungi and Gram-positive bacteria, activates Dif and Dorsal, two p65-like/NF-κB proteins [12,13]. On the contrary, the IMD pathway, which is preferentially triggered by virus and Gram-negative bacteria, activates Relish, a p100-like/NF-κB precursor protein [4,14,15]. Unlike the mammalian NF-κB, Relish processing does not require the ubiquitin/proteasome pathway, but needs to be cleaved by both the caspase Dredd and the IκB-kinase (IKK) complex to translocate into the nucleus

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and activate AMPs transcription [16]. In *Drosophila*, the IKK complex involved in the IMD pathway is consisted of two subunits including Ird5 and Kenny, which are homologues of mammalian IKKβ and IKKγ respectively [16,17]. As a key adaptor protein, IMD links the extracellular signal and the intracellular reaction to initiate the signal transduction in the IMD pathway, which is similar to the receptor-interacting serine-threonine protein 1 (RIP1) in the TNF-Receptor pathway [4,14]. IMD orthology has been isolated in crustaceans including pacific white shrimp (*Litopenaeus vannamei*) [18], Chinese shrimp (*Fenneropenaeus chinensis*) [19], and crayfish (*Procambarus clarkii*) [20]. Moreover, two IKK homologues (IKKβ and IKKε) have been characterized in pacific white shrimp (*L. vannamei*) [21] and mud crab (*Scylla paramamosain*) [22]. Relish and Dorsal, the essential members in the NF-κB family, have been identified in several crustaceans including Chinese mitten crab (*Eriocheir sinensis*) [23,24], pacific white shrimp (*L. vannamei*) [25,26], Chinese shrimp (*F. chinensis*) [27–29], giant freshwater prawn (*Macrobrachium rosenbergii*) [30], and giant tiger prawn (*Penaeus monodon*) [31]. However, it is still unclear whether IMD is present in crab species and if all the genes mentioned above are involved in the IMD signaling pathway. Moreover, little is known whether the IMD signaling pathway contributes to the immune defense against bacterial infection in crab species.

The mud crab, *S. paramamosain*, is a commercially important mariculture species which is found throughout the south-east Asian coastal areas especially in south-east estuary regions in China [32]. However, in recent years diseases break out frequently which have caused large economic losses in the crab aquaculture [33]. *Vibrio alginolyticus,* a species of Gram-negative bacteria, is one of the major bacterial pathogens in crab culture [34]. In the present study, we identified and characterized an *IMD* homologue and two invertebrate NF-κB family members, *Relish* and *Dorsal*, from mud crab *S. paramamosain*, and investigated their tissue expression profiles with or without *V. alginolyticus* challenge. Moreover, an *in vivo* RNA interference assay was applied to illuminate the regulation of IMD-regulated immune genes in *S. paramamosain*. Meanwhile, a bacteria clearance assay was conducted to evaluate the impact of IMD interference on the bacteria clearance ability in crabs. The results gained from this study may lead to a further understanding of the network for the IMD signaling pathway as well as its participation in the innate immune system in *S. paramamosain* and even in crustaceans.

2. Materials and methods

2.1. Experimental animals

Healthy mud crabs (*S. paramamosain*) weighing 75 ± 5 g were obtained from Hangzhou Bay of Zhejiang province (China). During the acclimatization, crabs were maintained in PVC tanks with a continuous flow of aerated artificial seawater (salinity 18 ppt, temperature 28 ± 2 °C). Crabs were fed with fresh manila clam *Ruditapes philippinarum* daily until three days before experiments.

2.2. RNA extraction and cDNA synthesis

Tissues of mud crabs were sampled immediately and preserved in liquid nitrogen. Total RNA was extracted from tissues with RNAiso Plus (Takara) reagent as described in the manufacture's protocol. RNA integrity and purity were analyzed by electrophoresis and NanoDrop 2000 spectrophotometer (Thermo Scientific). To synthesize cDNA for PCR and RT-PCR, all RNA samples were treated with gDNA Eraser and then subjected to cDNA using PrimeScript™ RT reagent Kit (Takara).

2.3. PCR amplification and cloning

To obtain the cDNA segments of *SpIMD*, *SpRelish* and *SpDorsal*, primers (Table 1) were designed based on the conserved sequences of *IMD*, *Relish* and *Dorsal* genes in other crustacean species obtained from NCBI GenBank database. For the PCR amplification, the following program was running: 1 cycle of denaturation at 94 °C for 5 min, 30 cycles of 94 °C, 30 s; 58 °C, 30 s; 72 °C, 2 min, and a final extension at 72 °C for 10 min. The PCR products were separated on 1.0% (w/v) agarose gels, recovered with Gel Recovery Kit (Sangon Biotech) and cloned into pMD19-T plasmid vectors (Takara). The recombinant plasmids were transformed into DH5a chemically competent *E. coli* (Takara) for sequencing.

2.4. Rapid amplification of cDNA ends (RACE)

To synthesize first-strand cDNA for RACE reactions, 1 μg RNA from hepatopancreas was used as template, and reverse transcribed using reagents provided in a SMARTer™ RACE cDNA Amplification Kit (Clontech) according to the manufacture's protocol. For 3′-RACE PCR amplification, specific primers were used for nested PCR combined with a universal primer. The amplification of the 5′ terminal was also performed using nested PCR with primer UPM. PCR products were isolated, purified, cloned, and sequenced as described above. Primers were listed in Table 1.

2.5. Bioinformatics analysis

Program BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to compare the obtained sequences to the sequence database. DNAStar software was utilized to find the open reading frames (ORF) of the DNA sequences and translate into amino acids. The putative domains were predicted by SMART website (http://smart.embl-heidelberg.de/). Multiple sequence alignments were generated using the CLUSTALW program (http://www.genome.jp/tools/clustalw/). Neighbor-joining (NJ) phylogenic trees were constructed using MEGA 5 software based on the deduced amino acid sequences.

2.6. Tissue distribution of SpIMD, SpRelish, SpDorsal

Tissues including heart, hemocytes, gill, epidermis, nerve, muscle, intestine, and hepatopancreas were dissected from *S. paramamosain*. Total RNA was extracted and first-strand cDNA was synthesized as described above. The relative mRNA expressions of *SpIMD*, *SpRelish* and *SpDorsal* in different tissues were determined by quantitative real-time PCR (qRT-PCR) using SYBR Premix Ex Taq™ II kit (Takara). The amplification of qRT-PCR was carried out according to the following program: first denatured at 95 °C for 30 s, then 95 °C for 5 s, 60 °C for 30 s followed by 45 cycles, and a final dissociation cycle of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. PCR amplifications were performed in triplicates for each sample. The relative mRNA expression was calculated using the $2^{-\Delta\Delta t}$ method [35], and the gene-specific qPCR primers were listed in Table 1.

2.7. Immune challenge with V. alginolyticus

V. alginolyticus was isolated on a thiosulfate-citrate-bile-sucrose (TCBS) agar plate, and cultured overnight in LB liquid medium at 28 °C, then collected by centrifugation at 3000*g* for 10 min, washed twice in PBS and adjusted to 10^9 CFU/mL. Same volume (100 μL) of *V. alginolyticus* (1×10^6 CFU/mL) and PBS (control group) was injected into crabs through the base of fourth paraeiopod. Hepatopancreas was sampled at different time intervals (0 h, 3 h, 6 h, 12 h, 24 h, 48 h and 72 h) after injection for RNA extraction and RT-PCR experiment. Primers of *SpIMD*, *SpIKKβ*, *SpIKKε*, *SpRelish* and *SpDorsal* for qRT-PCR experiment were listed in Table 1.

2.8. Interference of SpIMD in vivo by dsRNA injection

Gene specific primers were designed to obtain DNA templates