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Characterization of an immune deficiency homolog (IMD) in shrimp (*Fenneropenaeus chinensis*) and crayfish (*Procambarus clarkii*)

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ABSTRACT

The immune deficiency (IMD) signal pathway mediates immunity against Gram-negative bacteria in *Drosophila*. Recent studies show that the IMD pathway also involves in antiviral innate immune responses. The functions of the pathway in crustacean immunity are largely unknown. In this paper, two IMDs (*Fc*IMD and *Pc*IMD), one of the key elements of the IMD pathway, were identified from Chinese white shrimp *Fenneropenaeus chinensis* and red swamp crayfish *Procambarus clarkii*. Both proteins have a death domain located at the C-terminal. *Fc*IMD was mainly expressed in the gills and stomach and *Pc*IMD was mainly detected in the heart, hepatopancreas, and stomach. *Fc*IMD peaked in hemocytes at 12 h after white spot syndrome virus (WSSV) challenge and it peaked in the gills at 6 h after WSSV challenge, but it was decreased at 2 h and kept the low level to 24 h in hemocytes and no obviously change in gill after *Vibrio anguillarum* challenge. *Pc*IMD first decreased in hemocytes at 2 h and peaked at 12 h in hemocytes after *V. anguillarum* challenge. It was also upregulated in gill after bacterial challenge, peaked at 2 h, and decreased at 6 h, and then gradually increased at 12–24 h. *Pc*IMD has no significant change in hemocytes and gill after WSSV challenge. Western blot analysis detected *Fc*IMD protein in all tissues, and immunocytochemical analysis localized *Fc*IMD in the cytoplasm of hemocytes. RNA interference analysis showed that the IMD pathway was involved in regulating the expression of three kinds AMP genes, including *crustins*, *anti-lipopolysaccharide factors* and *lysozymes*, in shrimp and crayfish. They are *Cru 1*, *Cru 2*, *ALF 1*, *ALF 2* and *Lys 1* in crayfish, and *Cru1*, *Cru 3*, *ALF 6*, *ALF 8*, and *Lys2* in shrimp. These results suggest that although IMD distribution and expression patterns have some differences, the IMD pathway may have conserved function for AMP regulation in shrimp and crayfish immunity against Gram-negative bacteria.

1. Introduction

Invertebrates lack an adaptive immunity and rely entirely on innate immunity to fight foreign microbes through humoral and cellular immune responses (Hoffmann et al., 1999; Lanzrein et al., 1998). Humoral responses include the blood clotting system (Muta and Iwanaga, 1996), the prophenoloxidase activating system (proPO) (Soderhall and Cerenius, 1998), and the production of antimicrobial peptides (AMPs) (Bulet et al., 1999; Hoffmann et al., 1999).

In *Drosophila melanogaster*, AMP production is dependent on the Toll and immune deficiency (IMD) signal pathways (Tanji and Ip, 2005). The absence of the Toll and IMD pathway causes a deficiency in AMP production and the flies become more susceptible to even normally nonpathogenic bacteria (Tanji et al., 2007). Pathogen-associated molecular patterns (PAMPs) from Gram-positive bacteria and fungi induce the activation of the protease cascade and causes prospaetzle cleavage. Activated spaetzle binds to the Toll receptor, triggers the pathway, and induces a subset of AMPs, such as drosomycin expression (Tanji et al., 2007). In the IMD pathway, the diaminopimelic acid-type peptidoglycan from Gram-negative bacteria is recognized and binds to the peptidoglycan recognition protein (PGRP)-LE and PGRP-LC receptor complex and activate the adaptor protein IMD, which leads to the activation of Relish (Takehana et al., 2002). Activated Relish serves as a transcription factor that promotes the production of AMPs, such as diptericin, cecropins and drosocin (Lemaitre et al., 1995b; Stoven et al., 2003). IMD is a death domain–containing protein and

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mutation of IMD causes severe defects in resistance to Gram-negative bacteria, but has normal response to Gram-positive bacteria and fungi (Georgel et al., 2001; Lemaitre et al., 1995a). The IMD pathway has an antiviral role in insects (Avadhanula et al., 2009; Costa et al., 2009).

The cultivation of shrimp such as *Fenneropenaeus chinensis* and *Penaeus monodon*, has been beset with serious problems linked with *Vibrio* and white spot syndrome virus (WSSV) infection (Kang et al., 2004; Peng et al., 2001). The innate immunity of Chinese white shrimp has been widely studied from pattern recognition to AMP production in the past years (Li and Xiang, 2013; Wang and Wang, 2013a,b). The serine protease cascade including serine proteases and its inhibitors are also analyzed and find that they possibly participate in the signal cascades (Ren et al., 2009; Shi et al., 2008). A number of AMPs have been identified in Chinese white shrimp, including penaeidins (Kang et al., 2004, 2007), crustins (Sun et al., 2009; Zhang et al., 2007), a single WAP domain containing peptide (SWD) (Jia et al., 2008), and a double WAP domain containing protein (Du et al., 2009). The two important molecules of the Toll pathway, Toll and spaetzle, are also found in Chinese white shrimp, and spaetzle induces crustin two expression in crayfish (Shi et al., 2009; Yang et al., 2008). Thus far, shrimp IMD has only been found in *Litopenaeus vannamei* and its function in activating AMPs in vitro was investigated. The results show that IMD induces the expression of attacin A and penaeidin 4 in *Drosophila* Schneider 2 (S2) cells (Wang et al., 2009a). The functions of IMD pathway in crustacean in vivo remain largely unknown.

The bacterium *Vibrio anguillarum* and the WSSV are two important pathogens in shrimp and crayfish. *V. anguillarum* is a Gram-negative bacterium and the IMD pathway participates in anti-Gram-negative bacteria innate immunity in insects (Georgel et al., 2001). The IMD pathway is also involved in antiviral innate immunity (Costa et al., 2009). Thus, identifying the key molecules in the IMD pathway and understanding their regulation mechanism are necessary. In this paper, we identified the IMD in *F. chinensis* and *Procambarus clarkii*. The expression patterns after *Vibrio* and WSSV challenge were analyzed. An RNA interference assay was also performed to investigate the readouts of the IMD pathway in the shrimp and crayfish and to obtain further insight into this pathway in different crustaceans.

2. Materials and methods

2.1. Preparation of viral inoculum

The WSSV inoculum was prepared based on a previously described method (Wang et al., 2009c). WSSV was extracted from the gills of naturally heavily infected *F. chinensis* stored at −80 °C. The gill tissue from three shrimp (1 g) was homogenized in 10 mL of phosphate-buffered saline (PBS) (140 mmol/L NaCl, 2.7 mmol/L KCl, 10 KH_2PO_4), and then centrifuged at 5000 × *g* for 10 min at 4 °C. The supernatant was passed through a 450 nm membrane filter and used as the inoculum.

2.2. Immune challenge and isolation of total RNA from shrimp and crayfish

Adult shrimp (approximately 10–20 g per shrimp) were purchased from a professional fisherman in Xiaogang Fishery Market in Qingdao, Shandong Province, China, and then temporarily cultured in 500 L tanks filled with aerated seawater in the Institute of Oceanology, Chinese Academy of Science. Red swamp crayfish (about 10–15 g each) were obtained from Jinan Fishery Market in Jinan, Shandong Province, China and were cultured in laboratory tanks with fresh water at room temperature. *V. anguillarum* (approximately 3×10^7 cells per shrimp or per crayfish, *Vibrio* was a gift of Institute of Oceanology, Chinese Academy of Sciences) and WSSV (3.2×10^7 per shrimp or per crayfish), were injected into the abdominal segment of the shrimps (Wang et al., 2009d). Hemolymph was collected from the ventral sinus of the shrimp (15 shrimp at each time point) at 0, 2, 6, 12, and 24 h after *Vibrio* challenge and after WSSV challenge with a 1/10 volume of anticoagulant buffer (10% sodium citrate, pH 7). For crayfish, the anticoagulant buffer [0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, and 10 mM ethylenediamine tetraacetic acid (EDTA), pH 4.6] was used (Soderhall and Smith, 1983). The hemolymph was immediately centrifuged at 800 × *g* for 5 min (4 °C) to isolate the hemocytes. Total RNA was also isolated from other tissues of unchallenged shrimp and crayfish using Unizol reagent (Biostar, Shanghai, China). RNA was also extracted from the gills of shrimp and crayfish challenged with *V. anguillarum* and with WSSV at 2, 6, 12, and 24 h (three shrimp and three crayfish at each time point).

2.3. cDNA cloning of FcIMD

The primer *Fc*IMDF (5′-CTA CCC GTA CGA GGC TCA GGA CGG TAA-3′) was designed based on the published sequence of the *L. vannamei* IMD(ACL37048) gene to obtain the 3′ fragment of *FcIMD* with the 3′ anchor R primer (5′-GAC CAC GCG TAT CGA TGT CGA C-3′). After obtained the fragment of *Fc*IMD, the primer *Fc*IMDR (5′-GGT GTC GAT GCC CTT ATT TTC C-3′) was designed to amplify the 5′ coding region of *FcIMD* with the primer *Fc*IMDF1 (5′-GAT GGA TAA TAT TAA GAC AG-3′ based on the *L. vannamei* IMD sequence. The complete coding region with a poly A sequence was obtained by overlapping the two fragments. The *PcIMD* coding region was obtained by a pair of primers based on the *FcIMD* sequence (*Pc*IMDF: 5′-ATG GAT AAT ATT AAG ACA G-3′; *Pc*IMDR: 5′-TAT GTG CCT TCA GGG TGT A-3′).

2.4. Phylogenetic and sequence analysis of FcIMD and PcIMD

Similarity analysis of *Fc*IMD and *Pc*IMD was conducted using BLASTx (http://www.ncbi.nlm.nih.gov/). The corresponding cDNA were translated and the deduced proteins were predicted using ExPASy (http://www.au.expasy.org/). Signal sequencing and domain prediction were performed using SMART (http://smart.embl-heidelberg.de/). Multiple alignment of the IMD protein sequences and the DNA sequences of the IMD coding region from three shrimp and one crayfish were performed using MEGA 4 software (Kumar et al., 2008) and GENDOC software. Phylogenetic trees were constructed through the neighbor-joining method using MEGA 4 software (Kumar et al., 2008).

2.5. Tissue distribution and responses of FcIMD and PcIMD to bacterial and WSSV challenge

Tissue distribution and time course expression of *FcIMD* and *PcIMD* in hemocytes and gills were determined at 0, 2, 6, 12, and 24 h after *Vibrio* challenge and WSSV challenge via real-time polymerase chain reaction (PCR) using a pair of specific primers designed according to the *FcIMD* and *PcIMD* sequences. The protocol was designed according to our previous paper (Shi et al., 2008). The primers used in quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were *Fc*IMD-QRT-F: 5′-ACG AGG CTC AGG ACG GTA A-3′, *Fc*IMD-QRT-R: 5′-AGT GGA AGG TCG ATT GGG A-3′ for *FcIMD* and *Pc*IMD-QRT-F: 5′-CGT GCC CAG CGA TTT TCA-3′ and *Pc*IMD-QRT-R: 5′-TGC TGC GAG CGA GGG TTA-3’ for *PcIMD*.