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Identification and functional study of a shrimp Relish homologue

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ABSTRACT

Rel/NF-κB transcription factors play central roles in induction and regulation of innate immune responses. Here we describe the identification and functional analysis of a Relish homologue, LvRelish and its shorter isoform sLvRelish, from the Pacific white shrimp, *Litopenaeus vannamei*. The LvRelish gene has 22 exons in approximately 15 kb genomic sequence. The full-length cDNA of LvRelish is 4071 bp with an open reading frame that encodes 1207 amino acids. LvRelish contains a conserved Rel homology domain (RHD), a nucleus localization signal, an IκB-like domain (six ankyrin repeats), and a death domain, suggesting that it belongs to the class I NF-κB. sLvRelish cDNA is 1051 bp encoding 317 amino acids. It shares the RHD region with LvRelish. RT-PCR analysis showed that LvRelish and sLvRelish mRNAs were expressed at different levels in tissues. Western blot analysis showed that recombinant intact LvRelish could be cleaved into two fragments in S2 cells, and immunofluorescence assay showed that the plasmid-expressed LvRelish protein was seen both in the cytoplasm and the nucleus. Electrophoretic mobility shift assay showed that recombinant RHD of LvRelish in S2 cells bound specifically with *Drosophila melanogaster* κB motifs in vitro. Both the LvRelish and its RHD domain transactivated the reporter gene controlled by the 5′ flanking region of penaeidin 4, an antibacterial peptide of shrimp, suggesting that LvRelish can regulate the transcription of penaeidin 4 gene. Identification of LvRelish will help us better understand shrimp immunity and may help obtain more effective methods to prevent shrimp diseases.

1. Introduction

Rel/NF-κB transcription factors play central roles in important physiological and pathological processes such as apoptosis, proliferation and differentiation, as well as in innate immune responses [1–4]. NF-κB factor was first identified as a B-cell-specific transcription factor that binds the κB site in the immunoglobulin (Ig) light chain enhancer [5]. Subsequently, NF-κB activity has been found in all cell types. Rel/NF-κB factors have a well-conserved Rel homology domain (RHD) involved in DNA binding, dimerization and interaction with the inhibitor κB (IκB). When a cell receives signals, Rel/NF-κB factors are released from the IκB, and then rapidly enter the nucleus to activate expression of various downstream genes [1]. In mammals, five NF-κB factors, NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel have been identified. They can be divided into two classes, class I and class II, based on the sequences to the C-terminus of the RHD. The class I NF-κB factors, such as p105 and p100, have long C-terminal domains containing the IκB-like domain (with seven ankyrin repeats, ANKs), which acts to inhibit these NF-κB molecules. The class II NF-κB factors, such as c-Rel, RelB and RelA, contain the C-terminal transactivation domains.

In *Drosophila melanogaster*, three Rel/NF-κB factors, Dorsal, Dorsal-related immunity factor (Dif) and Relish, have been identified to control the transcription of effectors such as antibacterial and antifungal peptide genes [6]. Dorsal [7] and Dif [8], which belong to the class II NF-κB, are activated by the Toll pathway in response to infection by fungi and Gram-positive bacteria. While Relish, which belongs to the class I NF-κB, is triggered by the immunodeficiency (Imd) pathway in response to infection by Gram-negative bacteria [9]. After stimulation and induction, the Relish is cleaved into the N-terminal RHD and C-terminal ANKs. The RHD translocates into the nucleus and immediately activates the expression of antibacterial peptide genes, such as cecropin A1, attacin, and diptericin [10].

The Pacific white shrimp, *Litopenaeus vannamei*, is one of the most important economic penaeid shrimps worldwide. With the

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rapid production and development of *L. vannamei*, shrimp diseases, including bacterial and viral diseases, have become severe and threatened the shrimp industry in many of the shrimp-farming countries in the world since 1990s [11–13]. Since shrimps lack an adaptive immune system, they utilize innate immunity to defend against various invading microorganisms [14]. Immune-related proteins, such as anti-LPS factor [15,16], lysozyme [17] and antibacterial peptides like penaeidin [18–20], have been reported to participate in shrimp immune response. In addition, a shrimp Toll-like receptor has been cloned in our laboratory [21]. However, no Rel/NF-κB homologue has ever been identified in shrimp. Here we report identification and characterization of a Relish homologue, LvRelish and its shorter isoform sLvRelish, from *L. vannamei*.

2. Materials and methods

2.1. Cloning of LvRelish cDNA

Healthy white shrimps (weight 8–9 g, body length 8–10 cm) were obtained from Hengxing shrimp farm in Zhanjiang, Guangdong Province, China. Total RNA was extracted from the hepatopancreas using RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's instructions. cDNA was prepared using moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, USA) with an oligo$(dT)_{18}$ primer following the manufacturer's instructions. Two degenerate primers (DPRelF and DPRelR, Table 1) were designed based on the reported Relish sequences of insects. Touch-down PCRs were performed with denaturation at 94 °C for 3 min; 15 cycles of 94 °C for 35 s, 54–0.5 °C/cycle for 40 s, 72 °C for 50 s; and 28 cycles of 94 °C for 35 s, 46 °C for 40 s and 72 °C for 50 s, followed by a 3 min extension at 72 °C. The PCR products were cloned into pGEM-T Easy vector (Promega, USA) and sequenced. A 401 base pair (bp) fragment was obtained.

5′ and 3′-rapid amplification of cDNA ends (RACE) was performed using BD SMART RACE cDNA Amplification Kit (Clontech, Japan) with the primers for RACE (Table 1). 5′-RACE was carried out using primers 5′RPRelseq921R, 5′RPRelseq748R and Universal Primer A Mix (UPM) for LvRelish, primers 5′RPRelSseq282R, 5′RPRelSseq480R and UPM for sLvRelish. 3′ RACE was performed with primers 3′RPRelseq745F, 3′RPRelseq920F and 3′-RACE CDS Primer. Usually, nested or semi-nested PCR was employed.

2.2. Sequence analysis

Simple modular architecture research tool (SMART, http://smart.embl-heidelberg.de) was used to analyze the deduced amino acid sequence of LvRelish. The identity levels of LvRelish RHD, ANKs and death domain (DD) with corresponding domains of other Relish proteins were produced with ClusterW2 by using neighbor-joining (N-J) method in EBI (http://www.ebi.ac.uk/Tools/clustalw2/index).

2.3. Genomic DNA extraction and PCR amplification

Genomic DNA was isolated from *L. vannamei* muscle using genomic DNA extraction kit (Takara, Japan) following the instruction manual. DNA concentration was assessed by agarose gel electrophoresis and UV spectrophotometry.

To obtain the LvRelish genomic DNA sequence, genomic PCR was performed with 4 combinations of the primers for genomic structure (Table 1). Additionally, to obtain genomic DNA sequences adjacent to the 5′ and 3′ ends of LvRelish, genome walker libraries were constructed according to the manufacturer's instructions of Genome Walker DNA Walking kit (Clontech, Japan). The 8 primers, including AP1 and AP2, for the genomic DNA sequences adjacent to the 5′ and 3′ ends are listed in Table 1. The PCR products were cloned into pGEM-T Easy vector and sequenced.

2.4. Expression analysis by reverse transcription (RT)-PCR

cDNAs were obtained from shrimp tissues, including hepatopancreas, gill, muscle, heart, intestine, stomach, epidermis, hemocytes, eyestalk, brain and pyloric caecum. The primers used for LvRelish and sLvRelish cDNA fragments are listed in Table 1. The PCRs were performed using the following conditions: denaturation at 94 °C for 3 min, 32 cycles of 94 °C for 35 s, 60 °C for 40 s, 72 °C for 1 min, followed by an elongation at 72 °C for 3 min. As an internal loading control, the shrimp β-actin cDNA fragment was amplified with primers 5RTBactinseq81F and 3RTBactinseq733R (Table 1) with the following conditions: denaturation at 94 °C for 3 min, 28 cycles of 94 °C for 35 s, 60 °C for 40 s, 72 °C for 1 min, followed by an elongation at 72 °C for 3 min.

2.5. Construction of expression and reporter plasmids

For expression plasmids, Myc tag was introduced by PCR to the Relish coding sequence at the N-terminus with the primers: pAc5RelMycF and pAcRelFNotIR, pAc5RelMycF and pAcRelNotIRHDR (Table 1). The PCR products were digested with KpnI and NotI and ligated to KpnI/NotI digested pAc5.1/V5-His A expression vector (Invitrogen, USA) to produce two plasmids: pAcRelF and pAcRelRHD. Other expression plasmids: pAcDif, pAcDmDor, and pAcDmRel for *D. melanogaster* NF-κB factors without Myc-tag, were constructed in a similar way using primers listed in Table 1. A schematic diagram of the expression plasmids is shown in Fig. 6B.

For reporter plasmids, BglII and KpnI restriction sites were respectively introduced by PCR to the 5′ flanking regions of *L. vannamei* penaeidin (PEN) 2, PEN 4 and *Drosophila melanogaster* attacin genes with the primers: 5pGL3PEN2seq538 and 3pGL3PEN2seq1 for pGL3PEN2, 5pGL3PEN4-697 and 3pGL3PEN4-1 for pGL3PEN4, 5pGL3Attacin989 and 3pGL3Attacin1 for pGL3Atta (Table 1). The PCR product was digested with BglII and KpnI, and ligated to the BglII/KpnI digested, promoterless pGL3-luciferase expression vector (Promega, USA), which contains the firefly luciferase gene as a reporter. A schematic diagram of reporter plasmids is shown in Fig. 6A. Plasmids for transfection were prepared using EndoFree Plasmid Mini Kit (OMEGA, USA) according to the manufacturer's instruction.

2.6. Cell culture and transfection

Drosophila Schneider 2 (S2) cells were maintained at 27 °C in *Drosophila* SDM (Serum-Free Medium; Invitrogen, USA), supplemented with 10% fetal bovine serum (Invitrogen). For DNA transfection, cells were seeded overnight, and then plasmids were transfected by using the Cellfectin reagent (Invitrogen) according to the manufacturer's recommendation. For immunofluorescence assay, 1.5 μg pAc5RelMycF expression plasmid was added to S2 cells on coverslips in each well of a 12-well plate. For western blot analysis, expression plasmid was added at 3 μg per well to S2 cells in a 6-well plate. For Dual-luciferase reporter assays, 0.3 μg expression plasmid, 0.15 μg reporter gene plasmid and 0.015 μg pRL-TK renilla luciferase plasmid (as an internal control, Progema, USA) were used for cells in each well in a 96-well plate. All assays were performed with three independent transfections.

2.7. Immunofluorescence assay

At 48 h post transfection, S2 cells on coverslips were washed with PBS twice and fixed with ice-cold methanol for 10 min at room