Developmental and Comparative Immunology 38 (2012) 78–87

Contents lists available at SciVerse ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Litopenaeus vannamei inhibitor of apoptosis protein 1 (LvIAP1) is essential for shrimp survival

Jiann-Horng Leu [a,b,*], Yen-Chen Chen [c], Li-Li Chen [a,b], Kuan-Yu Chen [a], Huai-Ting Huang [a], Jan-Ming Ho [d], Chu-Fang Lo [e]

[a] Institute of Marine Biology, National Taiwan Ocean University, 2 Pei-Ning Road, Keelung 20224, Taiwan, ROC
[b] Center of Excellence for Marine Bioenvironment and Biotechnology, National Taiwan Ocean University, 2 Pei-Ning Road, Keelung 20224, Taiwan, ROC
[c] Graduate Institute of Biomedical Informatics, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan, ROC
[d] Institute of Information Science, Academia Sinica, 128 Academia Road, Section 2, Nankang, Taipei 115, Taiwan, ROC
[e] Institute of Zoology, College of Life Science, National Taiwan University, 1 Roosevelt Road, Section 4, Taipei 10617, Taiwan, ROC

ARTICLE INFO

Article history:
Received 4 February 2012
Revised 25 March 2012
Accepted 12 April 2012
Available online 5 May 2012

Keywords:
Litopenaeus vannamei
Apoptosis
Inhibitor of apoptosis protein
Haemocyte

ABSTRACT

The members of the inhibitor of apoptosis protein (IAP) family are involved in the regulation of diverse cellular processes, including apoptosis, signal transduction and mitosis. Here, we report the cloning and characterization of three IAP genes from Pacific white shrimp *Litopenaeus vannamei*: *LvIAP1*, *LvIAP2* and *LvSurvivin*. LvIAP1, the orthologue of *Penaeus monodon* IAP (PmIAP), consists of three BIR domains and one RING domain; LvIAP2 consists of two BIR domains and LvSurvivin has only one BIR domain. Expression profiling by absolute quantitative real-time RT-PCR revealed that of the three IAP genes, *LvIAP1* had the highest expression levels in almost all examined tissues and *LvSurvivin* had the lowest expression levels. Furthermore, among the examined tissues, the lymphoid organs most strongly expressed all three genes. When *LvIAP1* expression was silenced by injection of its corresponding dsRNA, the shrimp died within 48 h after injection, whereas injection of the other two dsRNAs did not cause shrimp death. In *LvIAP1*-silenced shrimp, the number of circulating haemocytes decreased dramatically because of extensive apoptosis. This suggested that *LvIAP1* is central to the regulation of shrimp haemocyte apoptosis.

1. Introduction

Apoptosis is a genetically regulated cell suicide program that removes surplus or abnormal cells, thereby playing crucial roles in development, homeostasis, immunity and insect metamorphosis (Opferman and Korsmeyer, 2003; Steller and Grether, 1994; Vaux and Korsmeyer, 1990). Because of its destructive effect on living cells, apoptosis is tightly controlled by multiple regulators, and the interaction between positive (pro-apoptotic) and negative (anti-apoptotic) regulators determines whether this program is activated by a death signal. These regulators and their underling mechanisms of regulation are highly conserved from mammals to nematodes (Danial and Korsmeyer, 2004). As anti-apoptosis regulators, inhibitor of apoptosis proteins (IAPs) can inhibit the activity of caspases, the main executors of the apoptosis program, and play important roles in regulating the progression of apoptosis in many species (Deveraux and Reed, 1999; Salvesen and Duckett, 2002).

IAPs were first described in insect baculoviruses (Crook et al., 1993; Birnbaum et al., 1994), wherein these genes inhibit infected insect cells from executing apoptosis, thereby enhancing viral multiplication. Since then, numerous cellular IAP homologues have been identified in yeast, nematodes, flies and higher vertebrates (O'Riordan et al., 2008). Members of the IAP family are recognized by the presence of one to three copies of a zinc-binding baculoviral IAP repeat (BIR) domain at their N-termini. The BIR domains, acting as the protein–protein recognition and interaction modules for IAPs, bind to caspases and a group of proteins that contain an IAP-binding motif (IBM) (Liston et al., 2003; O'Riordan et al., 2008). These IBM-containing proteins are IAP antagonists that counteract anti-apoptotic activity of IAPs to induce apoptosis. These IAP antagonists include *Drosophila* Reaper (Rpr), Grim and Hip as well as mammalian Smac/DIABLO. Normally, BIR domains bind and suppress caspases; however, under apoptotic conditions, IAP antagonists are activated and then bind to BIRs, thus displacing bound caspases that are then free to initiate the caspase activation cascade, leading to apoptosis (Liston et al., 2003; O'Riordan et al., 2008). Some IAPs have a really interesting new gene (RING) finger domain at their C-termini. The RING domain functions as an E3 ubiquitin ligase, which can recruit E2 ubiquitin-conjugating enzymes and transfer ubiquitin to target proteins that bind to IAPs.

* Corresponding author at: Institute of Marine Biology, National Taiwan Ocean University, 2 Pei-Ning Road, Keelung 20224, Taiwan, ROC. Tel.: +886 2 24622192x5316; fax: +886 2 24633152.
E-mail address: jiholeu@ntou.edu.tw (J.-H. Leu).

0145-305X/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.dci.2012.04.006

The ubiquitinated target proteins, including caspases, some IBM proteins and IAPs themselves, are subjected to proteasomal degradation or inactivation. Depending on the target, ubiquitination can serve both pro- and anti-apoptotic functions (Liston et al., 2003; Ditzel and Meier, 2002; Vaux and Silke, 2005).

IAPs, however, function beyond the regulation of apoptosis. IAPs with only one or two BIR domains play important roles in mitotic regulation that is conserved from yeast to mammals; these IAP members include mammalian Survivin, *Drosophila* Deterin, *C. elegans* CeIAP1 and yeast IAPs (Wei et al., 2008). Some IAPs are involved in signaling cascades, such as mammalian cellular inhibitor of apoptosis 1 (cIAP1) and cIAP2, both of which are components of the type-2 tumor necrosis factor receptor (TNFR2) complexes (Rothe et al., 1995) and play important roles in TNF-mediated induction of NF-κB signaling (Petersen et al., 2007; Varfolomeev et al., 2007). IAPs play roles in immune responses against microbial infection in both *Drosophila* and mammals (Srinivasula and Ashwell, 2008). In *Drosophila*, infection with gram-negative bacteria triggers the immune deficiency (IMD) pathway of innate immunity, leading to the expression of antimicrobial peptides (AMPs). Depletion of *Drosophila* IAP2 (DIAP2) by RNAi abrogated the expression of AMP in response to gram-negative bacterial infection in cultured cells (Kleino et al., 2005; Gesellchen et al., 2005). Importantly, *diap2*-null mutant *Drosophila* exposed to gram-negative bacteria failed to induce the synthesis of antimicrobial peptides and died (Leulier et al., 2006a; Huh et al., 2007).

Compared to other model organisms, the study of crustacean apoptosis is still at its infancy (Menze et al., 2010). We cloned and characterized the first crustacean IAP gene, *Penaeus monodon* IAP (*PmIAP*), from the black tiger shrimp *P. monodon* (Leu et al., 2008a). PmIAP contains three BIR domains and a RING domain. In insect cells, PmIAP can block apoptosis induced by IAP antagonist *Drosophila* Rpr, suggesting that PmIAP might use mechanisms similar to *Drosophila* and mammals to regulate apoptosis in prawns. However, no other crustacean IAP gene has been identified. In this study, we report our continuing effort in the identification and characterization of novel IAP genes from penaeid shrimp. By using the *PmIAP* gene as the query sequence, homology searches against the *Litopenaeus vannamei*-expressed sequencing tags (Lv ESTs) in the NCBI EST database (dbEST) identified three *PmIAP*-homologous genes: *LvIAP1*, *LvIAP2* and *LvSurvivin*. To further characterize these genes, we isolated their full-length cDNAs and determined their mRNA expression profiles. Lastly, we used RNA interference (RNAi) technology to silence their expression and found that one of them, *LvIAP1*, is essential for shrimp survival.

2. Materials and methods

2.1. Identification of PmIAP-related genes in L. vannamei ESTs

The Lv ESTs were downloaded from dbEST and uploaded to the website Bio301 (http://www.bc02.iis.sinica.edu.tw/bio301new/; Chen et al., 2012) for EST analysis. Bio301 is an automated functional annotation system for ESTs, which analyzes the input ESTs through steps including vector sequence trimming, EST clustering, homology searching and gene annotation. After analysis, a 'library' is created to store the analyzed results. Genes of interest can then be identified in this library either by full text searches for the annotated gene name or function or by sequence homology searches using BLAST. After the Lv EST library was created by Bio301, a TBLASTN search was performed using the amino acid sequence of *PmIAP* as the query to search against the translated nucleotide sequences in the library. The returned hits with *E* values < 10^{-8} were considered to be the possible *PmIAP*-related genes in *L. vannamei*. The identified hits and their characteristics are summarized in Table 1.

2.2. Isolation of full-length cDNAs

Based on the available Lv EST sequences, primers for 5′/3′-RACE were designed (Table 2) to isolate full-length cDNAs of the three genes using the SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories Inc.). First, total RNAs were extracted from the stomach, pleopods and lymphoid organs of shrimp using TRIzol reagent (Invitrogen, USA). Then, 100 μg of total RNAs from each organ were mixed together and subjected to poly(A)$^+$ RNA purification using the QuickPrep Micro mRNA Purification Kit (GE Healthcare). The SMARTer™ RACE cDNA Amplification Kit was then used to individually synthesize 5′- and 3′-RACE-ready cDNAs from 500 ng of mRNAs. For 5′-RACE, an aliquot of 5′-RACE-ready cDNA was subjected to nested PCR using two gene-specific primers (Table 2) and universal primers supplied in the kit. For 3′-RACE, an aliquot of 3′-RACE-ready cDNA was subjected to PCR using the gene-specific primers (Table 2) and universal primers provided in the kit. PCR was performed using the Advantage 2 Polymerase Mix (Clontech Laboratories Inc.); the PCR products were cloned into pGEM-T Easy vectors (Promega) and sequenced.

2.3. Amino acid sequence analysis

The deduced amino acid sequences of the three shrimp genes were subjected to ScanProsite analysis (http://www.expasy.org/tools/scanprosite/) to identify the structural motifs.

2.4. Tissue tropism of three IAP genes determined by absolute quantitative real-time PCR

Five shrimp weighing approximately 8 g each were dissected to collect various tissues/organs including the epithelium, gills, heart, haemocytes, hepatopancreas, intestine, lymphoid organs, nerves, pleopods and stomach. TRIzol reagent was used for total RNA extraction according to manufacturer's instructions. To begin cDNA synthesis, equal amounts of the same tissue/organ RNAs from the five shrimp were combined and 100 ng of this was treated with TURBO DNA-free™ DNase (Ambion Inc.) to remove any contaminating genomic DNA. The treated total RNAs were reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Reverse transcription reactions were primed with random primers supplied in the kit. The synthesized cDNAs were further diluted and subjected to absolute quantitative real-time PCR.

The plasmids used for generating standard curves were constructed by cloning the corresponding real-time PCR products into pGEM-T Easy vectors. The cloned fragments were verified by DNA sequencing. Plasmid DNA was quantified by spectrophotometry, and the gene copy number was determined according to the molar mass derived from the plasmid and amplicon sizes. The plasmid DNAs were serially diluted 10-fold to generate standard curves in quantities ranging from 10^2 to 10^6 copies.

Real-time PCR was conducted using the Fast SYBR Green Master Mix (Applied Biosystems) and performed using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). PCR was performed in a 10-μl reaction mixture containing primers at a concentration of 300 nM. The thermal cycling program was as follows: 2 min at 50 °C, 10 min at 95 °C and 45 cycles of 15 s at 95 °C and 20 s at 60 °C. A dissociation curve analysis (95 °C for 15 s, 60 °C for 30 s, 95 °C for 15 s) was included for each sample after PCR to examine the specificity of the PCR products. Three technical replicates were performed for each assay sample and standard dilution. After PCR, the *Ct* values for the