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Molecular cloning and characterization of an inhibitor of apoptosis protein (IAP) from the tiger shrimp, *Penaeus monodon*☆

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Abstract
The inhibitor of apoptosis proteins (IAPs) play important roles in both apoptosis and innate immunity. Here, we report the first cloning and characterization of a novel IAP family member, PmIAP, from *Penaeus monodon*. The full-length *PmIAP* cDNA is 4769 bp, with an ORF encoding a protein of 698 amino acids. The PmIAP protein contains three BIR domains and a C-terminal RING domain, and its mRNA was expressed in all analyzed tissues. In insect cells, PmIAP, together with *Spodoptera frugiperda* IAP, AcMNPV P35, and WSSV449 (or ORF390, an anti-apoptosis protein encoded by white spot syndrome virus), could all block the apoptosis induced by *Drosophila* Reaper protein (Rpr), whereas only P35 and WSSV449 could block the apoptosis induced by actinomycin D. Co-immunoprecipitation showed that PmIAP physically interacted with Rpr, and in an immunofluorescent analysis the two proteins produced co-localized punctate signals in the cytoplasm. Deletion analysis revealed that both the BIR2 and BIR3 domains of PmIAP could independently bind to and inhibit Rpr, whereas the BIR1 domain could not. These results strongly suggest that PmIAP blocks Rpr's pro-apoptotic activity through mechanisms that are evolutionarily conserved across crustaceans, insects, and mammals.
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1. Introduction

Apoptosis is a genetically programmed cellular suicide process that eliminates unwanted or diseased cells, and it plays important roles in embryogenesis, homeostasis, insect metamorphosis, and immunity [1–3]. Cells undergoing apoptosis display a series of morphological changes, including cell shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing, and, finally, formation of

Abbreviations: IAP, inhibitor of apoptosis protein; ORF, open reading frame; ActD, actinomycin D; WSSV, white spot syndrome virus; BIR, baculoviral IAP repeat; Rpr; reaper; IBM, IAP-binding motif; UTR, untranslated region.

☆GenBank accession number of *Penaeus monodon* inhibitor of apoptosis protein: EF114675.

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apoptotic bodies [4]. The concept of apoptosis was first introduced in 1972 [5]. Since then, hundreds of genes that control apoptosis have been identified in different species, which is good evidence that the mechanisms and the molecules involved in this suicide program have been highly conserved throughout metazoan evolution [6].

Inhibitor of apoptosis proteins (IAPs) are a conserved group of proteins that regulate apoptosis in both vertebrates and invertebrates. Since the first discovery of an IAP gene in a baculovirus [7], numerous cellular IAP homologs have been identified [8,9]. The hallmark of an IAP protein is the presence of one to three copies of a zinc-binding baculoviral IAP repeat (BIR) domain. These BIR domains bind directly to the caspases to inhibit them, and they are essential for the anti-apoptotic properties of the IAPs [10,11]. The interaction between BIR domains and caspases is negatively regulated by proteins that contain an IAP-binding motif (IBM), including *Drosophila* Reaper (Rpr), Grim, and Hip, and mammalian Smac/DIABLO. The only sequence homology shared by these IBM proteins is a short consensus sequence at their amino termini. This conserved sequence is the IBM, which is responsible for binding to the BIR domain and interfering with IAP-mediated caspase inhibition [12]. The IAPs also contain another zinc-binding domain known as the RING domain, which is invariably located at their C-termini. The RING domain has an ubiquitin E3 ligase activity, which attaches ubiquitin to proteins that bind to the IAPs, including caspases, some IBM proteins, and IAP itself. The ubiquitinated IAP and IBM proteins are then subject to proteasomal degradation, while the ubiquitinated caspases, instead of being degraded, become inactive [12–14].

In addition to acting as important regulators in apoptosis, recent evidence suggests that some IAPs also play important roles in innate immunity in both mouse and *Drosophila* [15–19]. In *Drosophila*, microbe infection initiates innate immune responses through two major, distinct signaling pathways: the Toll pathway and the immune deficiency (Imd) pathway. The Toll pathway is predominantly activated by fungal and Gram-positive bacterial infections, whereas the Imd pathway responds to infection by Gram-negative bacteria. The activation of these two pathways causes members of the nuclear factor kB (NF-kB) family to translocate to the nucleus to activate the transcription of antimicrobial peptide genes [20]. The importance of *Drosophila* IAP2 (DIAP2) in *Drosophila* innate immunity was first revealed by two independent studies using large-scale RNAi screening in cultured *Drosophila* cells to identify novel components in the Imd pathway. Both studies showed that *diap2* RNAi abrogated the Imd-signaling antimicrobial peptide response [16,17]. Moreover, *diap2* null mutant *Drosophila* fail to induce the synthesis of antimicrobial peptides and are highly susceptible to infection by Gram-negative bacteria [18,19].

In the present paper, based on annotations of a 5′EST database generated from a *Penaeus monodon* post-larvae cDNA library, we identified a cDNA clone that appeared to encode an IAP protein. Since no crustacean IAP has been reported, we therefore decided to clone the full-length cDNA sequence of this *P. monodon IAP* (*PmIAP*) and to characterize this protein. In a series of functional assays, other anti-apoptosis proteins were also included for comparison. One of these is a novel, recently reported [21], anti-apoptosis protein (WSSV449, or ORF390) encoded by a shrimp viral pathogen, white spot syndrome virus (WSSV).

2. Materials and methods

2.1. Identification of a cDNA clone of a *P. monodon* inhibitor of apoptosis protein (PmIAP)

A cDNA library constructed from WSSV-infected postlarvae of *P. monodon* was subjected to large-scale 5′ and 3′ end sequencing, and the sequencing results were used to generate a 5′ and 3′ EST database. Analysis of the 5′ EST database using BLASTX against the NCBI non-redundant (nr) database revealed that one cDNA clone, PmTwI09F1, contained a partial nucleotide sequence that was highly homologous to the RING domain of the *Bombyx mori* inhibitor of apoptosis protein. Complete sequencing of this clone by primer walking showed that it contained only part of the nucleotide sequence of a putative *P. monodon IAP* gene.

2.2. Isolating the 5′ end of PmIAP cDNA

Pleopod (swimming leg) mRNA was purified using a QuickPrep Micro mRNA Purification Kit according to the manual supplied by the manufacturer (GE Healthcare). Briefly, pleopods from adult shrimp were powdered in the presence of liquid nitrogen, and further homogenized in the extraction buffer supplied with the kit. The homogenate was diluted with elution buffer, clarified by centrifugation at 12 000*g* for 5 min, and then mixed with oligo(dT)-cellulose. After extensive washing with high- and low-salt buffers, the mRNA was eluted with elution buffer, precipitated with ethanol, and then quantified at A260. A 5′ RACE kit was then used to isolate the 5′ end of *PmIAP* cDNA according to the instructions provided by the manufacturer (Roche Molecular Biochemicals). Three gene-specific primers, I09F1-SP1, I09F1-SP2, and I09F1-SP3 (Table 1), were designed according to the nucleotide sequence of the PmTwI09F1 cDNA clone. The first-strand cDNA was synthesized from 100 ng pleopod mRNA using I09F1-SP1 primer, tailed at the 3′ end with dATPs using the terminal transferase, and then subjected to first-round PCR using oligo(dT)-anchor primer and I09F1-SP2 primer. The PCR product was diluted and subjected to second-round PCR using anchor primer and I09F1-SP3 primer. The final PCR products of 5′ RACE were cloned into the pGEM-T easy vector (Promega) and used to obtain the complete cDNA sequence of *PmIAP*.

2.3. Sequence analysis of PmIAP

The deduced amino acid sequence of PmIAP was analyzed with BLASTP against the NCBI nr database and ScanProsite. For multiple sequence alignment, the BIR1, 2, and 3 domains and RING domain from PmIAP were aligned using GeneDoc (ver. 2.6.002) with the corresponding domains from SfIAP (AX213188), DIAP1 (Q24306), DIAP2 (Q24307), and XIAP (P98170).

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