Short communication

Bioinformatic characterization and gene expression pattern of apoptosis inhibitor from Macrobrachium rosenbergii challenged with infectious hypodermal and hematopoietic necrosis virus

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Abstract

Apoptosis is genetically programmed cellular killing processes that execute unnecessary or infected cells. It plays an important role in embryogenesis, homeostasis, insect metamorphosis and immunity. Apoptosis inhibitor (MrIAP) was sequenced from the freshwater prawn Macrobrachium rosenbergii using Illumina Solexa Genome Analyzer Technique. MrIAP consisted of 1753 base pair nucleotides encoded 535 polypeptide with an estimated molecular mass of 60 kDa. MrIAP amino acid sequence contains IAP superfamily domain between 5 and 490. The deduced amino acid sequences of the MrIAP were aligned with the other IAP family members. The highest sequence similarity was observed in IAP-5 from ant Camponotus floridanus (67%) followed by IAP from body louse Pediculus humanus corporis (66%) and the lowest (62%) in IAP-5 isoform-5 from common chimpanzee Pan troglodytes and IAP-5 from Aedes aegypti. The IAP phylogenetic tree showed that MrIAP closely related to other arthropod blacklegged tick Ixodes scapularis, formed a sister group with IAP from a hemichordate acorn worm Saccoglossus kowalevskii and finally clustered together with IAPs from fish groups. The quantitative real time PCR analysis revealed that significantly (P < 0.05) highest expression was noticed in hepatopancreas and significantly (P < 0.05) lowest expression in pleopods. Based on the results of gene expression analysis, MrIAP mRNA transcription in M. rosenbergii challenged to infectious hypodermal and hematopoietic necrosis virus (IHHNV) was highly induced in hepatopancreas. The collective results of this study indicate that the MrIAP is an essential immune gene and influences the immune response against IHHNV infection in M. rosenbergii.

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1. Introduction

Apoptosis or programmed cell death is a cellular killing process in which impaired or damaged cells are eradicated from multicellular organisms [1]. This concept was first introduced by Kerr in 1972 [2]. From then on, numerous genes have been identified in different species which control apoptosis [3–5]. The exclusive morphological changes observed during apoptosis include cell shrinkage, membrane blebbing, DNA fragmentation and apoptotic body formation [6]. This genetically maintained mechanism plays an important role in development, tissue homeostasis and removal of redundant cells during embryogenesis. It also causes cell atrophy upon endocrine withdrawal or reduction of necessary growth factor or cytokines, tissue remodeling and repair and disposal of cells that have undergone genotoxic damage [7].

Inhibitor of apoptosis proteins (IAPs) which regulate apoptosis in both vertebrates and invertebrates is a conserved group of protein [3]. A close relationship observed between baculoviral IAPs and insect IAPs indicates that baculoviral IAPs may have been formed through gene transfer from host insect cells. Recently, IAPs have been listed out in several multicellular species from Drosophila to mammals. However IAPs are absent in plants, yeast and protozoans [8].

IAP protein has unique structure that contains 1–3 copies of baculoviral IAP repeat (BIR) domain [1]. It comprises almost 70 peptides and has a conserved arrangement of Cys/His which is formed in a particular pattern of ‘stable fold structure’ that can chelate zinc [9,10]. This BIR domain attaches to caspases and prevent the anti-apoptotic qualities of IAPs, which is important for the biological process [10]. The relationship between BIR domains and caspases is negatively maintained by proteins which contain an
Fig. 1. Nucleotide and deduced amino acid sequences of MrIAP. The nucleotide sequence is numbered from 5' end, and the single letter amino acid code is shown below the corresponding codon. The start codon (ATG) and the end codon (TAA) are bolded. cAMP- and cGMP-dependent protein kinase phosphorylation sites are highlighted in light gray color. Tyrosine kinase phosphorylation sites are highlighted in dark gray color. And amidation site is underlined. The termination code is marked with an asterisk.
IAP binding motif (IBM). This IBM is responsible for attaching to the BIR domain and involving with the inhibition of IAP-mediated caspase [10].

The C terminal region of IAP contains a typical structural feature called ‘RING finger domain’ [8]. It has a crucial ubiquitin E3 ligase activity, which joins to ubiquitin that attaches to the IAPs including caspases. This ubiquitinated IAPs and IBM proteins are subjected to proteasomal degradation. However the ubiquitinated caspases became active, instead of being degraded [8,11,12]. Interestingly, Huang et al. [13] reported that the RING domain of a lepidopteran insect Spodoptera frugiperda IAP was found to increase the pro-apoptotic activity of mammalian caspase-9. Hence it is suggested that this RING region operate as a trans-dominant inhibitor of endogenous proteins involved in apoptosis suppression [1].

A recent review [14] describes the different classes and family members of IAPs based on the structure and homology of BIR regions and RING domain. So far 8 IAPs reported in mammals, these IAPs become active, instead of being degraded [8,11,12]. Interestingly, Huang et al. [13] reported that the RING domain of a lepidopteran insect Spodoptera frugiperda IAP was found to increase the pro-apoptotic activity of mammalian caspase-9. Hence it is suggested that this RING region operate as a trans-dominant inhibitor of endogenous proteins involved in apoptosis suppression [1].

2. Materials and methods

2.1. M. rosenbergii

Specific pathogen free (SPF) M. rosenbergii (average body weight 10 g) were obtained from the Bandar Sri Sendayan, Negeri Sembilan, Malaysia. They were maintained in flat-bottomed glass tanks (300 L) with aerated and filtered freshwater at 28 ± 1℃ in the laboratory. M. rosenbergii were acclimatized for 1 week before challenged to IHHNV. A maximum of 15 individuals per tank were maintained during the experiment.

2.2. Identification and sequence analysis of MrIAP

A full length MrIAP gene was identified from the M. rosenbergii transcriptome unigenes obtained by Illumina’s Solexa sequencing technology. Briefly, unigenes obtained from the assembly of the illumina Solexa short reads from the RNA sequencing of the muscle, gill and hepatopancreas transcriptomes of M. rosenbergii were mined for sequences which had been identified as inhibitor of apoptosis gene through BLAST homology search against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast). The open reading frame (ORF) and amino acid sequence of MrIAP was obtained by using DNAssist 2.2. Characteristic domains or motifs were identified using the PROSITE profile database [21]. Identity, similarity and gap percentages were calculated using FASTA program [22]. The N-terminal transmembrane sequence was determined by DAS transmembrane prediction program (http://www.sbc.su.se/~miklos/DAS). Signal peptide analysis was done using the SignalP world wide P server (http://www.cbs.dtu.dk). Pair-wise and multiple sequence alignment were analyzed using the ClustalW version 2.2. Identiﬁcation of the potential transmembrane helices was performed employing the DAS transmembrane prediction program (http://www.sbc.su.se/~miklos/DAS). Signal peptide analysis was done using the SignalP world wide P server (http://www.cbs.dtu.dk). Pair-wise and multiple sequence alignment were analyzed using the ClustalW version 2.2 program [23]. The phylogenetic relationship of the MrIAP was determined using the neighbor-joining (NJ) method and Phylib (3,69).
Fig. 2. Multiple sequence alignments of MrIAP with five other homologous amino acid sequences. IAP-5 from zebra fish Danio rerio (NP_955834), IAP-5B from African clawed frog Xenopus laevis (NP_001080226), apoptosis inhibitor from body louse Pediculus humanus corporis (BAH56608), yellow fever mosquito Aedes aegypti (EAT35554) and IAP 5 isoform b from human Homo sapiens (NP_006586) are shown. Asterisk marks indicate identical amino acids and numbers to the right indicate the aa position of apoptosis inhibitor in the corresponding species. Conserved substitutions are indicated by (;) and semi-conserved substitutions are indicated by (.). Deletions are indicated by dashes. GenBank accession numbers for the amino acid sequences given in the parentheses.
2.3. Infectious hypodermal and hematopoietic necrosis virus challenge

For IHHNV induced mRNA expression analysis, the prawns were injected with IHHNV, as described by Dhar et al. [24]. Briefly, IHHNV infected prawn tail tissue, tested positive by nested PCR was homogenized in sterile 2% NaCl (1:10, w/v) solution and centrifuged in a tabletop centrifuge at 5000 rpm for 5 min at 4°C. The supernatant was filtered through 0.45 μm filter and used for injecting (100 μl per 10 g prawn) the animals. Samples were collected before (0 h), and after injection (3, 6, 12, 24 and 48 h) and were immediately snap-frozen in liquid nitrogen and stored at -80°C until the total RNA was isolated. Using a sterilized syringe, the haemolymph (0.2–0.5 ml per prawn) was collected from the prawn heart and immediately centrifuged at 3000 g for 10 min at 4°C to allow hemocyte collection for total RNA extraction. Tissue homogenate prepared from healthy tail muscle served as control. All samples were analyzed in three duplications and the results are expressed as relative fold of one sample as mean ± standard deviation.

2.4. Gene expression analysis by real time PCR

Total RNA was extracted from the tissues of each animal using TRI Reagent following manufacturer’s protocol (Guanzhou Dongsheng Biotech, China). Total RNA was treated with RNase free DNA set (5 Prime GmbH, Hamburg, Germany) to remove the contaminating DNA. The total RNA concentration was measured spectrophotometrically (NanoVue Plus Spectrophotometer, GE Healthcare UK Ltd, England). First-strand cDNA was synthesized from total RNA by M-MLV reverse transcriptase (Promega, USA) following the manufacturer’s protocol with AFLP primer (5'GGCGCCAGCGTCG ACTAGTAC(T)16(A/C/G)3'0).

The relative expression of MrAP in the hemocytes, pleopods, walking legs, eye stalk, gill, hepatopancreas, stomach, intestine, brain and muscle were measured by quantitative real time polymerase chain reaction (qRT-PCR). qRT-PCR was carried out using a ABI 7500 Real-time Detection System (Applied Biosystems) in 20 μl reaction volume containing 4 μl of cDNA from each tissue, 10 μl of Fast SYBR® Green Master Mix, 0.5 μl of each primer.
The obtained complete *M. rosenbergii* apoptosis inhibitor was submitted to NCBI GenBank database under the accession number HQ668090. The full length sequence of MrIAP was 1753 base pairs (bp), containing a 1605 bp open reading frame (ORF) that encoded a 535 amino acid residues with a calculated molecular weight of 60 kDa, a 57 bp 3’ untranslated region (UTR) and a 91 bp 3’ UTR (Fig. 1). ScanProsite analysis revealed that the predicted MrIAP protein contains a long IAP superfamily domain between 5 and 490. Other than this big domain, MrIAP protein contains 33 common motifs including 7 casein kinase II phosphorylation sites at 66–69, 145–148, 172–175, 191–194, 234–237, 348–351 and 404–407; 9 N-myristoylation sites at 133–138, 280–285, 306–311, 453–458, 500–505, 514–519, 519–524, 523–528 and 530–535; 10 protein kinase C phosphorylation sites at 256–258, 259–261, 401–403, 439–441, 447–449, 463–465, 482–484, 495–497, 515–517 and 520–522; 2 N-glycosylation sites at 402–405 and 461–464; 2 cAMP- and cGMP-dependent protein kinase phosphorylation sites at 42–45 and 465–468; 2 tyrosine kinase phosphorylation sites at 296–304 and 479–487 and 1 amidation site at 526–529.

The deduced amino acid (aa) sequences of the MrIAP were aligned with the other IAP family members and presented in Table 1. The results revealed that *Aedes aegypti* has the longest amino acid sequence (557 aa), even though conserved motifs were observed among the sequences, the length of the amino acids varied from species to species. The MrIAP aa identity, similarity and gap percentages were calculated using FASTA program. We analyzed the MrIAP sequence identity with other 19 homologous sequences (Table 1). The highest sequence similarity was observed in IAP-5 from ant *Camponotus floridanus* (67%) followed by IAP from body louse *Pediculus humanus corporis* (66%) and the lowest (62%) in IAP-5 isoform-5 from common chimpanzee *Pan troglodytes* and IAP-5 from *A. aegypti*. All individuals taken for similarity analysis showed not less than 60% similarity with MrIAP.

Neighbor-Joining (NJ) phylogenetic trees were produced based on phylogenetic analysis of MrIAP (Fig. 3) of representatives from invertebrates, pisces, amphibians, birds and mammals. The IAP phylogenetic tree showed that MrIAP closely related to other arthropod blacklegged tick *Ixodes scapularis*, formed a sister group with IAP from a hemichordate acorn worm *Saccoglossus kowalevskii* and finally clustered together with IAPs from fish groups. The genetic distance is 0.05.

To investigate the tissue distribution of MrIAP transcripts, total RNA extraction followed by cDNA were synthesized from various *M. rosenbergii* tissues and subjected to quantitative real time PCR analysis. Fig. 4A shows that the MrIAP transcript was expressed in all the tissues analyzed. Significantly (*P < 0.05*) highest expression was noticed in hepatopancreas and significantly (*P < 0.05*) lowest expression at pleopods. Based on the results of tissue distribution analysis, MrIAP mRNA expression in *M. rosenbergii* was induced in hepatopancreas following challenged to IHHNV. MrIAP mRNA expression in hepatopancreas was significantly greater than the control and after 3, 6, 12 and 24 h post IHHNV infection (*P < 0.05*),

### Table 1

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Fig. 3. A phylogenetic tree of MrIAP with 19 other organisms was reconstructed by the NJ method. The tree is based on an alignment corresponding to full length aa sequences, using ClustalW and Phylip (3.69). The numbers shown at the branches denote the bootstrap majority consensus values of 1000 replicates. The GenBank accession number, gene and species details are given in Table 1.

Fig. 4. Gene expression patterns of MrIAP by qRT-PCR. A: Tissue distribution of MrIAP in different tissues of *M. rosenbergii*. Data are expressed as a ratio to MrIAP mRNA expression in pleopod. The different alphabets are statistically significant at $P < 0.05$ level by one-way ANOVA and Tukey’s Multiple Range Test. B: The time course of MrIAP mRNA expression in hepatopancreas at 0, 3, 6, 12, 24, and 48 h post injection with IHHNV. Data are expressed as a ratio to MrIAP mRNA in sample from injected control group. The significant difference at $P < 0.05$ level by one-way ANOVA and Tukey’s Multiple Range Test of MrIAP expression between the challenged and the control group were indicated with different alphabets.
peaking up to 7.5 times above the control after 12 h post infection (Fig. 4B). Control groups were yielded no significant increase in expression levels.

4. Discussion

Certain diseases and its cure often involve the right intervention in mitotic or apoptotic processes [26]. According to Canlon et al. [27] and Bahekar et al. [28] deregulation of this mechanism resulting in cell loss can cause stroke, neuro-degeneration and hearing impairment. Commonly, regulation of programmed cell death (PCD) occurs inter alia in pro- and anti-apoptotic BCL-2 protein family members and IAPs through gene expression [8,10–12]. In this study, we have reported the infection of IHHNV on IAP prawn immune cells. The MrIAP was isolated from M. rosenbergii muscle, gill and hepatopancreas transcriptome database. The MrIAP is similar in the sequence composition to several invertebrates and vertebrates including fishes, amphibians, birds and mammals. The motif analysis of MrIAP is in accordance with the human IAP protein suggesting functional conservation [29]. MrIAP has the distinctive IAP superfamily domain which is responsible for the apoptosis suppression as reported by Holcik et al. [30] and Clem et al. [31]. This typical structural region in IAP family plays an important role in mediating cell survival and hypothesized to support protein–protein interaction [32,33]. The MrIAP ORF encodes a 535 amino acid protein with a predicted molecular weight of 60 kDa and shows 64% (protein) homology to MrIAP. From a phylogenetical point of view, IAPs including only one IAP superfamily domain, formed a cluster separated from that of IAPs containing two IAP superfamily domains, due to domain divergence [34]. Accordingly, MrIAP sequence is more similar to other IAPs containing single domain than to IAP which contains two.

IHHNV infection significantly (P < 0.05) induced apoptosis levels of the host immune cells, and the different time course of this induction varied. Rothe et al. [35] reported that IAPs expression can be stimulated through virus infection. And also virus infection can change the host cell gene expression. Further they reported that even in some cases the viral protein itself act as an apoptosis inhibitor [35]. In this study, we observed that MrIAP mRNA transcription is significantly (P < 0.05) expressed at different time points with respect to the IHHNV infection. The results of gene expression study showed that the MrIAP are involved in the immune function of M. rosenbergii and act against IHHNV infection. Similar results were reported in tiger prawn P. monodon [19], Chinese mitten crab E. sinensis [20] and the eastern oyster Crassostrea virginica [36] infected with white spot syndrome virus, Vibrio anguillarum and Perkinsus marinus respectively. The results of our studies indicate an increasing pattern of peaks is observed till about 12 h and afterwards MrIAP mRNA transcription starts to decline. This is in consistence with the studies by Hughes et al. [36]. The immune system is the first line of defense to protect the host in the first hours to days of infection. The host tries to eliminate the pathogen and heal the infection successfully [37]. However, the MrIAP mRNA expression of M. rosenbergii depends on both the pathogen and gene. Conclusively, the results of this study indicated that the MrIAP is an essential defense gene and influences the immune response against IHHNV infection in M. rosenbergii. Further research is necessary on the characterization of MrIAP at the protein level to reveal its role in M. rosenbergii immune system.

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References


