**Role of *Penaeus monodon* hemocyte homeostasis associated protein (PmHHAP) in
regulation of caspase-mediated apoptosis**

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Abstract

The viral responsive protein, *PmHHAP*, plays an important role in the control of hemocyte homeostasis in shrimps during viral infection. In this study, we further investigate the role of *PmHHAP* in the regulation of hemocyte apoptosis. RNA interference (RNAi) mediated gene silencing was used to suppress the *PmHHAP* expression and the change in hemocyte apoptosis was determined in the knockdown shrimp. Within circulating hemocytes, *PmHHAP* knockdown increased the number of annexin V-positive apoptotic cells and the combined caspase-3/-7 activity and induced the characteristic apoptotic DNA ladder. Furthermore, *PmHHAP* down-regulation was accompanied by significantly altered expression of apoptosis-related proteins including the effector caspases, *PmCaspase* and *PmCasp*. Yeast two-hybrid and co-immunoprecipitation assays showed that *PmHHAP* binds to the p20 domain of *PmCasp*. Moreover, the recombinant *PmHHAP* protein was able to reduce the caspase activity in the actinomycin D-treated hemocyte cells and r*PmCasp*-treated hemocyte cells. Taken together, our data indicate that *PmHHAP* regulates hemocyte homeostasis by inhibits apoptotic cell death through caspase activation.

1. Introduction

Hemocyte is considered as an immune-responsive cell that plays the most important role in the immunity of arthropods (Amparyup et al., 2013; Jiravanichpaisal et al., 2006; Johansson et al., 2000; Lavine and Strand, 2002; Tassanakajon et al., 2013). Hemocytes are constantly produced and released from the hematopoietic tissue (Hpt). However, their number in the circulation can transiently dramatically upon injury or in response to microbial infections (Jiravanichpaisal et al., 2001; Johansson et al., 2000; Persson et al., 1987; Smith and Söderhäll, 1983). Homeostasis, achieved by the continuous proliferation and apoptosis, is thus required for the maintenance of hemocyte balance in the circulation.

Hemocyte homeostasis is a complex process by which the hemocyte numbers are maintained at an appropriate level. It was previously shown that a hemocyte homeostasis-associated protein (*PmHHAP*) plays a role in hemocyte homeostasis during viral infection in the black tiger shrimp *Penaeus monodon* (Prapavorarat et al., 2010). Silencing of the *PmHHAP* transcripts resulted in a greatly decreased level of circulating hemocytes together with hemocyte deformation and lysis (Prapavorarat et al., 2010). In addition, suppression of *PmHHAP* also led to 100% mortality of the *PmHHAP* knockdown shrimp within 30 h post dsRNA injection, indicating the importance of *PmHHAP* in shrimp viability (Prapavorarat et al., 2010).

PmHHAP belongs to the zinc finger protein family, which are small regulatory protein domains characterized by a special secondary structure that is stabilized by a zinc ion coordinated with the Cys- and/or His- residues (Klug and Schwabe, 1995). Zinc finger domains can bind to DNA, RNA, proteins and lipids (Hall, 2005) and a role for zinc finger containing proteins in mediating apoptosis has been described previously (Krishna et al., 2003; Verhagen et al., 2001; Vitorino et al., 2014).

Apoptosis is an evolutionarily conserved cell suicide process that is initiated through the extrinsic caspase-dependent and intrinsic caspase-independent pathways (Galluzzi et al., 2012). The executioner of apoptosis are cysteine-dependent aspartate-directed proteases or caspases. Caspases are synthesized as inactive precursors containing three domains: an amino-terminal prodomain; a large subunit that contains the active-site cysteine (p20); and a carboxy-terminal small subunit (p10) (Riedl and Shi, 2004). In shrimp, several caspase genes have been identified, included *PjCaspase* from kuruma shrimp *P. japonicus* (Wang et al., 2008), *cap-3* and *Lvcaspase2-5* from white shrimp *P. vannamei* (Rijiravanich et al., 2008; Wang et al., 2013), *cap-3* from banana prawn *P. merguensis* (Phongdara et al., 2006) and two caspases, *PmCasp* and *PmCaspase*, from *P. monodon* (Leu et al., 2008a; Wongprasert et al., 2007).

Recently, a protein named crustacean hematopoietic factor (*Pl-CHF*) has been demonstrated as an essential factor that important in the crossroad between apoptosis and hemocyte differentiation in crayfish *Pacifastacus leniusculus*. *Pl-CHF* is a small cysteine-rich protein that most similar to the N-terminal part of the human CRIM1 protein. *In vivo* and *in vitro* RNAi experiments revealed severe losses of hemocytes and a higher apoptotic rate in the Hpt cells indicated the roles of *Pl-CHF* in preventing apoptosis of crayfish hemocytes and Hpt cells (Lin et al., 2011).

In shrimp, our previous study demonstrated that a *PmHHAP* plays an important role in hemocyte homeostasis (Prapavorarat et al., 2010). However, the mechanism underlying the function of *PmHHAP* in mediating hemocyte homeostasis in shrimp remains unclear. In the present study, the potential role of *PmHHAP* in the regulation of hemocyte homeostasis in *P. monodon* shrimps was evaluated using RNAi knockdown and protein-protein interaction assays. Our results suggest that *PmHHAP* controls hemocyte homeostasis in shrimp by inhibiting the caspase-dependent apoptotic pathway.

2. Materials and methods

2.1 Experimental shrimp

Specific pathogen-free (SPF) shrimp *P. monodon* were obtained from the Shrimp Genetic Improvement Center, BIOTEC, Thailand. Shrimp were maintained in tanks with aerated sea water (20 ppt) for 7 days before processing to allow acclimatization.

2.2 Preparation of double-stranded RNA (dsRNA)

Double-stranded RNAs (dsRNAs) of *PmHHAP* and GFP genes were generated using *in vitro* T7 RiboMAX[™] Express Large Scale RNA Production System (Promega) as previously described (Prapavorarat et al., 2010) with some minor modifications. Briefly, two PCR reactions were set up using the gene specific primers flanked by a T7 promoter sequence at the 5' ends (Table 1) to produce the sense and anti-sense strands DNA templates, separately. To generate dsRNA, 1 µg of each gel purified PCR products was used as a template for *in vitro* transcription according to the instruction (Promega). Equal amount of sense and anti-sense single-stranded RNAs were annealed to produce dsRNA and the remaining DNA templates were then digested with RNase-free DNase I (Promega). The quality of dsRNAs was verified by agarose gel electrophoresis with UV visualization following by the ethidium bromide staining and quantified by using UV spectrophotometer.

2.3 In vivo gene knockdown of *PmHHAP* and semi-quantitative RT-PCR analysis

SPF shrimp of ~2 g, fresh weight were used for *in vivo* RNAi experiments. Briefly, 25 µl of 150 mM NaCl solution containing 3 µg of *PmHHAP* or GFP dsRNA was injected into each shrimp. At 12 h post injection (hpi), hemocytes were collected from each shrimp and total RNA was then extracted using the TRI Reagent[®] (Molecular Research Center) followed

by DNase I (Promega) treatment. First-stranded cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instruction. The efficiency of *PmHHAP* gene transcript silencing was verified by semi-quantitative RT-PCR using the specific primer for *PmHHAP* gene (Table 1). A fragment of elongation factor 1- α (EF1 α) was also amplified and used as an internal control. One microlitre of cDNA was used as template for the PCR amplification in a 25 μ l reaction volume containing 1X reaction buffer, 100 μ M of dNTP, 0.2 μ M of each gene specific primers and 1 unit of RBC Taq DNA polymerase (RBC Bioscience). The thermal cycling was performed at 94 °C for 1 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and subsequently a final 72 °C for 5 min, except only 22 cycles were performed for the EF1 α gene. The PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis and visualized by UV-transillumination.

2.4 Apoptosis analysis in hemocyte cells of the *PmHHAP* silenced shrimp

DsRNA-mediated RNAi knockdown of *PmHHAP* gene was performed as described above. Hemolymph was withdrawn from the experimental and control shrimp at 12 hpi with the respective dsRNA and hemocytes were subsequently collected by centrifugation at 800 $\times$ g for 10 min at 4 °C followed by washing with the phosphate buffer saline (PBS, pH 7.4). The number of apoptotic hemocytes in the *PmHHAP* knockdown shrimp and controls were then analyzed using the annexin-V-FLUOS Staining Kit (Roche) according to the manufacturer's protocol. Briefly, the hemocyte number was counted under a light microscope, and then distributed into the wells of a 96-well plate at 10⁴ cells per well. Then, the hemocyte cells were incubated with 100 μ l annexin-V-FLOUS labeling solution for 10-15 min at room temperature and examined under a fluorescence microscope (Olympus). The positive green fluorescent signal of annexin-conjugated FITC from the apoptotic cells were scored as

apoptotic positive cells. The apoptotic hemocyte rate was expressed as the mean $\pm$ S.D. of three replicates.

2.5 Apoptotic DNA ladder detection in shrimp hemocyte

To elucidate whether *PmHHAP* is involved in the control of apoptosis in shrimp *P. monodon*, *in vivo* RNAi knockdown of *PmHHAP* gene transcripts was carried out as described above. Control shrimp were injected with GFP dsRNA. The hemocytes were collected from shrimps at 12 hpi and DNA was extracted using the Apoptotic DNA Ladder Detection Kit (Abcam) according to the manufacturer's protocol. Briefly, hemocyte cells were washed with PBS and pellet cells by centrifugation for 5 min at 800 $\times$ g. Cells were lysed with TE lysis buffer and then incubated with enzyme A at 37 °C for 10 min and with enzyme B at 50 °C for 30 min. The DNA was precipitated by isopropanol and washed with 70% (v/v) ethanol. DNA samples were analyzed on a 1.2% (w/v) agarose gel and visualized by under UV light after ethidium bromide staining.

2.6 Caspase activity assay in shrimp hemocyte

Activities of key member of the apoptotic pathway (caspase 3 and 7) were determined in hemocyte of *PmHHAP* knockdown shrimp. At 12 h post-dsRNA injection, the hemolymph was withdrawn from each shrimp as described above. After centrifugation, the cell pellets were washed with PBS and the total protein concentration of the cell suspension determined using Bradford Reagent (Bio-Rad). An equal concentration of shrimp hemocytes (as protein) from each of three shrimps per treatment was pooled and then analyzed for the caspase-3 and -7 activity using the Caspase-Glo 3/7 Assay Kit (Promega) according to the manufacturer's instruction. Briefly, 100 μ l of the pooled hemocyte cell suspension was seeded in the 96-well white plate, and then 100 μ l of Caspase-Glo reagents was added to each well, gently mixed

and incubated for 15 min. The luminescence of each sample was then measured in a plate-reading luminometer (Molecular devices). The data were reported as the mean $\pm$ S.D. of three replicates.

2.7 Analysis of apoptotic genes expression in *PmHHAP* knockdown hemocyte using Real-time RT-PCR

The transcript levels of the apoptotic related-genes were evaluated using quantitative real-time RT-PCR (qRT-PCR). The *PmHHAP*-silenced and control shrimp were prepared as described above. Shrimp hemocyte (three individuals each) was collected at 12 h post-dsRNA injection. Total RNA was extracted from the hemocyte, and used to synthesize single stranded cDNAs with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative real-time RT-PCR was performed using an equal amount of cDNAs in a CFX96 TouchTM Real-Time PCR Detection system using SsoFastTM EvaGreen[®] Supermix (Bio-Rad) with the following conditions: 95 °C for 8 min, 40 cycles of 95 °C for 10 s, 58 °C for 15 s and 72 °C for 30 s. The specific oligonucleotide primers used for amplification of *PmCasp* (DQ846887), *PmCaspase* (EF114674), cathepsin B (EF213113), cathepsin D (EF213114), defender against apoptotic death (DAD1) (EF581986) and survivin (GU903910) are shown in Table 1. The EF1 α was amplified as the internal control and reference standard to verify the quantitative real time PCR reaction. Each real-time PCR reaction was done in triplicate. The results were presented as the relative expression ratios of the target genes expressed in the hemocyte of *PmHHAP* silenced shrimp versus the GFP control shrimp, with normalization to a reference gene (EF1 α) transcript levels in the same cDNA sample.

2.8 Yeast two-hybrid assay

The yeast two-hybrid assay for detecting interaction between *PmHHAP* and the p20 domain of *PmCasp*, p10 domain of *PmCasp* or *PmCasp* were carried out basically according to the strategies from the Matchmaker GAL4 Two-Hybrid System (Clontech). The coding sequences of *PmHHAP*, p20-*PmCasp*, p10-*PmCasp* and *PmCasp* were amplified from total RNA extracted from *P. monodon* hemocytes using SuperScript One-Step RT/Platinum Taq Mix (Invitrogen) with the specific primer pairs *PmHHAP*-F-*NdeI*/*PmHHAP*-R-*XhoI*, F-p20-*NdeI*/R-p20-*BamHI*, *PmCasp*-p10-F-*NdeI*/R-*PmCasp*-*BamHI* and F-*PmCasp*-*NdeI*/R-*PmCasp*-*BamHI*, respectively (Table 1). The amplification reaction and PCR temperature profiles were carried out as described previously (Lertwimol et al., 2014). Except for *PmHHAP*, which was digested with *NdeI* and *XhoI*, all amplified products were digested with *NdeI* and *BamHI* restriction nucleases. The bait plasmid was generated by cloning the purified *PmHHAP* fragment into the *NdeI*/*SalI* site downstream of GAL4 DNA-binding domain (BD) of pGBKT7 (Clontech), while prey plasmids were constructed by cloned the p20-*PmCasp*, p10-*PmCasp* and *PmCasp* fragments into the *NdeI*/*BamHI* sites downstream of GAL4 DNA-activation domain vector (AD) of pGADT7 (Clontech). The constructs were subsequently sequenced to confirm gene sequences and correct reading frames. Yeast two-hybrid assays were then performed by co-transforming the bait and prey plasmids simultaneously into the yeast cells *Saccharomyces cerevisiae* strain AH109 using the lithium acetate/dimethyl sulfoxide method (Gietz et al., 1995). Yeast cells were grown on low stringency media lacking leucine and tryptophan (-L/-W) and the binding was tested in the high stringency medium lacking adenine, histidine, leucine and tryptophan (-A/-H/-L/-W) supplemented with X- α -gal (Apollo Scientific).

2.9 Co-immunoprecipitation

To confirm the protein-protein interaction of *PmHHAP* and the p20 domain of *PmCasp*, co-immunoprecipitation (co-IP) was carried out. The recombinant (r) *PmHHAP* protein and p20 domain (rP20) of *PmCasp* were produced in *E. coli*. The *NdeI/BamHI* digested p20- *PmCasp* fragment (described above) was inserted in-frame with the *NdeI/BamHI* sites of pET15b expression vector (Novagen). The recombinant plasmid (pET15b-rP20-*PmCasp*) was transformed into competent *E. coli* XL1-Blue and positive recombinant clones were analyzed by DNA sequencing. The selected recombinant plasmid was then transformed into *E. coli* BL21 (DE3) cells. The His₆-fusion proteins of *PmHHAP* (Prapavorarat et al., 2010) and rP20 of *PmCasp* were expressed by induction with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and used in the co-IP between r*PmHHAP* and rP20 of *PmCasp*. The polyclonal *PmHHAP* antibody was immobilized and cross-linked with protein A-sepharose CL4B (GE Healthcare) using dimethyl pimelimidate. Fifty microlitres of cross-linked bead was equilibrated with binding buffer (1X PBS, pH 7.4 and 1 M NaCl) and then 40 μ g of r*PmHHAP* and/or 60 μ g of rP20 of *PmCasp* were added and incubated at 4 °C overnight. The proteins were eluted with 100 mM glycine (pH 2.5). The immunoprecipitated complexes were then separated by SDS-PAGE (18% (w/v) acrylamide) and subjected to western blot analysis. The r*PmHHAP* and rP20 of *PmCasp* were detected using anti-His tag antibody (1:5000; Millipore) and goat anti-mouse IgG-AP conjugate (1:10,000; Millipore). The positive bands were detected using NBT/BCIP solution.

2.10 Analysis of PmHHAP-suppressed caspase activation in shrimp hemocyte

In order to investigate whether *PmHHAP* can suppress caspase activity in shrimp hemocyte, the caspase activity was detected using Caspase-Glo 3/7 assay (Promega). Hemocytes were collected from five individual shrimp, homogenized in PBS and the total protein concentration of each resultant shrimp hemocyte lysate supernatant (HLS) was

measured by the Bradford method. Each shrimp HLS was separated into four groups (150 µg of HLS per group made up to a final volume of 100 µl with PBS), the first group was incubated with 5 µM of *rPmHHAP*, the second group with 5 µM of control protein (bovine serum albumin, BSA) and other groups contain only HLS. After incubation of proteins with actinomycin D (ActD; 50 µg/µl), Caspase-Glo reagents (100 µl) was added and luminescent signals were measured at 15 min using microplate reader (Molecular devices). Moreover, to investigate whether *PmHHAP* can inhibit the caspase activation directly by *PmCasp*, the caspase activity detected using Caspase-Glo 3/7 assay (Promega) was performed. After prepared the HLS, shrimp HLS were separated into 4 groups (150 µg of HLS per group made up to a final volume of 100 µl with PBS), the first group was incubated with 10 µM of *rPmHHAP*, the second group with 7 µM of *rPmCasp*, the third group with 10 µM of *rPmHHAP* and 7 µM of *rPmCasp* and the remaining groups contain HLS with and without BSA. After incubation of proteins for 30 minutes, Caspase-Glo reagents (100 µl) was added and measuring the luminescent signals after 15 min using microplate reader (Molecular devices). The results were expressed as the mean $\pm$ S.D. of three replicates.

3. Results

3.1 Silencing of the *PmHHAP* gene by dsRNA injection

To understand the functional role of *PmHHAP* in shrimp hemocyte apoptosis, the gene silencing was performed to suppress the *PmHHAP* transcript and protein levels in shrimp. After 12 h of dsRNA injection, total RNA from the hemocytes of knockdown and control shrimp was prepared and subjected to semi-quantitative RT-PCR and Western blot analyses. Successful gene silencing in shrimp was achieved by injection of *PmHHAP* dsRNA (3 µg/g shrimp) (Fig. 1A). According to the *PmHHAP* / EF1 α ratio of DNA band intensity in the knockdown shrimp compared to the control shrimp, approximately 88% reduction of

PmHHAP transcript level was observed. Similarly, a marked decrease in the *PmHHAP* protein level was found in the knockdown shrimp (Fig. 1B).

3.2 Apoptosis is induced in hemocyte of PmHHAP-knockdown P. monodon

The level of apoptosis in hemocytes of the *PmHHAP*-knockdown and control (GFP dsRNA-treated) shrimp was determined by the annexin-V-FLUOS staining assay. Hemocyte cells of the *PmHHAP*-silencing shrimp (ds*PmHHAP*) clearly displayed an enhanced FITC-conjugated annexin-V signal compare to the control samples (Fig. 2A). The apoptotic rate, as the proportion of annexin-V-positive cells, was increased 2.4-fold in the *PmHHAP* knockdown shrimp compared to that in the control shrimp injected with GFP dsRNA (Fig. 2B).

3.3 Apoptotic DNA fragmentation occurred in the PmHHAP-knockdown shrimp hemocytes

DNA fragmentation is a key characteristic of late apoptosis. To confirm that suppression of *PmHHAP* induces apoptosis in shrimp hemocyte, the gene silencing experiment was performed as described above. After 12 h post dsRNA injection, analysis of DNA fragmentation in hemocyte of the knockdown shrimp was evaluated by agarose gel electrophoresis. The characteristic of DNA ladder pattern of apoptosis was detected in the hemocytes of the *PmHHAP* knockdown shrimp (Fig. 3) but not in the control shrimp, suggesting the role of *PmHHAP* in controlling apoptosis in hemocytes.

3.4 Activation of caspase activity in hemocytes from PmHHAP-knockdown P. monodon

Based on the above results, we hypothesized that the elevated annexin V-positive (apoptotic) cells and DNA fragmentation levels in hemocyte *PmHHAP* knockdown shrimps

is associated with the activation of caspase activity levels. To determine whether or not *PmHHAP* is involved in the caspase-dependent apoptosis, the activation of caspase-3 and -7 in hemocyte cells of the knockdown shrimp was analyzed. It was found that the activities of caspase-3 and -7 in hemocyte of the *PmHHAP* silenced shrimp was increased 3.9-fold at 12 hpi, compared with the control shrimp (Fig. 4). This supports that hemocyte apoptosis was significantly induced by *PmHHAP* dsRNA knockdown, and is congruent with the results of the annexin V-positive apoptotic cells and DNA fragmentation.

3.5 PmHHAP suppression results in the altered expression of effector caspases

To investigate whether *PmHHAP* transcript effect to the mRNA expression of apoptotic-related genes in shrimp, dsRNA-mediated gene knockdown of *PmHHAP* was performed. The different apoptotic related-genes such as two effector caspases *PmCasp* and *PmCaspase*, cathepsin B, cathepsin D, defender against apoptotic death (*PmDAD1*) and survivin were selected for quantitative real-time RT-PCR based evaluation of their relative expression levels. After silencing of *PmHHAP*, the expression of *Pmcasp* was slightly decreased while the *Pmcaspase* was significantly down-regulated (3.4-fold) (Fig. 5A). Moreover, the expression of cathepsin B and cathepsin D showed significantly decreased about 5-fold and 7.5-fold, respectively (Fig. 5A). Transcript levels of anti-apoptotic genes, *PmDAD1* and survivin, were significantly lower by about 3.6-fold and 2.9-fold, respectively (Fig. 5A). Because decreased *PmHHAP* expression levels can lead to an increase caspase activity in shrimp, the effect of suppressing *PmHHAP* on the expression level of two isoforms of effector caspases in shrimp *P. monodon*, *PmCasp* (Wongprasert et al., 2007) and *PmCaspase* (Leu et al., 2008a) that share 30% sequence identity, was further evaluated using the quantitative real-time PCR analysis. It was found that the gene silencing of *PmHHAP* observed at 12, 18, and 24 h post dsRNA injection (Fig. 5B) was accompanied with a

reduction of *PmCaspase* transcript levels at the same time points (Fig. 5B). Interestingly, the mRNA level of *PmCasp* was significantly increased at 18 h in hemocyte of the *PmHHAP* knockdown shrimp (Fig. 5B). These results suggested that suppression of *PmHHAP* can altered the transcription of effector caspases in the apoptosis pathway.

3.6 *PmHHAP* interacts with *PmCasp*

Yeast two-hybrid system was performed to detect the interaction of *PmHHAP* with the effector caspase, *PmCasp*, or with p20 domain or p10 domain of *PmCasp*. The results showed that *PmHHAP* was able to interact with the p20 domain of *PmCasp* (Fig. 6A), but not with a mature protein or a p10 domain of *PmCasp*, indicating that *PmHHAP* is capable of interacting with *PmCasp* via its p20 subunit. Furthermore, the co-immunoprecipitation assay was applied to verify the existence of the physical association between *PmHHAP* and p20 subunit of *PmCasp*. The result confirmed the interaction of *PmHHAP* with the p20 subunit of *PmCasp* (Fig. 6B). These results implied that *PmHHAP* might function with *PmCasp* to control apoptosis in shrimp.

3.7 Inhibition of caspase activation in shrimp hemocyte by *rPmHHAP*

The ability of *PmHHAP* to inhibit the apoptosis induced by actinomycin D was evaluated in shrimp HLS. The caspase activation analysis was performed by incubating the *rPmHHAP* and shrimp hemocyte lysate (HLS) with actinomycin D, followed by measuring the caspase activity. The results revealed that the caspase-3 and -7 activities were significantly decreased (25%), compared with the control group (BSA) (Fig. 7A). Moreover, no significant differences in caspase-3 and -7 activities were noticed among the groups of actinomycin D-treated HLS in the presence or absence of BSA. However, the level of caspase activity in actinomycin D-treated HLS was significantly increased (50%), compared to non-

treated HLS. In addition, the inhibition of apoptosis by *rPmHHAP* through caspase activation was performed by incubating the *rPmHHAP* and *rPmCasp* with shrimp hemocyte lysate (HLS), then measuring the caspase activity. It was found that the caspase-3 and -7 activities in *rPmHHAP* and *rPmCasp* treated-HLS were significantly decreased (67%), compared with the *rPmCasp*-treated HLS (Fig. 7B). No significant differences in caspase-3 and -7 activities was observed among the groups of HLS treated with and without *rPmHHAP* and with BSA. This result suggested that *PmHHAP* likely inhibits shrimp hemocyte apoptosis via inhibition of *PmCasp* activity.

4. Discussion

Apoptosis, comprising caspase-dependent and caspase-independent pathways, plays a critical role in biological processes including development, maintaining cells population homeostasis and host defense to pathogenic infection (Bortner et al., 1995; Fuchs and Steller, 2011; Henson and Hume, 2006). Normally, apoptosis is influenced by intracellular signals that directly act on targets within the cells and extracellular signals which required the binding of ligand to the receptor to activate apoptosis (Elmore, 2007). This pathway is initiated by the activation of caspases and results in morphological and biochemical changes and finally demolishes the cell by phagocytosis (Martinvalet et al., 2005). To date, the molecular mechanisms of apoptosis in animals have been well studied (Galluzzi et al., 2012; Kumar, 2007; Riedl and Shi, 2004). In host defense mechanism, apoptosis plays a key role in antiviral in animals by limiting viral multiplication through the destruction of virally infected cells. In many cases, the progeny of virus was decreased in the apoptotic cells and virus multiplication can be interfering by host cells apoptosis (Vandergaast et al., 2011; Koyama et al., 2000). Nevertheless, some viruses have the ability to prevent host cell apoptosis by activation of viral and/or host cell anti-apoptotic proteins and so prolong the viability of the

host cells sufficiently in order to enhance viral replication, assembly and release (Everett and McFadden, 1999; Koyama et al., 2000, 2003).

During the process of apoptosis, the morphological features and biochemical changes occur such as chromatin condensation, a degradation of chromosomal DNA into DNA fragments (DNA ladder), formation of apoptotic bodies, nuclear pore disruption and the appearance of phosphatidylserine on the cell surface (Martinvalet et al., 2005; Koyama et al., 2000) as well as the activation of caspases, the key regulators of apoptosis (Saraste et al., 2000). Thus, several methods are used to characterize apoptosis cells including detection of DNA fragmentation, assay of caspase activity and detection of the membrane alteration (Bossy-Wetzel and Green, 2000; Wyllie, 1980).

It has previously been shown that *PmHHAP*, which is up-regulated after WSSV infection, plays an important role in shrimp hemocyte homeostasis during viral infection (Prapavorarat et al., 2010). Based on the function of *PmHHAP* in hemocyte homeostasis, we speculate that *PmHHAP* may be involved in the regulation of hemocyte apoptosis. In the present study, the putative role of *PmHHAP* as an anti-apoptosis protein was elucidated by RNAi-mediated gene silencing. Suppression of *PmHHAP* mRNA in shrimp hemocyte caused the translocation of phosphatidylserine to the outer layer of the membrane, as shown by the increased proportion of annexin-V positive cells. Moreover, the apoptotic DNA fragmentation and the increasing caspase 3/7 activity in *PmHHAP* knockdown hemocyte suggested that *PmHHAP* likely acts as an anti-apoptotic protein.

Homology analysis of the deduced amino acid sequence revealed that *PmHHAP* displayed 50 and 65% amino acid similarity to zinc finger CCHC domain-containing protein 24 (*Zcchc24*) of human and insect, respectively (Fig. S1). The *Zcchc24* protein is composed of two major domains of different zinc fingers including zf-CCHC and zf-3CxxC

superfamilies, but little is known about the function of this novel protein (Vitorino et al., 2014). Zinc finger proteins (ZFPs) are one of the largest protein superfamilies in animals and are involved in diverse functions, such as DNA/RNA-binding proteins or factors and protein–protein interactions (Laity et al., 2001). Recently, the expression pattern of a *Zcchc24* protein during early development of frogs and chickens was analyzed, where it was suggested that it might be involved in the chicken heart formation (Bento et al., 2011) and in the regulation of apoptosis in heart, somite and branchial arch formation of frogs (Vitorino et al., 2014). However, the expression and biological function of *Zcchc24* protein homologues in insect are not known. In shrimp, analysis of *PmHHAP* protein sequence by SMART program indicated that *PmHHAP* contains a zinc finger domain of zf-3CxxC superfamily that is similar to the C-terminal zinc finger domain (zf-3CxxC) of *Zcchc24* proteins. Generally, the zf-3CxxC domains are identified in several chromatin-associated proteins and are reported to be involved in DNA/ chromatin modifications (Birke et al., 2002; Blackledge et al., 2010). Whether shrimp *PmHHAP* indeed acts as a chromatin-associated protein was not addressed in this study, but the high sequence similarity of *PmHHAP* and *Zcchc24* proteins in the zinc finger domain support the hypothesis that *PmHHAP* is involved in the regulation of apoptosis.

There are several genes involve in apoptosis pathway. Cathepsin B, a cysteine proteinase has been reported to be involved in apoptosis (Chwieialski et al., 2006; Liu et al., 2006). In shrimp, cathepsin B was reported to response to *Vibrio harveyi* infection in *P.monodon* (Pongsomboon et al., 2008). Cathepsin D, an aspartic proteinase has been reported to induces apoptosis in mammalian cell by working together with caspase and p53 (Chwieralski et al., 2006). In contrast, the role of cathepsin in crustacean apoptosis is still unknown. The defender against apoptotic death (DAD1) is a negative regulator of apoptosis that has been reported to respond to YHV infection in *P.monodon* (Molthathong et al., 2008).

Survivin is an inhibitor of apoptosis that not only prevents apoptosis but also plays a role in mitotic regulation (Leu et al., 2008b; Chen et al., 2012). In crustaceans, to date, a number of apoptotic-associated proteins have been characterized including caspases (Lertwimol et al., 2014; Leu et al., 2008a, 2010, 2013; Phongdara et al., 2006; Rijiravanich et al., 2008; Wang et al., 2008, 2013; Wongprasert et al., 2007), inhibitor of apoptosis proteins (IAP) (Leu et al., 2008b, 2012), the mitochondrial related voltage-dependent anion channel (VDAC) (Wang et al., 2010), fortilin (TCTP) (Bangrak et al., 2004), and gC1qR (Watthanasurorot et al., 2010, 2013). In white shrimp *Litopenaeus vannamei*, silencing of *LvIAP1* caused 100% shrimp mortality and dramatically decreases of hemocytes because of excessive apoptosis (Leu et al., 2012). Interestingly, the effect of knockdown *LvIAP1* was similar to that of knockdown *PmHHAP* (Prapavorarat et al., 2010).

Recently, a crustacean hematopoietic factor (CHF) was functionally characterized in the crayfish *Pacifastacus leniusculus*. CHF is crucial for the survival of hemocyte and hematopoietic tissue cells by preventing their apoptosis and plays an important role in hemocyte homeostasis and in astakine-induced hematopoiesis of crayfish (Lin et al., 2011). Although *PlCHF* and *PmHHAP* proteins have similar functions in hemocyte homeostasis via inhibition of apoptosis, their respective nucleotide/amino acid sequences are totally different.

Caspases are the major mediators of apoptosis (Thornberry, 1998). Caspases are classified into two different subgroups, initiator and effector caspases (Saivesen, 1999). In shrimp *P. monodon*, two effector caspase genes (*PmCaspase* and *PmCasp*) have been reported (Wongprasert et al., 2007; Leu et al., 2008a). *PmCasp* is involved in response to WSSV-infection (Wongprasert et al., 2007), but *PmCaspase* is not (Leu et al., 2008a). According to the present study, *PmHHAP* appeared to be involved in hemocyte apoptosis, thus, *PmHHAP* might alter the expression of several apoptotic-related genes and we focus on these two caspases in *P. monodon*. Suppression of *PmHHAP* transcripts by RNAi resulted in

a significant decrease of *PmCaspase*, whereas *PmCasp* expression was significantly increased at 18 hpi. This result suggested that *PmHHAP* transcript could altered the mRNA expression of effector caspases but how these two genes/proteins directly function together needs further investigation. In previous study, caspase interaction proteins were characterized especially the inhibitor of apoptosis (IAP) which can bind directly and inhibit caspase activity (Deveraux et al., 1997). In mammalian XIAP can interact with p10 domain of caspase-3 and caspase-9 and inhibit their activity (Verhagen et al., 2001). In *Drosophila*, DIAP1 interact with caspase Dronc and Drive via BIR domain (Tenev et al., 2005), while DIAP2 can bind with caspase Drice (Leulier et al., 2006). In shrimp *P. monodon*, the white spot syndrome virus protein WSSV449 or AAP-1 has been shown to bind and cleaved by *PmCaspase* to inhibit caspase activity (Leu et al., 2008a). Recently, two novel anti-apoptotic proteins of white spot syndrome virus, WSSV134 and WSSV322, were reported to bind to p20 domain of *PmCasp* (Lertwimol et al., 2014). Surprisingly, yeast two-hybrid and co-immunoprecipitation analyses indicated that *PmHHAP* protein can interact directly with p20 domain of an effector *PmCasp*. However, the role of *PmCasp*, *PmHHAP* and the two anti-apoptotic proteins of WSSV in the regulation of apoptosis during WSSV infection needs to be investigated further. Nevertheless, the data presented here support that *PmHHAP* plays an important role in controlling hemocyte homeostasis during viral infection by inhibiting apoptosis in shrimp likely via interaction with the effector caspase.

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Figure Legends

Figure 1. Successful RNAi-mediated gene silencing of *PmHHAP* transcripts and protein levels in *P. monodon* hemocytes. (A) Semi-quantitative RT-PCR analysis of the silencing efficiency of *PmHHAP* dsRNA at the transcript level. Shrimp injected with GFP dsRNA served as controls. EF1 α was amplified and used as an internal control. The average relative expressions are representative of three independent repeat ± 1 S.D. (*error bars*). Significant difference compared with control indicated by an asterisk (* $p < 0.05$). (B) Western blot analysis of the *PmHHAP* and β -actin proteins in the *PmHHAP* silenced hemocytes. β -actin was used as the loading control. Each lane represents the protein from the pooled sample of three individual shrimp.

Figure 2. Detection of apoptosis in hemocyte of *PmHHAP*-silenced *P.monodon* by annexin V staining. Hemocytes were collected from the *PmHHAP* knockdown and control GFP dsRNA injected shrimp at 12 h after dsRNA injection and stained with annexin-V-FLUOS (A). The propidium iodide (PI) was used to counter stain the hemocyte nucleus. The annexin-V positive cells were counted under the microscope and the apoptosis rate of *PmHHAP*-silenced shrimp and that of the GFP control was reported (B). The proportion of annexin-V positive cells, counted under a fluorescent microscope, in *PmHHAP*-silenced and GFP control shrimps, shown as the mean ± 1 S.D., and derived from triplicate experiments. Asterisk indicates a statistical significant difference between means (*** $p < 0.001$).

Figure 3. Analysis of apoptosis DNA ladder of the *PmHHAP*-silenced shrimp. DNA was prepared from hemocytes of the *PmHHAP*-silenced shrimp and the GFP-injected shrimps (control) after 12 h post dsRNA injection according to the apoptotic DNA ladder kit

instruction and electrophoresed on 1.2% (w/v) agarose gel. The figure shown is representative of the triplicate experiments. Lane M is a 1 kb molecular weight DNA marker.

Figure 4. Caspase 3/7 activity in hemocyte of *PmHHAP*-silenced *P. monodon*. Hemocytes were collected from *PmHHAP* and GFP dsRNA-injected shrimps, and the caspase 3/7 activity was measured. The column represents the mean $\pm$ 1 S.D. derived from three separate shrimps. Asterisk indicates a statistical significant difference between means (* p < 0.05).

Figure 5. Effect of *PmHHAP* silencing on expression of apoptotic related genes in shrimp. (A) The effect of *PmHHAP* knockdown at 12 hpi on the expression level of genes involved in apoptosis (*Pmcasp*, *Pmcaspase*, cathepsin B, cathepsin D, DAD1 and survivin) were evaluated by quantitative real-time RT-PCR. (B) The effect of *PmHHAP* gene knockdown on the transcript expression level of *PmHHAP*, *PmCaspase* and *PmCasp* at each time point (hpi) was evaluated by quantitative real-time RT-PCR. EF1 α was served as the internal reference gene to normalize the amount of cDNA template. The data represent the mean $\pm$ 1 S.D. derived from three independent experiments.

Figure 6. Interaction of *PmHHAP* with *PmCasp* protein. (A) The protein-protein interaction between *PmHHAP* and the mature protein, p20 and p10 domains of *PmCasp*, as determined by yeast two-hybrid assay. The *PmHHAP* was in-frame with the BD domain of the pGBKT7 plasmid, whilst the mature protein, p20 and p10 domains of *PmCasp* were in-frame with the AD domain of pGADT7, respectively. Interaction (+) is indicated by blue yeast colonies and no interaction (-) by no growth. BD and AD constructs co-transformed in yeast are indicated. (B) The protein-protein interaction between *PmHHAP* and the p20 domain of *PmCasp* revealed by Co-immunoprecipitation assay. The immunoprecipitated

complex between r*Pm*HHAP and p20 domain (rP20) of *Pm*Casp was detected using the polyclonal anti-*Pm*HHAP antibody. The proteins were separated by SDS-PAGE and analyzed by Western blotting with anti-His antibody.

Figure 7. *In vitro* inhibition of caspase activation by r*Pm*HHAP. (A) Hemocyte lysate supernatant (HLS) was incubated with r*Pm*HHAP in the presence or absence of actinomycin D. Control groups were HLS only and HLS incubated with BSA. (B) Hemocyte lysate supernatant (HLS) was incubated with r*Pm*HHAP, r*Pm*Casp and co-incubated with both r*Pm*HHAP and r*Pm*Casp, control group was HLS with and without BSA protein control. Total caspase activity was detected using the Caspase-Glo 3/7 assay kit. The results are expressed as the mean $\pm$ S.D. of three replicates. Means with a different lowercase letter are significantly different ($p < 0.05$).

Table 1

Nucleotide sequence of the primers used.

Primer	Sequence (5'-3')	GenBank accession number
Real-time RT-PCR		
<i>Pm</i> HHAP-F	5' GCAACAGGAGAACCTGTGGATA 3'	HQ130431
<i>Pm</i> HHAP-R	5' GGTTCAGAAATCGCCTCCTATA 3'	HQ130431
<i>Pm</i> Casp-F	5' TAAACTTCACGGCTGAACGG 3'	DQ846887
<i>Pm</i> Casp-R	5' TCAGCATGGATGGGAATCAC 3'	DQ846887
<i>Pm</i> Caspase-F	5' CGTGGTTTCATTTCAGTCGCTG 3'	EF114674
<i>Pm</i> Caspase-R	5' AACCTTTCGCATCAGGGTTG 3'	EF114674
Cathepsin B-F	5' ATGAGTGATCGGCAGTGTATCC 3'	EF213113
Cathepsin B-R	5' GATGCAGATCACTCTCGTAGTC 3'	EF213113
Cathepsin D-F	5' GACCAAGTCCTCCACATACAAG 3'	EF213114
Cathepsin D-R	5' GTGACTCCATCAACAGCAATCC 3'	EF213114
<i>Pm</i> DAD1-F	5' ACTCCTTCCTTCTGGGTTCA 3'	EF581986
<i>Pm</i> DAD1-R	5' AAATCAGCAAAGCCTCGTTC 3'	EF581986
Survivin-F	5' CCTGACCTCGTCCGTTTCGTTTG 3'	GU903910
Survivin-R	5' GGTGTCTCACTCTCTGCCTTTG 3'	GU903910
EF1 α -F	5' GGTGCTGGACAAGCTGAAGGC 3'	
EF1 α -R	5' CGTCCGGTGATCATGTTCTTGATG 3'	
Gene silencing		
<i>Pm</i> HHAPi-F	5' GCAACAGGAGAACCTGTGGATA 3'	HQ130431
<i>Pm</i> HHAPi-R	5' GGCCTTGTGACGTTCTTCCATT 3'	HQ130431
T7 <i>Pm</i> HHAPi-F	5' TAATACGACTCACTATAGGGCAACAGGAG AACCTGTGGATA 3'	HQ130431
T7 <i>Pm</i> HHAPi-R	5' TAATACGACTCACTATAGGGCCTTGTGAC GTTCTTCCATT 3'	HQ130431
GFP-F	5' ATGGTGAGCAAGGGCGAGGA 3'	U55761
GFP-R	5' TTACTTGTACAGCTCGTCCA 3'	U55761
GFPT7-F	5' TAATACGACTCACTATAGGATGGTGAGCAA GGGCGAGGA 3'	U55761
GFPT7-R	5' TAATACGACTCACTATAGGTTACTTGTACAG CTCGTCCA 3'	U55761
Yeast two-hybrid		
<i>Pm</i> HHAP-F-NdeI	5' CATATGAGCGCCGAAATGAAAACC 3'	HQ130431
<i>Pm</i> HHAP-R-XhoI	5' CTCGAGGCGTTTATAGGAGGCGATTCT 3'	HQ130431
F- <i>Pm</i> Casp-p20-NdeI	5' GGAATTCCATATGCCGCGGGGCGCTGCTCT 3'	DQ846887
R- <i>Pm</i> Casp-p20- BamHI	5' CGCGGATCCGTCGGGGCCCCTGCATGCCTG 3'	DQ846887
<i>Pm</i> Casp-p10-F-NdeI	5' GGAATTCCATATGTACCCAGGATTCCCGGC 3'	DQ846887
F- <i>Pm</i> Casp-NdeI	5' GGAATTCCATATGAGCGACGCCGACGACTC 3'	DQ846887
R- <i>Pm</i> Casp-BamHI	5' CGCGGATCCTCAGAAGTAAATTTACGAAG 3'	DQ846887

Figure 1

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