A novel viral responsive protein, namely hemocyte homeostasis-associated protein (HHAP), was characterized for its role in the response of shrimp to white spot syndrome virus infection. The full-length cDNAs of HHAP from the black tiger shrimp (PmHHAP), Penaeus monodon, and the fresh water crayfish (PIHHAP), Pacifastacus leniusculus, were obtained and showed high sequence identity to a hypothetical protein from various organisms, with the highest identity to the hypothetical protein TcgaG2_TC006773 from the red flour beetle, Tribolium castaneum (54% amino acid sequence identity). Transcripts of PmHHAP were expressed in various shrimp tissues with the highest expression in hematopoietic tissue, whereas the transcripts of PIHHAP were found in the hematopoietic and nerve tissues. Upon white spot syndrome virus infection, a high up-regulation level of shrimp hemocytic HHAP mRNA and protein was observed by real-time reverse transcription-PCR and immunofluorescence microscopy, respectively. Gene silencing of PmHHAP by RNA interference resulted in a significant decrease in the number of circulating hemocytes and 100% shrimp mortality within 30 h of the double-stranded PmHHAP RNA injection (but not in control shrimp), indicating that HHAP is essential for shrimp survival. Interestingly, severe damage of hemocytes was observed in vivo in the PmHHAP knockdown shrimp and in vitro in shrimp primary hemocyte cell culture, suggesting that PmHHAP plays an important role in hemocyte homeostasis. Thus, it is speculated that the up-regulation of PmHHAP is an important mechanism to control circulating hemocyte levels in crustaceans during viral infection.

White spot syndrome virus (WSSV), a major shrimp pathogen, critically affects the shrimp aquaculture worldwide, causing white spot syndrome with the onset of a rapid and mass mortality within 2–7 days post infection (1, 2). A clinical sign of white spot syndrome typically is the development of many white spots on the carapace of the infected shrimp (2). It has a broad host range (3) and can infect various tissues (4, 5). WSSV is the type species of the genus Whissoviridae (6). It contains a circular double-stranded DNA of ~305 kb and is an enveloped rod-shaped particle with a single filamentous appendage-like tail at one end of the nucleocapsid (6, 7). The average size of the virus is ~298 ± 21 nm long and 107 ± 8 nm in diameter (4).

Invertebrates, including crustaceans, rely on an effective innate immune system to fight against invading pathogens, which is composed of cellular and humoral immune responses. The cellular responses include phagocytosis, nodule formation, and encapsulation, whereas the humoral responses involve the prophenoloxidase-activating system, the clotting cascade, and activity of immune-related proteins such as antimicrobial peptides, antiviral peptides, proteases, and protease inhibitors (8–10). So far, several research reports on the viral defense mechanisms in crustaceans have identified some potential molecules as likely to be involved in the antiviral immunity (see Liu et al. (11), for a review). Nevertheless, the information concerning viral infection and antiviral mechanisms in crustaceans is still mostly unknown.

The hemocyte is a major immune-responsive cell in crustaceans, because it produces many immune effectors, and participates in a number of immune activities (8). Crustacean hemocytes are generally classified into three types: hyaline (agranular), semigranular (small granular), and granular (large granular) hemocytes (12, 13). It is believed that the hyaline hemocyte is associated with phagocytosis (14, 15), whereas the granular cells principally function in apoptosis, melanization, encapsulation, and nodulation (16–19). Apoptosis, which occurs after viral infections, plays an important role in the antiviral mechanism of crustaceans. However, this also leads to a significant reduction in the number of circulating hemocytes, probably resulting in a decline of antiviral immunity as well as mortality of crustaceans (20–24). Therefore, maintenance of the hemocyte level in the blood-circulating system, including the rapid production of new hemocytes from hematopoietic tissue, is essential for the survival of the animals as is the capacity to protect against pathogenic invaders.

§ This work was supported by grants from The Commission on Higher Education, and the Thailand National Center for Genetic Engineering and Biotechnology and by a student fellowship (to A. P. and T. V.) from the Royal Golden Jubilee PhD Program, Thailand Research Fund. This work was also supported by the Thai Government Stimulus Package 2 (TSSK2555), under the Project for Establishment of Comprehensive Center for Innovative Food, Health Products, and Agriculture.

1 To whom correspondence should be addressed: Center of Excellence for Molecular Biology and Genomics of Shrimp, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand. Tel.: 66-2-218-5439; Fax: 66-2-218-5414; E-mail: anchalee.k@chula.ac.th.

2 The abbreviations used are: WSSV, white spot syndrome virus; dsRNA, double-stranded RNA; GFP, green fluorescence protein; HHAP, hemocyte homeostasis-associated protein; LB, Luria-Bertani; PBS, phosphate-buffered saline; PI, Pacifastacus leniusculus; Pm, Penaeus monodon; HHAP, recombinant HHAP; RNAi, RNA interference; SPF, specific pathogen-free; SSH, suppression subtractive hybridization; RNAi, RNA interference; RACE, rapid amplification of cDNA ends; RT, reverse transcription.
A Novel Protein in Hemocyte Homeostasis in P. monodon

To gain more insight into viral infection and/or antiviral mechanisms in crustaceans, we applied suppression subtractive hybridization (SSH) to identify viral responsive genes in the hemocytes of WSSV-challenged *Peneaus monodon* at the early and late phases of the infection (our unpublished data). Among these genes, a gene encoding a protein with significant similarity to the hypothetical protein TcasGA2_TCC006773 from the red flour beetle, *Tribolium castaneum* (GenBank™ accession number EFA09058), was further investigated, because it was one of the highly up-regulated genes found in the SSH libraries. In this report, we characterize this novel viral responsive gene/protein that was found for the first time in crustaceans, which appears to be involved in hemocyte homeostasis. Therefore, it was named as “hemocyte homeostasis-associated protein (HHAP).”

**EXPERIMENTAL PROCEDURES**

**Shrimp and Crayfish Cultivation**—Specific pathogen-free (SPF) black tiger shrimp, *P. monodon*, of ~20 g bodyweight were obtained from a farm in Nakhon Si Thammarat Province, Thailand. The animals were reared in laboratory tanks at ambient temperature (28 ± 4 °C) and maintained in aerated water with a salinity of 20 ppt for at least 7 days before use. For RNAi experiments, *P. monodon* of ~5 g in weight were purchased from Torsång, lake Vättern, Sweden, and maintained in laboratory tanks with aerated water at 10 °C.

### Identification of a Full-length cDNA of Shrimp and Crayfish HHAP

The hemolymph of shrimp of ~20 g body weight was drawn from the ventral sinus using a sterile 1-ml syringe with 150 μl of 10% (w/v) sodium citrate solution. The hemolymph was immediately centrifuged at 5000 x g for 5 min at 4 °C to separate the hemocytes from the plasma. Total RNA was isolated from the hemocytes using the TRI Reagent™ - The SMART™ RACE cDNA amplification kit (Clontech) with the *Pm*HHAP (expression) primers (Table 1) according to the manufacturer’s instruction. The RACE product was purified using NucleoSpin® Extract II kit (Clontech) according to the manufacturer’s protocol, and cloned into the RBC T & A Cloning Vector (RBC Bioscience). Then the recombinant plasmid was transformed into *Escherichia coli* DH5α-competent cells (RBC Bioscience). The positive clones were commercially sequenced by Macrogen Inc., Seoul, South Korea.

### Experimental Procedures

**Shrimp and Crayfish Cultivation**—Specific pathogen-free (SPF) black tiger shrimp, *P. monodon*, of ~20 g bodyweight were obtained from a farm in Nakhon Si Thammarat Province, Thailand. The animals were reared in laboratory tanks at ambient temperature (28 ± 4 °C) and maintained in aerated water with a salinity of 20 ppt for at least 7 days before use. For RNAi experiments, *P. monodon* of ~5 g in weight were purchased from Torsång, lake Vättern, Sweden, and maintained in laboratory tanks with aerated water at 10 °C.

### TABLE 1

| Primer name | Primer sequence (5’ to 3’) | Usage |
|-------------|----------------------------|-------|
| *Pm*HHAP1 (RNAi) | Forward: GACACACGAGAAGACCTCTGATA | Gene silencing |
| *Pm*HHAP2 (RNAi) | Forward: GCACAAGGAGACTGCTGATA | Gene silencing |
| GFP1 (RNAi) | Forward: TTACATTACCTCAATAGGTCGCTG | Gene silencing |
| GFP2 (RNAi) | Forward: AATGGGAGATTAGGTCGCTG | Gene silencing |
| VP28 | Forward: TATCAGCTCAGCTTGGTGAACACTT | RT-PCR |
| β-Actin | Forward: ATGGGAGATTAGGTCGCTG | Real-time PCR |
| β-Actin (real-time PCR) | Forward: AATGGGAGATTAGGTCGCTG | Real-time PCR |
| *Pm*HHAP (degenerate) | Forward: AARACGATCGACACAT | Degenerate PCR |
| *Pm*HHAP (5’ RACE) | Forward: AATGGGAGATTAGGTCGCTG | Full-length cDNA identification |
| *Pm*HHAP (3’ RACE) | Forward: AATGGGAGATTAGGTCGCTG | Full-length cDNA identification |
| *Pm*HHAP (Nested 3’ RACE) | Forward: AATGGGAGATTAGGTCGCTG | Full-length cDNA identification |
| 40 S ribosomal protein | Forward: AATGGGAGATTAGGTCGCTG | RT-PCR |

**JULY 9, 2010 | VOLUME 285 • NUMBER 28 • JOURNAL OF BIOLOGICAL CHEMISTRY**
A Novel Protein in Hemocyte Homeostasis in P. monodon

The total RNA was extracted from each tissue using the TRI Reagent (Molecular Research Center). After DNase (Fermentas) treatment, the total RNA (1 μg) was converted to single-stranded cDNA with the ImPromp-II™ reverse transcription system (Promega, Madison, WI) according to the manufacturer’s instruction. The HHAP gene expression level in each tissue was identified by RT-PCR using 1 μl of cDNA template with either the PmHHAP-1 primers or PmHHAP (RT-PCR) primers (Table 1) for the PmHHAP and PmHHAP genes, respectively. The β-actin and 40 S ribosomal protein partial gene fragments were amplified using the β-actin and 40 S ribosomal protein-specific primers (Table 1), respectively, as internal controls for shrimp and crayfish, respectively. The PCR conditions consisted of 94 °C for 3 min, followed by 31 (for PmHHAP), 40 (for PmHHAP), and 25 (for β-actin and 40 S ribosomal protein) cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and then a final extension at 72 °C for 5 min. The PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis.

Production of Recombinant PmHHAP and Anti-PmHHAP Polyclonal Antibody—The open reading frame of PmHHAP conjugated with a 5′ Xhol and a 3′ Ndel restriction site was amplified from the PmHHAP-recombinant plasmid by PCR with the PmHHAP (expression) primers (Table 1) for the following: 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s, and then a final extension at 72 °C for 5 min. The PCR product was purified using a NucleoSpin® Extract II kit (Clontech), and cloned into the pET-22b(+) expression vector (Novagen). The recombinant plasmid was transformed into E. coli Rosetta (DE3)-competent cells by electroporation. The transformed E. coli was grown in Luria-Bertani (LB) medium, and the protein expression was induced with 1 mm isopropyl 1-thio-β-d-galactopyranoside. The overexpression of recombinant PmHHAP (rPmHHAP) was analyzed by SDS-PAGE (18% (w/v) acrylamide) followed by Coomassie Brilliant Blue staining. The rPmHHAP was found to be principally located in inclusion bodies, and so it was solubilized with 20 mM sodium phosphate buffer, pH 7.4, containing 8 M urea, 500 mM sodium chloride, and 20 mM imidazole at room temperature overnight. The solubilized protein was purified using the affinity column containing nickel-Sepharose™ 6 Fast Flow (Amersham Biosciences) according to the manufacturer’s suggestion, and subsequently, the protein was refolded by dialyzing with 20 mM Tris-HCl, pH 8.0, containing 4, 2, and 0 mM urea, respectively, for 3 h in each step. The purity of the recombinant protein was analyzed by SDS-PAGE as above. The purified rPmHHAP was used to generate rabbit polyclonal antibodies by a commercial service at Biomedical Technology Research Unit, Chiang Mai University, Thailand.

Western Blot Analysis of PmHHAP—Total protein was extracted from the hematopoietic tissue of each shrimp using the TRI Reagent® (Molecular Research Center). The purified rPmHHAP, and the total protein from hematopoietic tissue, were separated by SDS-PAGE (18% (w/v) acrylamide) and transferred onto a PROTRAN™ nitrocellulose transfer membrane (Whatman) by a semi-dry blotter. The membrane was immersed in blocking buffer (1 × phosphate-buffered saline (PBS), pH 7.4, containing 5% (w/v) skim milk and 0.05% (v/v) Tween 20) at room temperature overnight and then incubated with a 1:1,000 dilution of rabbit anti-rPmHHAP antibody in blocking buffer at 37 °C for 2 h. The membrane was washed three times with washing buffer (PBS, pH 7.4, containing 0.05% (v/v) Tween 20) and incubated with a 1:10,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG antibody in blocking buffer at room temperature for 1 h. Then the membrane was washed three times with washing buffer, and the color was developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (Fermentas) according to the manufacturer’s protocol.

PmHHAP mRNA Expression in Unchallenged and WSSV-challenged Shrimp Hemocytes—WSSV was prepared from the gills of WSSV-challenged P. monodon according to the method described by Du et al. (25) and then diluted in lobster hemolymph medium, prepared as described by Paterson and Stewart (26). 100 μl of the diluted WSSV solution (~80 viral copies/μl) was injected into each shrimp (~20 g body weight), a viral dose that had been previously determined as that which would induce a cumulative mortality of ~50% within 3 days post injection (data not shown). Control shrimp were injected with 100 μl of virus-free lobster hemolymph medium. Hemocytes of shrimp (three individuals each) were collected at 24, 48, and 72 h post injection, and total RNA was extracted from the hemocytes using the TRI Reagent® (Molecular Research Center) followed by DNase (Fermentas) treatment, and used to synthesize single-stranded cDNA with the ImPromp-II™ reverse transcription system (Promega). Real-time RT-PCR was performed using an equal amount of cDNAs in iCycler IQ™ Real-Time detection system using an IQ™ SYBR Green Supermix (Bio-Rad) with the following conditions: 95 °C for 9 min, 40 cycles of 95 °C for 30 s, 58 °C for (PmHHAP) or 60 °C (for β-actin) for 30 s, and 72 °C for 45 s. The PmHHAP and β-actin (real-time PCR) primers were used in the experiment (Table 1). The results are presented as the average relative expression ratio of PmHHAP expressed in the hemocytes of the sample (WSSV-challenged) shrimp versus the control (unchallenged) shrimp, with normalization to the reference gene (β-actin). The relative expression ratios of PmHHAP were calculated as described by Pfaffl (27).

Isolation and Tissue Culture Pretreatment of Primary Hemocyte Cultures—Hemolymph was drawn from each shrimp (~20 g body weight) using a sterile 1-ml syringe with 500 μl of anti-
coagulant, pH 5.6 (0.82% (w/v) sodium chloride, 0.55% (w/v) citric acid, 1.98% (w/v) glucose and 0.88% (w/v) sodium citrate; adjusted to pH 5.6 with sodium hydroxide solution). The hemolymph-anticoagulant mixture was then centrifuged at 200 × g for 10 min at room temperature to separate the hemocytes from the plasma. The hemocyte pellet was resuspended in 1 ml of L-15 culture medium (1.6× Leibovitz L-15 medium (Invitrogen) supplemented with 20% (v/v) fetal bovine serum, 1% (w/v) glucose, 0.4% (w/v) sodium chloride, 100 IU/ml penicillin, and 100 μg/ml streptomycin (pH 7.6); adjusting the osmotic pressure to 750 ± 15 mosm/kg with sodium chloride solution). The hemocyte number was counted under a light microscope and then distributed into the wells of a 96-well plate at 10^5 cells per well. The total volume in each well was adjusted to 150 μl with L-15 culture medium, and the culture plate was incubated at 28 °C for 24 h, whereupon the pretreated hemocytes were ready to be used in experiments.

PmHHAP mRNA Expression in Unchallenged and WSSV-challenged Shrimp Primary Hemocyte Cultures—After the pretreatment of primary hemocyte cultures as described above, 50 μl of L-15 culture medium was removed from each well and replaced with 50 μl of L-15 culture medium with (WSSV-challenged hemocytes) or without (SPF control hemocytes) ~10^6 WSSV viral copies, and reincubated at 28 °C for a further 24 h. Total RNA was extracted from the hemocytes using the TRI Reagent® (Molecular Research Center) followed by DNase (Fermentas) treatment, and then used to synthesize single-stranded cDNA. RT-PCR was performed as described above using the PmHHAP-1 and β-actin primers (Table 1). The PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis, and the differential expression level was reported as relative to that of β-actin.

Protein Expression and Subcellular Localization Analysis of PmHHAP in Shrimp Hemocytes—The diluted WSSV solution (100 μl, 8 × 10^3 WSSV copies) was injected into shrimp (~20 g body weight) while unchallenged shrimp were injected with 100 μl of virus-free lobster hemolymph medium. Sixty hours post injection, hemolymph was drawn from each shrimp using a sterile 1-ml syringe with 500 μl of anticoagulant, pH 5.6. The anticoagulant-hemolymph (50 μl) mixture was added into a 24-well plate containing a coverslip and 200 μl of L-15 culture medium in each well, and the mixture was incubated at room temperature for 2 h. Subsequently, the medium was removed, and then 300 μl of 4% (w/v) paraformaldehyde in PBS, pH 7.4, was added instead. The plate was incubated at room temperature for 20 min, washed three times with PBS, pH 7.4, and then immersed in PBS, pH 7.4, and kept at 4 °C until the next treatment.

The fixed hemocytes were incubated with a 1:500 dilution of rabbit anti-PmHHAP polyclonal antibody in PBS, pH 7.4, containing 1% (v/v) fetal bovine serum, at 37 °C for 1 h while the negative control (non-infected) hemocytes were incubated with PBS, pH 7.4 containing 1% (v/v) fetal bovine serum. Blots were then washed three times with PBS, pH 7.4, and then incubated with a 1:500 dilution of secondary antibody solution (Alexa Fluor 488 goat anti-rabbit IgG antibody) in PBS, pH 7.4, at room temperature for 1 h in the dark and washed three times with PBS, pH 7.4. Subsequently, the nucleus was stained with a 1:500 dilution of TO-PRO-3 iodide (Invitrogen) in PBS, pH 7.4, at room temperature for 1 h in the dark, and washed three times with PBS, pH 7.4. The coverslips containing the stained and fixed hemocyte samples were then coated with Prolong Gold Antifade Reagent (Invitrogen) before detecting the fluorescent signal by confocal microscopy.

Production of PmHHAP and GFP dsRNA—Two types of PmHHAP DNA templates with a single T7 promoter at the 5’ ends were PCR-amplified from the PmHHAP-recombinant plasmid by two separate PCR reactions using the PmHHAP1 and PmHHAP2 (RNAi) primer sets (Table 1) with the following conditions: 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s, and then a final extension at 72 °C for 5 min. The two types of PCR product templates were in vitro transcribed using T7 RiboMAX™ Express Large Scale RNA Production System (Promega) to produce two complementary single-stranded RNAs according to the manufacturer’s instruction. Then, equal amounts of each of the complementary single-stranded RNAs were mixed together and incubated at 70 °C for 10 min, and then slowly cooled down at room temperature to allow annealing to form dsRNA. The PmHHAP dsRNA solution was treated with 2 units of RQ1 RNase-free DNase (Promega) at 37 °C for 15 min, and then purified by standard phenol-chloroform extraction.

Green fluorescent protein (GFP) DNA templates with a single T7 promoter at the 5’ ends were PCR-amplified from the pd2EGFP-1 vector (Clontech) using the GFP1 and GFP2 (RNAi) primers (Table 1) with the following conditions: 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min, and then a final extension at 72 °C for 5 min. Subsequently, GFP dsRNA was produced as above.

Analysis of PmHHAP Gene Silencing Mediated by RNAi—Shrimp were separated into four groups with three shrimp per group. 25 μl of 150 mM sodium chloride solution alone (group 1) or containing 10 μg of GFP dsRNA (group 2) or 5 and 10 μg of PmHHAP dsRNA (groups 3 and 4, respectively) was injected into each shrimp (~5 g body weight) of each respective group. Twenty-four hours post injection, hemocytes were collected, and total RNA was extracted from them using the TRI Reagent® (Molecular Research Center) followed by DNase (Fermentas) treatment, and then single-stranded cDNA was synthesized with the ImPromp-II™ reverse transcription system (Promega). RT-PCR was performed to evaluate the degree of gene transcript silencing using the PmHHAP-1 and β-actin primers (Table 1), as mentioned above. The PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis, and PmHHAP gene expression level was reported as relative to that of β-actin.

Investigation of Shrimp Mortality after PmHHAP Gene Knockdown—Shrimp were separated into four groups with 10 shrimp per group. Twenty-five μl of 150 mM sodium chloride solution alone (group 1) or containing 8 μg of GFP dsRNA (group 2) or 4 and 8 μg of PmHHAP dsRNA (groups 3 and 4, respectively) was injected into each shrimp (~4 g body weight) in each respective group. Shrimp mortality was observed and recorded every 6 h post injection for 36 h, and the results are reported as average cumulative percent mortality + 1 S.D. from three experiments.
Identification of Shrimp Circulating Hemocyte Numbers after PmHHAP Gene Knockdown—Shrimp were separated into three groups with 15 shrimp per group. Twenty-five μl of 150 mM sodium chloride solution (group 1) and 150 mM sodium chloride solution containing 4 μg of GFP dsRNA (group 2) or 4 μg of PmHHAP dsRNA (group 3) was injected into each shrimp (≈4 g body weight) in each group. At 12 and 24 h post injection, 50 μl of hemolymph was drawn from three to five shrimp using a sterile 0.5-ml syringe with 50 μl of anticoagulant, pH 5.6. The total hemocyte number in the hemolymph was counted with a hemocytometer under a light microscope, and the average total hemocyte number of each group at each time point was calculated and recorded. The experiment was done in triplicate, and the results are shown as the average total hemocyte number ± S.D. from triplicate samples.

Morphological Analysis of PmHHAP-depleted Hemocytes by Scanning Electron Microscopy—25 μl of 150 mM sodium chloride solution containing 5 μg of PmHHAP dsRNA or GFP dsRNA was injected into each shrimp (≈5 g body weight), and after either 12 or 24 h, the hemolymph was drawn from each group of shrimp using a sterile 1-ml syringe without anticoagulant, and immediately dropped onto a coverslip. Proteins in the hemolymph were washed out with 0.1M sodium phosphate buffer, pH 7.2, and the coverslip was then immersed in 0.1M sodium phosphate buffer, pH 7.2, containing 2.5% (w/v) glutaraldehyde at room temperature for 1 h. The coverslip was then washed with 0.1M sodium phosphate buffer, pH 7.2, followed by distilled water, and then dehydrated through immersion for 3 min each in a series of 30, 50, 70, 90, 100, 100, and 100% (v/v) ethanol. Finally, the samples were dried using a critical point dryer and coated with gold using ion sputter. The hemocyte morphology was then observed by scanning electron microscopy.

RNAi-mediated PmHHAP Transcript Knockdown in Vitro in Primary Hemocyte Cultures—After pretreatment of primary hemocyte cultures, as described above, the L-15 culture medium was completely removed from the wells and replaced with 100 μl of serum-free L-15 transfection reagent (1.6× Leibovitz L-15 medium (Invitrogen) supplemented with 1% (w/v) glucose, 0.4% (w/v) sodium chloride,
A Novel Protein in Hemocyte Homeostasis in P. monodon

RESULTS

The Full-length cDNA of Shrimp and Crayfish HHAP and Sequence Analysis—The partial sequence of the P. monodon shrimp HHAP (PmHHAP) cDNA was previously obtained from the SSH library of WSSV-challenged P. monodon hemocytes. Full-length cDNA was then identified using 5’ RACE (Fig. 1A), and the size of the PmHHAP mRNA obtained was confirmed by Northern blot analysis showing the corresponding size of ~650 bp (data not shown). The BLAST homology search (28) indicated that the putative predicted protein sequence encoded by PmHHAP had a significant amino acid sequence similarity (54%) to the hypothetical protein TcS2A2_TC006773 from the red flour beetle, T. castaneum. From a protein-structural analysis, the protein is expected to be a DNA-binding protein (99.2% possibility; DBS-Pred, available on-line) (31). The full HHAP sequence was also determined from the freshwater crayfish, P. leniusculus (Fig. 1B). The pairwise alignment of shrimp and crayfish HHAP amino acid sequences exhibited 47% identity, and they share the eight conserved cysteine residues (Fig. 1C).

HHAP Expression in Shrimp and Crayfish Tissues—The tissue distribution of the HHAP transcripts in shrimp and crayfish were examined by RT-PCR using the respective gene-specific primers. The shrimp PmHHAP was transcribed in all tested tissues but was highly expressed in the heart, hematopoietic, and intestine tissues (Fig. 2A). 0.25% (v/v) Lipofectamine™ 2000 (Invitrogen), pH 7.6, and the osmotic pressure was adjusted to 750 ± 15 mosm/kg with sodium chloride solution) containing 1 μg of either PmHHAP or GFP dsRNA, respectively. The culture plate was incubated at 28°C for 48 h, and then the hemocyte morphology was observed by light microscopy. To evaluate the degree of PmHHAP gene knockdown, PmHHAP transcript levels were evaluated. Total RNAs were extracted from the hemocytes using the TRI Reagent® (Molecular Research Center), followed by DNase (Fermentas) treatment, and then used to synthesize single-stranded cDNA with the ImProm-II™ reverse transcription system (Promega). The gene knockdown was analyzed by RT-PCR using the PmHHAP-2 and β-actin primers (Table 1). PCR conditions for the PmHHAP-2 primers consisted of 94 °C for 3 min, 33 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and then a final extension at 72 °C for 5 min. The PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis, and PmHHAP gene expression level was reported as relative to that of β-actin.

Several cysteine residues are common to all isoforms of HHAP, and the pairwise alignments of shrimp and crayfish HHAP amino acid sequences indicated 47% identity, revealing eight conserved cysteine residues (Fig. 1C). The pairwise alignment of shrimp and crayfish HHAP amino acid sequences exhibited 47% identity, and they share the eight conserved cysteine residues (Fig. 1C).
A Novel Protein in Hemocyte Homeostasis in *P. monodon*

Transcript levels in hemocytes were at relative low levels. For the freshwater crayfish, *P. leniusculus*, *PmHHAP* transcript profiles were different, being found in hematopoietic and nerve tissues, but at very low levels compared with that found in *P. monodon* shrimp tissues and not at detectable levels in the other tissues, including hemocytes (Fig. 2B).

*PmHHAP Is Found among Hematopoietic Proteins—*To detect *PmHHAP* protein levels in hematopoietic tissues, a polyclonal antibody against the recombinant protein, *rPmHHAP*, was produced. Firstly, the *rPmHHAP* with histidine tag (Leu-Glu-His$_6$) at the C terminus was expressed in a bacterial expression system by using isopropyl 1-thio-β-D-galactopyranoside induction for 4 h (Fig. 3, lane 1). After affinity chromatography purification, a purified *rPmHHAP* was obtained (Fig. 3, lane 2), and this was used to generate an anti-*rPmHHAP* antibody in immunized rabbits. Western blot analysis indicated that the antibody reacted specifically with the *rPmHHAP* (Fig. 3, lane 4). The crude protein extracted from the shrimp hematopoietic tissue is shown in Fig. 3, lane 3. Western blot analysis with anti-*rPmHHAP* antibody identified the endogenous *PmHHAP* as one of the proteins in the hematopoietic tissue of *P. monodon* shrimp (Fig. 3, lane 5) with an of ~1 kDa lower molecular mass than the recombinant protein.

*PmHHAP mRNA Is Up-regulated in Response to WSSV Infection—* *PmHHAP* was previously identified as a highly up-regulated gene in the hemocytes of WSSV-infected *P. monodon* by SSH. To confirm the differential expression of *PmHHAP* mRNA upon WSSV infection, *PmHHAP* transcript levels were evaluated by real-time RT-PCR. The results clearly demonstrated that *PmHHAP* mRNA expression was highly up-regulated in shrimp hemocytes after WSSV challenge, increasing by ~3.0-, 8.3-, and 33.3-fold at 24, 48, and 72 h post infection, respectively, as compared with the gene expression in non-infected SPF hemocytes (Fig. 4A). Likewise, in the shrimp primary hemocyte cell culture, up-regulation of *PmHHAP* transcript levels in WSSV-infected *in vitro* hemocytes was also detected when compared with non-infected SPF hemocyte cells (Fig. 4B). These results strongly suggest that *PmHHAP* transcripts are significantly up-regulated in the mature *P. monodon* shrimp hemocytes in response to WSSV infection.

**Differential Expression and Subcellular Localization of *PmHHAP* in Shrimp Hemocytes**—The expression of *PmHHAP* protein in hemocytes was evaluated with immunofluorescence and confocal microscopy. The hemocyte nucleus was stained with TO-PRO-3 iodide (blue fluorescent dye) while *PmHHAP* were recognized with anti-*rPmHHAP* antibody and visualized as a green (Alexa Fluor 488) fluorescent dye (Fig. 5). The *PmHHAP* signal in the WSSV-infected hemocytes was much stronger than that in the non-infected hemocytes, whereas the control

---

**FIGURE 5.** *PmHHAP* protein expression analysis in non-infected and WSSV-infected *P. monodon* hemocytes by immunofluorescence microscopy. The hemocytes from non-infected and WSSV-infected shrimp were stained with anti-*rPmHHAP* (green) and nuclei (blue) as detailed under "Experimental Procedures." The fluorescent signal was detected by confocal microscopy. Images shown are representative of at least three such fields of view per sample and three independent samples.

**FIGURE 6.** A, gene-specific knockdown of *PmHHAP* transcript levels in WSSV-uninfected *P. monodon* hemocytes. The hemocytes from shrimp injected with 150 mM sodium chloride solution alone (NaCl) or containing GFP dsRNA (2 µg/g of shrimp) or *PmHHAP* dsRNA (1 and 2 µg/g of shrimp) for 24 h were collected and used to analyze *PmHHAP* gene expression levels by RT-PCR with β-actin as the internal reference. B, cumulative mortality analysis of *PmHHAP*-depleted shrimp. Shrimp were injected with NaCl alone or containing GFP dsRNA (2 µg/g of shrimp) or *PmHHAP* dsRNA (1 and 2 µg/g of shrimp), and their mortality was recorded every 6 h thereafter for 36 h. Data are reported as the average ± 1 S.D. and are derived from three independent trials.

---
where primary antibodies were not added had no signal (Fig. 5). Taken together, the results reveal that the hemocytic PmHHAP is not only up-regulated at the mRNA level but also at the protein level following a WSSV infection. Moreover, it was also found that PmHHAP was mainly distributed in the cytoplasm, and only partly located in a nuclear compartment of the hemocytes (Fig. 5).

Double Strand RNAi-mediated Knockdown of PmHHAP Gene Expression Resulted in Shrimp Mortality—RNAi has become a powerful tool to identify gene function by silencing the expression of specific genes with dsRNA and then analyzing their loss-of-function phenotype. Here, the systemic injection of 5.0 μg (1 μg of dsRNA/g of shrimp) and 10.0 μg (2 μg of dsRNA/g of shrimp) of PmHHAP dsRNA into shrimp of ~5 g of body weight could knock down PmHHAP gene expression by ~46 and 80%, respectively, whereas an injection of sodium chloride solution or GFP dsRNA had no effect on the gene silencing (Fig. 6A). Surprisingly, the knocked down shrimp displayed a very rapid mortality, 12 h post PmHHAP dsRNA injection (Fig. 6B). The shrimp challenged with 1 and 2 μg of PmHHAP dsRNA per gram of shrimp displayed a cumulative mortality of ~20 and 70% at 18 h post dsRNA injection, respectively, and reached 100% mortality within 36 and 30 h post injection, respectively. In contrast, the control groups had no shrimp deaths over the 36-h time course studied (Fig. 6B). Accordingly, the results indicate that PmHHAP is indispensable for the survival of shrimp.

Significant Decrease in Circulating Mature Hemocyte Numbers after PmHHAP Gene Knockdown—After PmHHAP dsRNA injection, peripheral (circulating) hemocytes in the shrimp hemolymph were reduced following dsRNA injection, whereas an injection of GFP dsRNA did not lead to any decrease in the hemocyte number (Fig. 7A), and this affect was more marked at 24 h after injection than after 12 h suggesting a possible continuous decline in levels until death. Evaluation of these data in terms of the concentration of circulating hemocytes in the knocked down shrimp reveals the same pattern, namely a 2- and 4-fold lower level of circulating hemocytes, compared with the control shrimp, at 12 and 24 h post PmHHAP dsRNA injection, respectively (Fig. 6B).

Morphology Changes in PmHHAP-depleted Hemocytes—All the observed hemocytes from the control group of shrimp, that is those that were injected with NaCl alone or containing GFP dsRNA for 12 and 24 h, were completely normal, and no morphological changes were observed. In contrast, clear changes in the morphology of the hemocytes were detected in the hemolymph of shrimp after PmHHAP dsRNA-mediated gene knockdown (Fig. 8). At 12 h post PmHHAP dsRNA injection, some hemocytes appeared abnormal and deformation was initiated, and by 24 h after the dsRNA injection, cell lysis and fragmentation into apoptotic-like bodies were observed (Fig. 8). Moreover, in vitro PmHHAP knockdown in the shrimp primary hemocyte cell culture corroborated the above in vivo results, with severe cell damage occurring in the PmHHAP-depleted hemocytes while most of the control hemocytes still looked healthy (Fig. 9). Altogether, our results reveal an essential function of PmHHAP in hemocyte homeostasis.

DISCUSSION

From our previous study, several WSSV-responsive genes were identified from an SSH cDNA library of P. monodon hemocytes, several of which were of unknown function. Among these genes, a novel viral responsive protein, named

3 A. Prapavorarat and A. Tassanakajon, unpublished data.
here HHAP, was highly up-regulated. A BLASTX search of the NCBI GenBank™ database revealed that the deduced amino acid sequence of PmHHAP significantly matched a hypothetical protein homolog from various invertebrates and vertebrates, with the highest similarity to the hypothetical protein TcasGA2_TC006773 from the red flour beetle, T. castaneum, with 54% amino acid sequence identity. In this study, the full HHAP sequence was determined from two crustacean species; the black tiger shrimp, P. monodon (PmHHAP), and the freshwater crayfish, P. leniusculus (PlHHAP). These two sequences exhibited 47% amino acid sequence identity to each other and shared eight conserved cysteine residues. Moreover, protein-structure analysis suggested that HHAP is likely to be a DNA-binding protein (99.2% possibility). The high predicted amino acid sequence identity of the HHAP sequences found in various organisms, and thus the potentially conserved function, and the possible DNA-binding properties, imply the potential importance of the HHAP protein.

The tissue distribution of HHAP transcripts in SPF P. monodon and P. leniusculus shrimp were different. Within P. monodon, PmHHAP mRNA expression was detectable in a wide variety of tissues, but mainly in the hematopoietic tissue and a low expression level in hemocytes. However, in P. leniusculus crayfish PlHHAP transcripts were only detected in nerve and hemopoietic tissues at low levels, and not at all in other tissues, including hemocytes. Moreover, the endogenous PmHHAP was also detected as one of the major proteins in the shrimp hematopoietic tissue (but not in circulating (mature) hemocytes themselves). In crustaceans, the hematopoietic tissue is reported to be an organ in which hemocyte proliferation and maturation take place (32–34). Hemocytes are produced and partially differentiated into young hemocytes in the hematopoietic tissue, and then become functional or mature hemocytes as they are released into circulation (33, 34). The high expression levels of HHAP transcripts in hematopoietic tissue may suggest a possible role in hemocyte production or maturation (hematopoiesis). Formerly, a new function of transglutaminase in crayfish was disclosed, in that it participates in preventing differentiation of hematopoietic stem cells (35). In addition, an endogenous...
cytokine-like factor, astakine, first described in crayfish (36), was required for cell proliferation and differentiation in the hematopoietic tissue of two crustaceans (36, 37), and ATP synthase in the plasma membrane of hematopoietic cells possibly functions as a receptor for astakine (38). Although there are some recent attempts to unveil crustacean hematopoiesis, the mechanisms regarding hemocyte synthesis and differentiation, the release of hemocytes into the circulation, and the maintenance of circulating hemocytes in crustaceans are still very indistinct.

To uncover the functions of HHAP, RNAi knockdown of the PmHHAP transcript expression in P. monodon shrimp was performed. The resultant partial average down-regulation (46 and 80% for the two doses tested) of PmHHAP transcript levels in circulating hemocyte cells correlated with a significant (2- to 4-fold) depletion of circulating hemocytes and a rapid mortality in the knocked down shrimp. The damage to hemocytes was demonstrated by scanning electron microscope (Fig. 8), and also observed in vitro in the shrimp primary hemocyte cell cultures (Fig. 9). These results suggest that PmHHAP plays an important role in hemocyte homeostasis, at least in P. monodon, because its knockdown initiates the deformation and lysis of hemocytes and a greatly decreased number of circulating hemocytes, as well as subsequent increased shrimp mortality. Notably in shrimp, the reduced hemocyte number is unlikely to be a direct cause of the significant increase in shrimp mortality, because depletion of up to 95% circulating hemocytes usually does not affect shrimp survival as new hemocytes are rapidly produced and released into the circulation. Therefore, we speculated that death in the HHAP knockdown shrimp might result from an inability of shrimp to recover circulating hemocytes due to loss of a critical function of HHAP that remains unclear and needs further investigation. Nevertheless, HHAP is clearly important in both shrimp viability and hemocyte homeostasis.

Hemocytes are widely admitted as a major cell type that plays several essential roles in the innate immunity (8). The number of circulating hemocytes in various crustaceans has been observed to decrease after viral infection (20, 21), or after challenge with bacterial components (34, 39), but this leads to a rapid recovery in the circulating hemocyte level by the induction of hemocytic maturation in hematopoietic tissue and the release of new hemocytes into the circulation (32, 34). From our results in P. monodon shrimp primary cell culture, the up-regulation of PmHHAP in WSSV-infected cultured hemocytes (Fig. 4B) clearly indicates that the increase of HHAP expression in the hemocytes of WSSV-infected shrimp is a result of gene up-regulation in the mature hemocytes, not from gene expression of HHAP in new hemocytes released from the hematopoietic tissue.

The high up-regulation level of hemocytic PmHHAP, both at the mRNA and at the protein level, in response to WSSV infection may suggest that PmHHAP is involved in the shrimp's immune response against viral infections. In the past, a few reports have proposed possible mechanisms for maintaining circulating hemocyte levels and protecting the infected shrimp from death, such as the rapid production and release of new hemocytes from hematopoietic tissue after infection (32, 34) and the control of over viral-triggered apoptosis by the action of the translationally controlled tumor protein (40). Our data clearly indicate that PmHHAP has a direct role in hemocyte persistence; thus, it is reasonable to speculate that the up-regulation of PmHHAP is a mechanism for maintaining the hemocyte level in the circulation by preventing too rapid hemocyte degradation and is also a way to control immune homeostasis in crustaceans during a viral infection.

Acknowledgments—We thank Prof. Timothy W. Flegel, Dr. Kallaya Sritunyalucksana, and Piya Chat Syncamrt for their help in immunofluorescence microscopy and Robert D. J. Butcher for critically reading the manuscript.

REFERENCES
1. Flegel, T. W. (2006) Aquacult. 258, 1–33
2. Chou, H., Huang, C., Wang, C., Chiang, H., and Lo, C. (1995) Dis. Aquat. Org. 23, 165–173
3. Lo, C., Ho, C., Peng, S., Chen, C., Hsu, H., Chiu, Y., Chang, C., Liu, K., Su, M., Wang, C., and Kou, G. (1996) Dis. Aquat. Org. 27, 215–225
4. Wang, C. S., Tang, K. F., Kou, G. H., and Chen, S. N. (1997) J. Fish Dis. 20, 323–331
5. Chang, P., Lo, C., Wang, Y., and Kou, G. (1996) Dis. Aquat. Org. 27, 131–139
6. Vlak, J. M., Bonami, J. R., Flegel, T. W., Kou, G. H., Lightner, D. V., Lo, C. F., Loh, P. C., and Walker, P. W. (2004) VIIIth Report of the International Committee on Taxonomy of Viruses, Elsevier, Amsterdam, The Netherlands
7. Yang, F., He, J., Lin, X., Li, Q., Pan, D., Zhang, X., and Xu, X. (2001) J. Virol. 75, 11811–11820
8. Jiravanichpaisal, P., Lee, B. L., and Söderhäll, K. (2006) Immunobiology 211, 213–236
9. Hoffmann, J. A., Kafatos, F. C., Janeway, C. A., and Ezekowitz, R. A. (1999) Science 284, 1313–1318
10. Iwanaga, S., and Lee, B. L. (2005) Mol. Biol. 38, 128–150
11. Liu, H., Söderhäll, K., and Jiravanichpaisal, P. (2009) Fish Shellfish Immunol. 27, 79–88
12. Tsing, A., Arcieri, J. M., and Brehelin, M. (1989) J. Invertebr. Pathol. 53, 64–77
13. Bauchau, A. G. (1980) In Invertebrate Blood Cells, pp. 385–420, Academic Press, New York
14. Smith, V. J., and Söderhäll, K. (1983) Cell Tissue Res. 233, 295–303
15. Smith, V. J., and Söderhäll, K. (1983) Biol. Bull. 164, 299–314
16. Sung, H. H., Chang, H. J., Her, C. H., Chang, J. C., and Song, Y. L. (1998) J. Invertebr. Pathol. 71, 26–33
17. Pech, L. L., and Strand, M. R. (2000) J. Insect Physiol. 46, 1565–1573
18. Kobayashi, M., Johansson, M. W., and Söderhäll, K. (1990) Cell Tissue Res. 260, 13–18
19. Persson, M., Vey, A., and Söderhäll, K. (1987) Cell Tissue Res. 247, 409–417
20. van de Braak, C. B., Botterblom, M. H., Huisman, E. A., Rombout, J. H., and van der Knaap, W. P. (2002) Dis. Aquat. Org. 51, 149–155
21. Wongprasert, K., Khabood, K., Glumukarn, S. S., Meeratana, P., and Wityahuanchamukkul, B. (2003) Dis. Aquat. Org. 55, 3–13
22. Wang, L., Zhi, B., Wu, W., and Zhang, X. (2008) Dev. Comp. Immunol. 32, 706–715
23. Granja, C. B., Araguren, L. F., Vidal, O. M., Aragón, L., and Salazar, M. (2003) Dis. Aquat. Org. 54, 73–78
24. Wang, W., and Zhang, X. (2008) Fish Shellfish Immunol. 25, 522–527
25. Du, H., Fu, L., Xu, Y., Kil, Z., and Xu, Z. (2007) Aquaculture 262, 532–534
26. Paterson, W. D., and Stewart, J. E. (1974) J. Fish Res. Board Can. 31, 1051–1056
27. Pfaffl, M. W. (2001) Nucleic Acids Res. 29, e45
28. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
29. Ahmad, S., Gromiha, M. M., and Sarai, A. (2004) Bioinformatics 20,
30. Ferrè, F., and Clote, P. (2005) *Nucleic Acids Res.* **33**, W230–W232
31. Schultz, J., Milpetic, F., Bork, P., and Ponting, C. P. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 5857–5864
32. van de Braak, C. B., Botterblom, M. H., Liu, W., Taverne, N., van der Knaap, W. P., and Rombout, J. H. (2002) *Fish Shellfish Immunol.* **12**, 253–272
33. Chaga, O., Lignell, M., and Söderhäll, K. (1995) *Anim. Biol.* **4**, 59–70
34. Söderhäll, I., Bangyeekhun, E., Mayo, S., and Söderhäll, K. (2003) *Dev. Comp. Immunol.* **27**, 661–672
35. Lin, X., Söderhäll, K., and Söderhäll, I. (2008) *BMC Immunol.* **9**, 58
36. Söderhäll, I., Kim, Y. A., Jiravanichpaisal, P., Lee, S. Y., and Söderhäll, K. (2005) *J. Immunol.* **174**, 6153–6160
37. Hsiao, C. Y., and Song, I. L. (2010) *Fish Shellfish Immunol.* **28**, 77–86
38. Lin, X., Kim, Y. A., Lee, B. L., Söderhäll, K., and Söderhäll, I. (2009) *Exp. Cell Res.* **315**, 1171–1180
39. Lorenzon, S., Guarrini, S. D., Smith, V. J., and Ferrero, E. A. (1999) *Fish Shellfish Immunol.* **9**, 31–50
40. Bangrak, P., Graedist, P., Chotigeat, W., and Phongdara, A. (2004) *J. Biotechnol.* **108**, 219–226

*A Novel Protein in Hemocyte Homeostasis in P. monodon*