VIRULENCE
2022, VOL. 13, NO. 1, 936–948
https://doi.org/10.1080/21505594.2022.2078471

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

OPEN ACCESS Check for updates

Proteomics analysis reveals a critical role for the WSSV immediate-early protein IE1 in modulating the host prophenoloxidase system

Chuanqi Wang[a,*], Menghao Wei[a,*], Gaochun Wu[a], Lixuan He[a], Jinghua Zhu[a], Jude Juventus Aweya[a], Xiuli Chen[b], Yongzhen Zhao[b], Yueling Zhang[a], and Defu Yao[a]

[a]Institute of Marine Sciences and Guangdong Provincial Key Laboratory of Marine Biotechnology, Shantou University, Shantou, China; [b]Guangxi Academy of Fishery Sciences, Guangxi Key Laboratory of Aquatic Genetic Breeding and Healthy Aquaculture, Nanning, China

ABSTRACT

White spot syndrome virus (WSSV) is a large, enveloped, double-stranded DNA virus that threatens shrimp aquaculture worldwide. So far, the mechanisms of WSSV-host interactions are ill-defined. Recent studies have revealed that IE1, an immediate-early protein of WSSV, is a multifunctional modulator implicated in virus–host interactions. In this study, the functions of IE1 were further explored by identifying its interacting proteins using GST-pull down and mass spectrometry analysis. A total of 361 host proteins that potentially bind to IE1 were identified. Bioinformatics analysis revealed that the identified IE1-interactors wereinvolved in various signaling pathways such as prophenoloxidase (proPO) system, PI3K-AKT, and MAPK. Among these, the regulatory role of IE1 in shrimp proPO system was further studied. The Co-immunoprecipitation results confirmed that IE1 interacted with the Ig-like domain of *Penaeus vannamei* proPO or proPO-like protein (hemocyanin). Additionally, we found that knockdown of IE1 reduced viral genes expression and viral loads and increased the hemocytes' PO activity, whereas recombinant IE1 protein inhibited the PO activity in a dose-dependent manner. Finally, we demonstrated that WSSV could suppress the hemocytes' PO activity at the early infection stage. Collectively, our current data indicate that IE1 is a novel viral regulator that negatively modulates the shrimp proPO system.

ARTICLE HISTORY
Received 15 February 2022
Revised 28 April 2022
Accepted 12 May 2022

KEYWORDS
WSSV; immediate-Early protein; shrimp; proPO system

Introduction

White spot syndrome virus (WSSV), the only member of the genus *Whispovirus* in the family *Nimaviridae*, is a highly infectious and lethal virus that infects all major cultured shrimp species in the world [1]. It is a large, enveloped, double-stranded DNA (dsDNA) virus with a circular genome of approximately 300 kbp. Since the first outbreak in the early 1990s, WSSV has spread globally to all the main shrimp-farming areas, causing mass mortalities and enormous economic losses in shrimp aquaculture [2]. Although the complete genome of various WSSV isolates has been successfully sequenced for many years [3–5], most viral proteins show no homology to any known proteins or motifs, and therefore their biological functions during viral infection are still ill-defined and remain to be explored.

Like other DNA viruses, the WSSV genome is expressed sequentially via immediate-early (IE), early (E), and late (L) phases [6]. The IE genes are expressed prior to *de novo* viral protein synthesis and depend only on the transcription and translation machinery of the host cells. They usually encode essential regulatory proteins implicated in initiating virus replication and/or modulating the host's physiological environment to benefit viral replication [7–10]. Until now, although twenty-one IE genes from WSSV genome have been identified using microarray analysis [11–13], only a few IE proteins have been functionally characterized. For example, it had been shown that WSV403 acted as a E3 ubiquitin ligase involved in virus latency infection [14]. WSV051 utilized the crayfish small ubiquitin modifier (SUMO) system to promote WSSV propagation [15]. WSV083, as a serine/threonine-protein kinase, inhibited cell adhesion by attenuating the phosphorylation of the shrimp Focal adhesion kinase (FAK) [16]. A recent study reported that WSV083 could also bind and phosphorylate shrimp *T*-cell factor (Tcf), which led to Tcf degradation via the ubiquitin-proteasome pathway and consequently inhibited the Tcf-mediated antiviral effect [17].

CONTACT Defu Yao dfyao@stu.edu.cn
*These authors contributed equally to this study.
Supplemental data for this article can be accessed online at https://doi.org/10.1080/21505594.2022.2078471.

Among the WSSV IE proteins identified, IE1, also named as WSV069, is currently the most intensively studied. It has been previously shown that IE1 possessed the properties of transactivation, dimerization and DNA-binding [18], suggesting that it may function as a transcription factor during WSSV infection. Interestingly, IE1 could annex shrimp TATA-box binding protein (TBP) to augment its transactivation activity [19], while exploited thioredoxin to restore its reduced DNA binding activity due to oxidizing conditions [20]. On the other hand, a growing number of studies have recently shown that IE1 can also interact with several host cellular proteins to manipulate cell functions for supporting viral replication. For instance, IE1 stimulated G1/S transition by competitively interacting with the shrimp Retinoblastoma protein (Rb), thereby providing a hospitable cellular environment for efficient viral genome replication [21]. Additionally, IE1 could bind and activate the signal transducer and activator of transcription (STAT) and c-Jun NH2-terminal kinase (JNK) to enhance viral genes expression [22,23]. Moreover, IE1 associated with shrimp β-catenin and Chibby, two important regulators of Wnt signaling pathway [24], to inhibit the antiviral effect of Wnt cascade via blocking the nucleus translocation of β-catenin [25,26]. From the foregoing, IE1 is a multifunctional regulator that plays crucial roles in WSSV-host interactions.

In the present study, the potential biological functions of IE1 were further explored by using GST-pull down coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. A total of 361 candidate IE1-interactors from penaeid shrimp were identified, which are mainly implicated in regulating various cellular processes or signaling pathways such as prophenoloxidase (proPO) system, PI3K-AKT, MAPK, Focal adhesion and cell cycle. Furthermore, we confirmed that IE1 interacted with the Ig-like domain of *Penaeus vannamei* proPO or proPO-like protein (hemocyanin), which consequently suppressed the PO activity and promoted viral propagation. Our present data provides additional insights into the functions of IE1 and contributes to a better understanding of the WSSV-host interactions.

Materials and methods

Shrimp and virus

The penaeid shrimp (*Penaeus vannamei*), about 5–8 g, were purchased from Huaxun Aquatic Product Corporation (Shantou, China). All shrimp were kept in air-pumped circulating seawater at room temperature for at least 2 days before all experiments. The intact WSSV stock (China strain, GenBank accession number: AF332093.3) was prepared from infected *Procambrus clarkii* using differential centrifugation, and the concentration of the purified virions was evaluated by spectrophotometry according to previous descriptions [27,28]. All animal experiments were performed in line with the guidelines and approval of the Animal Research and Ethics Committees of Shantou University, Guangdong, China.

Plasmid construction

For prokaryotic protein expression, the open reading frame (ORF) of IE1, amplified from WSSV genomic DNA, was digested by restriction enzyme *Eco*R I and *Xho* I (Takara Bio Inc, China) and inserted into the pGEX-6p-1 vector (Solarbio, China) to generate the plasmid pGEX-6P-IE1. For eukaryotic protein expression, the ORFs of IE1 and enhanced green fluorescent protein (EGFP) were cut by *Kpn* I and *Xho* I, and ligated into the pIZ-V5-His vector (Invitrogen, USA), which produced V5-tagged IE1 and EGFP expression plasmids (pIZ-V5-IE1 and pIZ-V5-EGFP). Similarly, the ORFs of *Pv*proPO1 (GenBank No. EU284136.1), Ig-like domain of *Pv*proPO1, and *Pv*proPO2 (GenBank No. EU373096.1) were fused with a FLAG tag at the *N*-terminus, followed by cleavage with *Hpa* I and *Hin*d III and finally ligated into the pIEx-4 vector (Novagen, USA) to produce plasmids pIEx-FLAG-*Pv*proPO1, pIEx-FLAG-*Pv*proPO1-Ig-like, and pIEx-FLAG-*Pv*proPO2. The ORF of *P. vanmamei* hemocyanin (*Pv*HMC) (GenBank No. X82502), fused with EGFP and FLAG tag at the C-terminus, was cut by *Bam*H I and ligated into the pIZ-V5-His vector using the EasyGeno single assembly cloning kit (TransGen Biotech, China) to construct the plasmid pIZ-*Pv*HMC-EGFP-FLAG. Primers used for plasmid construction are listed in Table 1.

Expression and purification of recombinant glutathione-S-transferase (GST) and GST-IE1 protein

The GST and GST-IE1 expression plasmids (pGEX-6p-1 and pGEX-6p-1-IE1) were transformed into *Escherichia coli* BL21, respectively. The transformed bacteria were cultured at 37°C for 4 h in Luria-Bertani (LB) medium (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.4) containing 100 μg/mL ampicillin (GenStar, China) and then induced using 1 mM isopropyl-B-D-thiogalactopyranoside (IPTG) (GenStar, China) for 16 h at 16°C. After protein induction, the bacteria were resuspended using TBS buffer (50 mM Tris, 150 mM NaCl, 1% TritonX-100, 10% glycerol, 1 mM PMSF, pH 8.0), and then lysed by a brief sonication on ice. Subsequently, the bacteria cell lysate was centrifuged, and the supernatant was collected

Table 1. Primers used in this study.

Primer name	Sequence (5′-3′)
For plasmid construction	
GST-IE1-F	CCGGAATTCATGGCCTTTAATTTTGAAGACTC
GST-IE1-R	CCGCTCGAGTTATACAAAGAATCCAGAAATCTCATC
IE1-V5-F	CGGGGTACCATGGCCTTTAATTTTGAAGACTC
IE1-V5-R	CCGCTCGAGCGTACAAAGAATCCAGAAATCTCATC
EGFP-V5-F	CGGGGTACCATGGTGAGCAAGGGCGAGGAG
EGFP-V5-R	CCGCTCGAGCGCTTGTACAGCTCGTCCATGCC
Flag-*Pv*proPO1-F	ATCGTTAACAATGGACTACAAGGACGACGATGACAAGATGGCGAACGACCAGCAGCGTC
Flag-*Pv*proPO1-R	CCCAAGCTTTCAGTTCAGTTTGTCGCCCAGGAAC
Flag-*Pv*proPO1-Ig-like-F	GGCATCGTTAACACAGGAGTACAAGTTGACCCAGC
Flag-PvproPO1-Ig-like-R	CCCAAGCTTTCACTTATCGTCGTCATCCTTGTAATCGTTCAGTTTGTCGCCCAGG
Flag-*Pv*proPO2-F	ATCGTTAACAATGGACTACAAGGACGACGATGACAAGATGGACAAGAGTCGGAAGAACC
Flag-*Pv*proPO2-R	CCCAAGCTTTCAGTCTCGGTTCAGCCTCTCGG
*Pv*HMC-EGFP-Flag	TTGGTACCGAGCTCGATGAGGGTCTTAGTGGTTCT
*Pv*HMC-EGFP-Flag	CACACTGGACTAGTGTAATGAATGTGTTCACCATGATTG
For qPCR assay	
qIE1-F	GCACAACAACAGACCCTACCC
qIE1-R	GAAATACGACATAGCACCTCCAC
qWSV051-F	GAAGAGCTTGTCGACGTGCC
qWSV051-R	TCTGTGGATTTTCTGATGGGT
qWSV079-F	GGGTGATTACGATTCGGACA
qWSV079-R	ACGGAAGAACCACACAGGGG
qWSV514-F	GTTTGATTATGCGGGTGCTTT
qWSV514-R	CCCTCTCCGTCCCCGCTCATA
qWSVICP11-F	ACCTTCCAGACTGACGCCG
qWSVICP11-R	CATTGAGGACAAAAGTAGATCCC
qVP28-F	AAACCTCCGCATTCCTGTGA
qVP28-F	TCCGCATCTTCTTCCTTCAT
qEF1α-F	TATGCTCCTTTTGGACGTTTTGC
qEF1α-R	CCTTTTCTGCGGCCTTGGTAG
For RNAi assay	
dsRNA-IE1-F	GAAGACTCTACAAATCTCTTT
dsRNA-IE1-R	CTTGCACCTACACGCATTACA
dsRNA-IE1-T7-F	GGATCCTAATACGACTCACTATAGGGAAGACTCTACAAATCTCTTT
dsRNA-IE1-T7-R	GGATCCTAATACGACTCACTATAGGCTTGCACCTACACGCATTACA
dsRNA-EGFP-F	*CGTAAACGGCCACAAGTT*
dsRNA-EGFP-R	TTCACCTTGATGCCGTTC
dsRNA-EGFP-T7-F	GGATCCTAATACGACTCACTATAGGCGTAAACGGCCACAAGTT
dsRNA-EGPF-T7-R	GGATCCTAATACGACTCACTATAGGTTCACCTTGATGCCGTTC

and further incubated with GSH magnetic beads (Beaverbio, China) for protein purification. The purity of the recombinant GST and GST-IE1 proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining.

GST pull-down and LC-MS/MS analysis

In order to identify the potential cellular proteins interacting with IE1, GST pull-down assay was performed as previously described with minor modification [29]. Briefly, the hemocytes were collected from the WSSV-infected shrimp and lysed in cell lysis buffer (50 mM Tris, 150 mM NaCl, 1% TritonX-100, 10% glycerol, 2 mM EDTA, 1 mM Phenylmethylsulfonyl fluoride [PMSF] and 1×cocktail protease inhibitor, pH7.4). After a brief sonication, the hemocytes lysates were centrifugated at 20,000 × g for 20 min at 4°C to collect the supernatants. Next, the supernatant proteins (about 5 mg total protein) were pre-cleared by incubating with GSH magnetic beads (Beaverbio, China) for 1 h at 4°C and then incubated with GST or GST-IE1 protein conjuncted GSH magnetic beads overnight at 4°C. Thereafter, the beads were thoroughly washed using cell lysis buffer and bound proteins were eluted from the beads by boiling with 1× SDS protein loading buffer (50 mM Tris, 100 mM dithiothreitol [DTT], 2% SDS, 0.1% bromophenol blue, 0.1% β-mercaptoethanol, 10% glycerol, pH 6.8). The prepared protein samples were analyzed by SDS-PAGE and subsequent silver staining.

For protein identification, the differential brands containing the potential IE1-interacting proteins were excised and delivered to Shanghai Applied Protein Technology (APT) for LC-MS/MS analysis. The gels were cut into small pieces and sequentially subjected to protein reduction, alkylation, and digestion, followed by LC-MS/MS analysis using a Q-Exactive mass spectrometer (Thermo Scientific, USA) as previously described [29]. The generated MS/MS spectra were searched against our *in-house P. vannamei* transcriptome data [30] using the Byonic

software (version1.7.0_25) to identify the peptide and protein. All procedures and methods used in the LC-MS/MS followed the standard guideline of APT.

Bioinformatics analysis

The Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway entry ID of each identified IE1-interacting proteins were retrieved from our *in-house P. vannamei* transcriptome data. The GO entry ID was subjected to GO functional classification using Web Gene Ontology Annotation Plot (WEGO) program (https://wego.genomics.cn), while the KEGG pathway entry ID was further analyzed by KEGG pathway database (https://www.genome.jp/kegg/). The functional domains of *Pv*proPO1, *Pv*proPO2 and *Pv*HMC were analyzed using Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Co-Immunoprecipitation (Co-IP) assay

High-five cells were seeded into six-well culture plate at the density of about 1×10^6 cells/well and then maintained in Express Five SFM medium (ThermoFisher Scientific, USA) overnight. For DNA transfection, 1 μg of the FLAG-tagged *Pv*proPO1, *Pv*proPO1-Ig-like, *Pv*proPO2 and *Pv*HMC expression plasmids (pIEx-FLAG-*Pv*proPO1, pIEx-FLAG-*Pv*proPO1-Ig-like, pIEx-FLAG-*Pv*proPO2, and pIZ-*Pv*HMC-EGFP-FLAG) were separately co-transfected with 1 μg of pIZ-V5-IE1 or pIZ-V5-EGFP using FuGENE HD transfection reagent (Promega, USA) according to the manufacturer's instructions. At 48 h post transfection, the cells were harvested and lysed with 200 μL of Western and IP cell lysis buffer (Beyotime, China) for 20 min on ice. The cell lysate supernatant was then collected by centrifugation at 16,000 × g for 10 min at 4°C, After that, a fraction of cell lysate supernatant (20 μL) was used to detect protein expression by Western blot, while the rest was incubated with 5 μL of anti-FLAG M2 magnetic beads (Sigma-Aldrich, USA) for overnight at 4°C. Finally, the beads were washed three times using cell lysis buffer, and further incubated with 20 μL of 3×FLAG peptide (1 mg/mL) to elute the bound proteins for Western blot analysis.

Western blot analysis

The protein samples were separated by SDS-PAGE and transferred onto PVDF transfer membrane (Millipore, USA). The membranes were blocked with 5% (w/v) skim milk dissolved in TBST buffer (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.6) at room temperature for 1 h, and then incubated with the primary antibodies (*i.e.*, anti-V5, anti-FLAG, anti-IE1 and anti-Tubulin) for overnight at 4°C. After three times wash, the membranes were further incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (ThermoFisher Scientific, USA) at room temperature for 1 h. Proteins on the membranes were subjected to react with Immobilon Western Chemiluminescent HRP Substrate (Millipore, USA) and visualized using Amersham Imager 600 (GE Healthcare, USA). The anti-V5 and anti-FLAG antibodies were bought from Sangong Biotech (Shanghai). The anti-tubulin antibody was purchased from Sigma-Aldrich. The anti-IE1 antibody was prepared *in-house* by immunizing the mouse with recombinant IE1.

WSSV challenge and determination of the PO activity

For WSSV challenge experiment, the shrimp were intramuscularly injected with 100 μL of WSSV virions (approximately 1×10^5 copies) using a sterile syringe with a 22-gauge needle, while the control group was injected with an equivalent volume of sterile PBS. At 0, 3, 6, 12 and 24 h post-infection, hemolymph per group was collected into 300 μL of precooled anti-coagulant solution (258 mM Sodium citrate dihydrate, 328 mM Sodium citrate, 110 mM glucose, 140 mM NaCl, pH 6.0) using the sterile syringe, and then immediately centrifugated at 500×g for 10 min at 4°C to pellet the hemocytes. For determination of the PO activity, the obtained hemocytes were washed two times using anti-coagulant solution and used for measurement of the PO activity as described previously with some modification [31,32]. In brief, hemocytes were lysed and sonicated in cell lysis buffer (415 mM NaCl, 100 mM glucose, 10 mM cacodylic acid, 5 mM $CaCl_2$, pH7.0). The hemocytes lysate supernatant (HLS) was collected by centrifugation at 20,000×g for 20 min at 4°C and total protein concentration was then determined using BCA protein assay kit (Sangon Biotech, China). Next, HLS (100 μl) was mixed with 65 μl of freshly prepared L-DOPA (3 mg/mL) (Sangon Biotech, China). After incubation for 30 min at room temperature, the absorbance at 490 nm was monitored using a microplate reader, and the PO activity was determined as ΔA490/mg total protein/min.

RNAi assay

In order to explore the role of IE1 on the shrimp proPO activation, RNAi experiment was performed as previously described with minor modification [19]. Briefly, the double-strand RNA (dsRNA) of IE1 and EGFP, designated as

dsRNA-IE1 and dsRNA-EGFP, were synthesized *in vitro* using a commercial RNA synthesis kit (New England Biolabs, USA) with primers shown in Table 1. Shrimps were randomly divided into two groups, with each of the experimental and control group intramuscularly injected with 10 μg dsRNA-IE1 and dsRNA-EGFP, respectively. At 12 h post-dsRNA injection, each shrimp were further injected with 1×10^5 WSSV virions. At 24 h post infection, hemocytes per group were collected and used for the measurement of the PO activity as described above. Meanwhile, the total RNA, genomic DNA and whole cell lysates were prepared from hemcoytes and subjected to evaluate the knockdown efficiency of IE1 using Western blot analysis as described above, as well as determine the viral genes expression and viral loads using qPCR.

Detection of viral genes expression and viral loads by qPCR

To analyze the knockdown efficiency of IE1 and further determine whether IE1 depletion affects the expression of the other viral genes, relative qPCR experiment was carried out. Briefly, the total RNA was extracted from hemocytes using the RNAFast200 kit (FeiJie, Shanghai, China), and cDNA was synthesized using the TransScript one-step gDNA removal and cDNA synthesis superMix (Trans Gen Biotech, China) according to the manufacturer's instructions. The qPCR reactions contained 10 μL of 2×RealStar Green power mixture (GenStar, China), 1 μL each of forward and reverse primers, 1 μL of cDNA template plus 7 μL of Milli-Q water. The qPCR assays were performed on a LightCycler 480 (Roche, Switzerland) with the following cycling conditions: one cycle at 95°C for 5 min and 35 cycles of 95°C for 15 s and 60°C for 30 s. Each sample per group was carried out in triplicate, with the *Pv*EF1α used as the internal control. For viral loads detection, the genomic DNA was isolated from hemoctyes using Marine Animal DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. The viral loads were detected using absolute qPCR as previously described [33]. All the primers used in this study are listed in Table 1.

Effect of recombinant IE1 protein on the PO activity

To further whether IE1 has a direct inhibitory effect on the PO activity, the purified recombinant GST-IE1 (0.2 μg or 2 μg) protein or the same amount of GST protein (negative control) was incubated with 70 μg of HLS for 5 min at room temperature. L-DOPA was then added into to the reaction mixture. At 30 min post incubation, the absorbance at 490 nm was monitored, and the PO activity was determined as described above.

Results

Identification of the host cellular proteins that potentially interact with IE1

Recently, several studies have demonstrated that IE1 is a versatile regulator that is essential for WSSV replication [19–23,25,26]. In order to further gain insight into the biological functions of IE1 during viral infection, in this study, we conducted GST-pull down and LC-MS/MS analysis to identify the cellular host proteins that may interact with IE1. At first, the recombinant GST-IE1 and GST proteins were expressed and purified from *E. coli* cells (Figure 1a–c). For GST pull-down experiment, the GSH magnetic beads bound with the GST-IE1 or GST (negative control) were incubated with HLS for overnight at 4°C. After a thorough wash, the bound proteins were eluted from the beads, followed by SDS-PAGE and silver staining analysis. As indicated in Figure 1(d), compared with control, there were 4 differential bands with molecular protein sizes at >90 kDa, 50–90 kDa, 30–40 kDa, and 20–25 kDa (indicated by box B1-B4). Finally, the four differential gels were then dissected and subjected to LC-MS/MS analysis. The MS/MS search results showed that the differential bands (B1-B4) were identified as 98, 110, 104, and 49 proteins, respectively (Supplementary Table S1). Therefore, we identified a total of 361 host proteins that potentially bind to IE1 in this study.

Functional classification analysis of the potential IE1-interacting proteins

To clarify the biological functions of the identified IE1-interacting proteins, the GO annotation analysis was performed. As shown in Figure 2(a), of the 361 identified IE1-interacting proteins, a total of 264 proteins had been annotated, which were classified into three GO terms including biological process, molecular function and cellular component. In biological process, the identified proteins were mainly implicated in cellular process (73.5%) and metabolic process (56.8%). For cellular component and molecular function, most of the identified proteins were localized in cell part (67.8%) and organelle (50.8%),

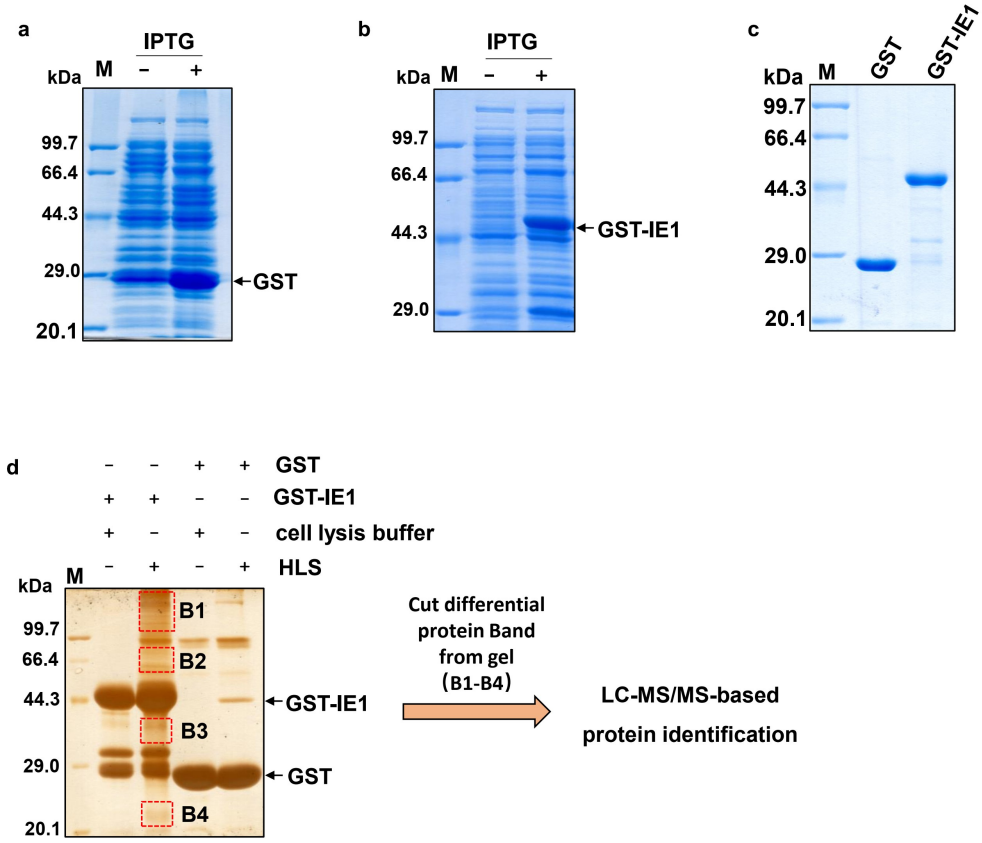

Figure 1. Identification of the host cellular proteins potentially interacting with WSSV IE1. (a) and (b) Expression of recombinant GST and GST-IE1 proteins. The GST or GST-IE1 expression plasmid transformed *Escherichia coli* cells were induced using 0 or 1 mM IPTG and then lysed for SDS-PAGE analysis. (c) SDS-PAGE analysis of the purified recombinant GST and GST-IE1 proteins. (d) GST pull-down and LC-MS/MS analysis of the candidate IE1-interacting proteins. The GST and GST-IE1-bound GSH magnetic beads were incubated with the hemocytes lysate supernatant (HLS). After incubation and wash, the bound proteins on the beads were eluted and then subjected to SDS-PAGE and silver staining analysis. The differential bands were excised from the gel and used for LC-MS/MS analysis. M: protein marker.

and possessed binding (69.3%) and catalytic activity (63.3%). In addition, the identified IE1-interacting proteins were further analyzed using KEGG pathway analysis. The results showed that the identified proteins were involved in modulating various signaling pathways such as proPO system, PI3K-AKT, MAPK, Focal adhesion and cell cycle (Figure 2b). Notably, the proPO system, an important component of the innate immune system in invertebrates [34], was the most annotated signaling pathway (Figure 2b and Supplementary Table S2), suggesting that IE1 might play vital roles in controlling the proPO activation in shrimp.

IE1 binds to Ig-like domain of PvproPO and PvHMC

According to the results of LC-MS/MS identification, a total of 14 proPO system-related proteins were identified to be putative IE1-interactors with the identification of *Pv*proPO1, *Pv*proPO2 and *Pv*HMC containing the most number of unique peptides (Table 2). Bioinformatic analysis revealed that *Pv*proPO1, *Pv*proPO2, and *Pv*HMC were highly homologous. The *Pv*proPO1 and *Pv*proPO2 had 72% identity in protein sequence, while *Pv*proPO1/2 and *Pv*HMC showed 28% sequence identity (Figure 3a). In addition, the three proteins were composed of the same functional domains including an all-alpha domain in the N terminus, a Copper-containing domain in the midsection and an Ig-like domain in the C terminus (Figure 3a). The data indicated that *Pv*proPO1/2 and *Pv*HMC play similar roles in the proPO system, and they were therefore selected to further determine whether they can interact with IE1 using Co-IP experiment. The FLAG-tagged *Pv*proPO1, *Pv*proPO2, and *Pv*HMC proteins were co-expressed with the V5-tagged IE1 or EGFP (negative control) in the insect High-five cells, respectively. As indicated in Figure 3(b–d), when using anti-FLAG or anti-V5 antibody in Western blot analysis, the expression of *Pv*proPO1, *Pv*proPO2,

Table 2. Summary of the identified proPO system proteins that potentially binds IE1.

Protein name	ProteinID	Pepcount	UniquePep	Coverage	MW
Prophenoloxidase-1	CL2821.Contig1	14	14	18.08%	78,170.71
Alpha 2 macroglobulin	CL1530.Contig1	12	12	7.57%	167,057.3
Prophenoloxidase-2	CL2821.Contig2	5	5	6.08%	78,810.5
Hemocyanin	Unigene13144	4	4	4.53%	74,988.91
Serine protease 3	Unigene20940	4	3	11.14%	41,037.38
Clip domain serine proteinase 1	Unigene16701	3	3	6.96%	41,946.03
Serine protease 1	Unigene9185	3	2	6.01%	39,113.96
Prophenoloxidase activating enzyme	CL4501.Contig2	2	2	3.71%	56,046.14
QM protein	Unigene16939	2	2	5.45%	25,531.53
Serine protease snake	CL4163.Contig1	2	2	5.43%	38,122.1
Serine proteinase	Unigene14592	2	2	5.41%	36,386.68
Prophenoloxidase activating enzyme 3	Unigene1965	2	2	4.72%	41,021.6
Serine protease Mas	Unigene8002	1	1	2.25%	37,865.99
Clip domain serine proteinase 2	Unigene25426	1	1	3.74%	31,657.49

*Pv*HMC, IE1 and EGFP could be detected in all input samples. Next, the FLAG-tagged proteins were then immunoprecipitated using anti-FLAG M2 magnetic beads, and the IP samples were subjected to Western blot analysis using anti-V5 antibody. The results showed that the FLAG-tagged *Pv*proPO1, *Pv*proPO2, and *Pv*HMC could be immunoprecipitated with the V5-tagged IE1, whereas EGFP could not be immunoprecipitated (Figure 3b–d). Furthermore, to figure out which functional domain of *Pv*proPO and *Pv*HMC was responsible for IE1 binding, we constructed the expression plasmids of all-alpha domain, Copper-containing domain, and Ig-like domain of *Pv*proPO1 and Co-IP was performed as described above. Unfortunately, only the Ig-like domain of *Pv*proPO1 could be successfully expressed in cells, while the all-alpha domain and Copper-containing domain of *Pv*proPO1 could not be expressed. The Co-IP result showed that IE1 could bind to the Ig-like domain of *Pv*proPO1 (Figure 3e). Given that the Ig-like domain of *Pv*proPO and *Pv*HMC is highly conserved, the data here suggest that IE1 could physically interact with the Ig-like domain of the *Pv*proPO and *Pv*HMC.

IE1 inhibits the PO activity and promotes viral replication

To explore how IE1 modulates the proPO system, as well as its effect on virus replication, RNAi experiment was carried out in this study. As shown in Figure 4(a), the dsRNA-IE1 or dsRNA-EGFP (negative control) were injected into shrimp to knockdown IE1 expression during WSSV infection, followed by detection of viral genes expression, viral loads and PO activity. The qPCR results showed that the mRNA and protein level of IE1 in WSSV-infected hemocytes was dramatically downregulated after injection with dsRNA-IE1 compared with the control dsRNA-EGFP (Figure 4b–c), indicating that a successful *in vivo* gene silencing of IE1 during viral infection. It was showed that the suppression of IE1 could significantly

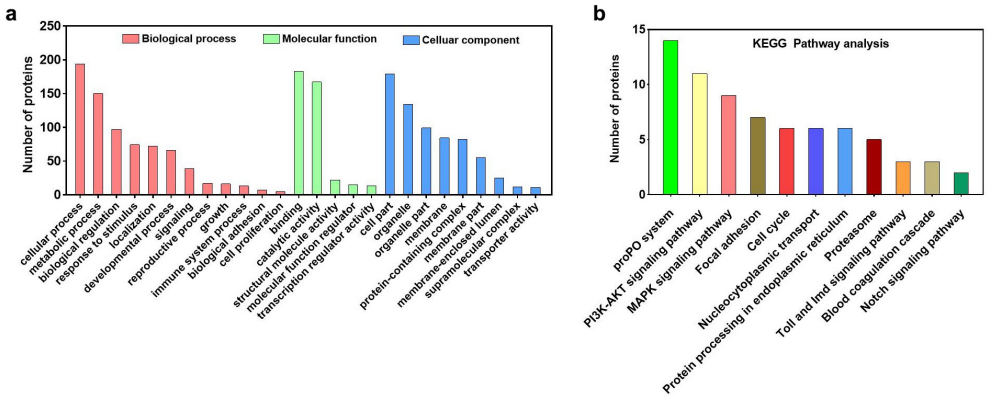

Figure 2. Functional classification analysis of the potential IE1-interacting proteins. (a) and (b) GO and KEGG pathway analysis of the identified IE1-interacting proteins. The GO and KEGG pathway entry ID of each identified IE1-interacting proteins were retrieved from our *in-house P. vannamei* transcriptome data, and then subjected to GO and KEGG pathway analysis using WEGO program and KEGG pathway database.

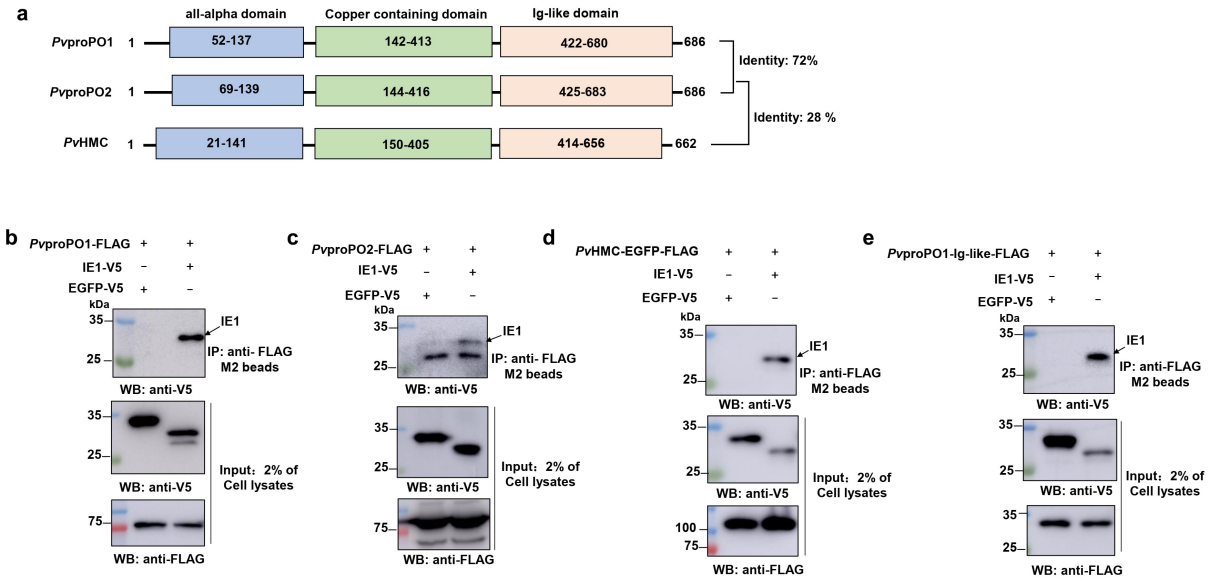

Figure 3. IE1 binds to the Ig-like domain of *Pv*proPO and *Pv*HMC. (a) Schematic comparison of *Pv*proPO1, *Pv*proPO2 and *Pv*HMC in protein sequence identity and functional domains. (b-e) Co-IP analysis of the interactions between IE1 and *Pv*proPO/*Pv*HMC. The FLAG-tagged PvproPo1/pvpropo2/pvhmc/pvpropo1-Ig-like protein was co-expressed with the V5-tagged IE1 or EGFP (negative control) in High-five cells. At 48 h post-transfection, cells were harvested and lysed. The whole cell lysate (Input samples) were obtained by centrifugation and incubated with anti-FLAG M2 magnetic beads. The bound proteins (IP samples) were eluted from the beads using 3×FLAG peptide. Finally, the input (2%) and IP samples were subjected to western blot analysis with the indicated antibodies.

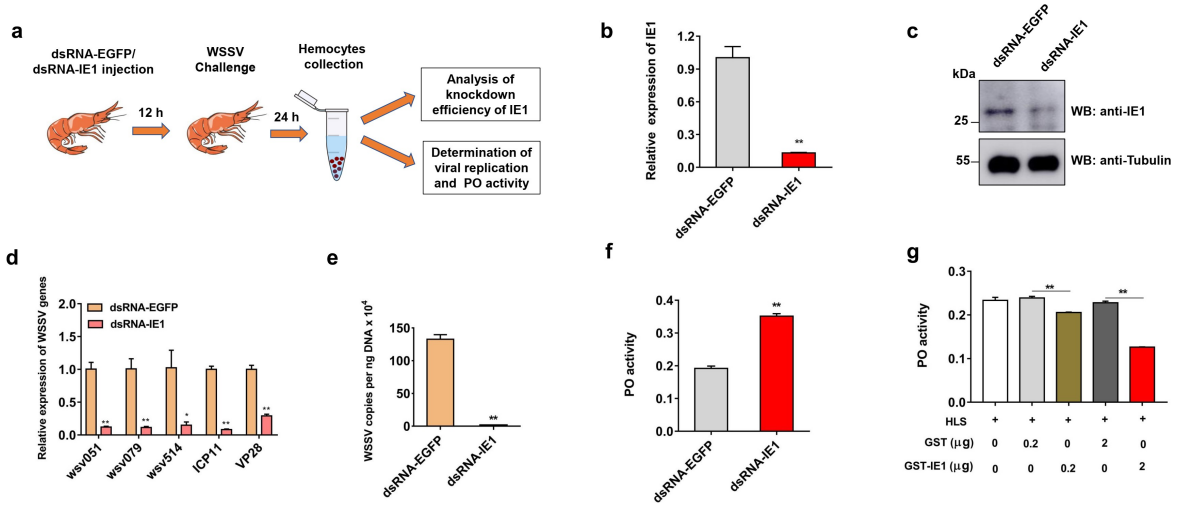

Figure 4. IE1 inhibits PO activity and promotes viral replication in shrimp hemocytes. (a) Workflow of RNAi of IE1 during WSSV infection. (b-c) Knockdown efficiency analysis of IE1. hemocytes in dsRNA-IE1 or dsRNA-EGFP-injected shrimp were collected and used to prepare cDNA and protein lysates for qPCR and western blot analysis. (d) Detection of WSSV genes after IE1 silencing by relative qPCR. The mRNA expression of IE1 (WSV069), WSV051, WSV079, WSV514, ICP11 (WSV230), and VP28 (WSV421) was normalized to *Pv*EF1α and calculated using the $2^{-\Delta\Delta CT}$ method. (e) Detection of viral loads by absolute qPCR. The genomic DNA was extracted from hemocytes in IE1 or EGFP-silenced shrimp, followed by absolute qPCR analysis, the WSSV copy number in 1 ng of shrimp genomic DNA was then calculated. (f) Determination of the PO activity after IE1 suppression. The hemocyte lysate supernatant (HLS) prepared from IE1 or EGFP-silenced shrimp were incubated with L-DOPA. After reaction for 30 min at room temperature, the absorbance at 490 nm was monitored, and the PO activity was determined as ΔA490/mg total protein/min. (g) Effect of recombinant IE1 protein on the PO activity. The HLS was incubated with recombinant GST-IE1 (0.2 μg or 2 μg) or the equal amount of GST protein (negative control) for 5 min at room temperature. After further reaction with L-DOPA, the PO activity was then determined by measuring the absorbance at 490 nm. All the experiments were carried out in independent triplicates, and the data were shown as Mean ± SD. The significance difference was analyzed using the student's *t*-test (*p <0.05, ** p <0.01).

decrease the expression of viral genes (i.e., WSV051, WSV079, WSV514, ICP11 and VP28) (Figure 4d) and viral loads (Figure 4e), suggesting that IE1 plays an important role in viral replication. Next, when the PO activity in hemocytes was determined using L-DOPA as the substrate, shrimp depleted with IE1 had a remarkable increase (1.83-fold) in PO activity compared with the control EGFP-silenced shrimp (Figure 4f). Furthermore, the direct inhibition of IE1 protein on the PO activity was also analyzed *in vitro*. The result showed that the PO activity of HLS prepared from non-WSSV infected shrimp was significantly reduced when recombinant IE1 protein was added compared with GST (Figure 4g). This inhibition was dose-dependent, as more decreased PO activity was detected when the dosage of recombinant IE1 protein increased (Figure 4g). These results suggest that IE1 can directly inhibit the PO activity and promote viral replication.

WSSV represses the PO activity at the early infection stage

Given that IE1 is an immediately early protein of WSSV, we sought to determine whether WSSV repressed the PO activity in the early infection stage. As indicated in Figure 5(a), at the early infection time points, the mRNA expression of IE1 was detected at as early as 3 h and increased gradually from 6 to 24 h post infection. Meanwhile, compared with the PBS control, the PO activity in WSSV-infected shrimp was reduced by 1.78-fold at 3 h post infection, and continued to decrease by 2-, 1.78- and 4-fold, respectively, at 6, 12 and 24 h (Figure 5b). These results therefore suggest that WSSV could suppress the shrimp PO activity, presumably from the early infection stage.

Discussion

For DNA viruses, they usually encode IE proteins that utilize and/or manipulate the host's cellular machinery for completing the replication cycle. Recently, a growing number of studies have shown that IE1, an immediate-early protein encoded by WSSV, interacts with several host cellular proteins (i.e. Rb, STAT, JNK, β-catenin and Chibby) to modulate various cellular processes or signaling pathways and subsequently facilitate virus replication [21–23,25,26]. However, the IE1-mediated interaction networks in shrimp remain poorly understood. Therefore, in this study, we used GST pull-down and LC-MS/MS to investigate the host cellular proteins that bind IE1. A total of 361 cellular proteins from the penaeid shrimp were identified to interact with IE1 (Supplementary Table S1) potentially. Bioinformatics analysis revealed that the identified IE1-interacting proteins were involved in regulating various cellular processes or signaling pathways such as proPO system, PI3K-AKT, MAPK, Focal adhesion and cell cycle (Figure 2b), indicating the critical roles played by IE1 in these cell functions. Interestingly, previous studies have shown that IE1 modulates the MAPK and cell cycle via binding of the shrimp Rb and JNK proteins [21,23]. Our present data further hinted us that the involvement of IE1 in the regulation of MAPK and cell cycle is presumably intricate, and the detailed molecular mechanisms need further studies in the future.

Given that the IE1-interacting proteins are mainly annotated in the proPO system (Figure 2b and Table 2), the involvement of IE1 in regulation of this process was further investigated in this study. It is well known that the proPO system, which mediates the melanization, is a crucial innate immune defense

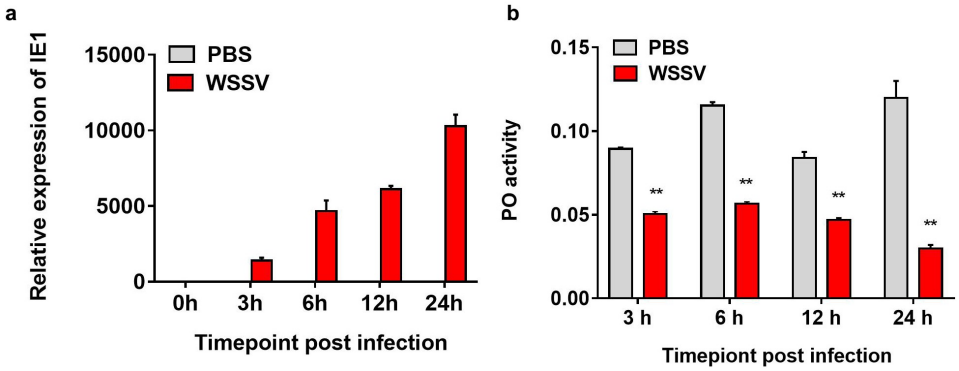

Figure 5. WSSV suppresses the shrimp PO activity at the early stage of infection. (a) qPCR analysis of IE1 expression during WSSV infection. (b) Detection of PO activity after WSSV infection. The shrimp was divided into two group, and then intramuscularly injected with WSSV or PBS (negative control). At 0, 3, 6, 12 and 24 h post-infection, the hemocytes per group were collected and used for qPCR and determination of PO activity. The data referred to the means ± SD of triplicate assays (**, p <0.01).

system in invertebrates [35]. The proPO system is activated through a serine proteinase cascade. Once microbes are invading, the microbes-derived elicitors such as lipopolysaccharide (LPS) trigger a serine proteinase cascade including the proPO-activating enzymes (PPAE), ultimately leading to the cleavage of the proPO zymogen into the active PO enzyme. The latter factor oxidizes phenols into quinones that act as precursor to produce the pigment melanin and toxic reactive intermediates for killing and restraining the invading microbes. In shrimp, there are many studies reporting that the proPO system plays crucial roles in immune defenses against bacterial and viral infections [36]. It was showed that RNAi-mediated suppression of PPAEs significantly increased shrimp's susceptibility to *Vibrio harveyi* infection [37,38]. Similarly, it was found that knockdown of PPAEs and proPOs increased viral loads and cumulative shrimp mortality after WSSV infection [39,40]. However, mounting evidence have recently suggested that the pathogenic microbes, especially virus, have evolved with various strategies to evade or counter the antiviral melanization for their own benefit. For example, it was reported that the WSSV-induced miR-315 attenuated the activation of proPO system via suppression of PPAE3 expression [41]. In addition, WSSV encoded proteins WSSV453 and WSSV164 (also known as WSV394 and WSV108 in China WSSV strain) could inhibit the proPO activation by direct binding and inhibition of the shrimp PPAEs and proPOs, respectively [42,43]. In this study, we further demonstrated that another viral protein IE1 could interact with the Ig-like domain of the proPO or proPO-like proteins (*Pv*proPO1/2 and *Pv*HMC) (Figure 3b–e), which are key enzymes/regulators in the shrimp proPO system [44–46]. Further analysis showed that *in vivo* RNAi-mediated knockdown of IE1 increased the hemocytes' PO activity in WSSV-infected shrimp (Figure 4f), while incubation of recombinant IE1 protein with HLS reduced the PO activity *in vitro* in dose-dependent manner (Figure 4g). These results indicate that IE1 is a novel viral regulator that negatively modulates the shrimp proPO system during WSSV infection. In addition, given that the viral proteins WSSV164 and IE1 were both identified to be IE proteins [12], couple with the fact that WSSV453 could also be expressed at the early stage of infection [42], we reasoned that WSSV might inhibit the proPO system in the early period. To confirm this, the PO activity of shrimp hemocytes was analyzed at early

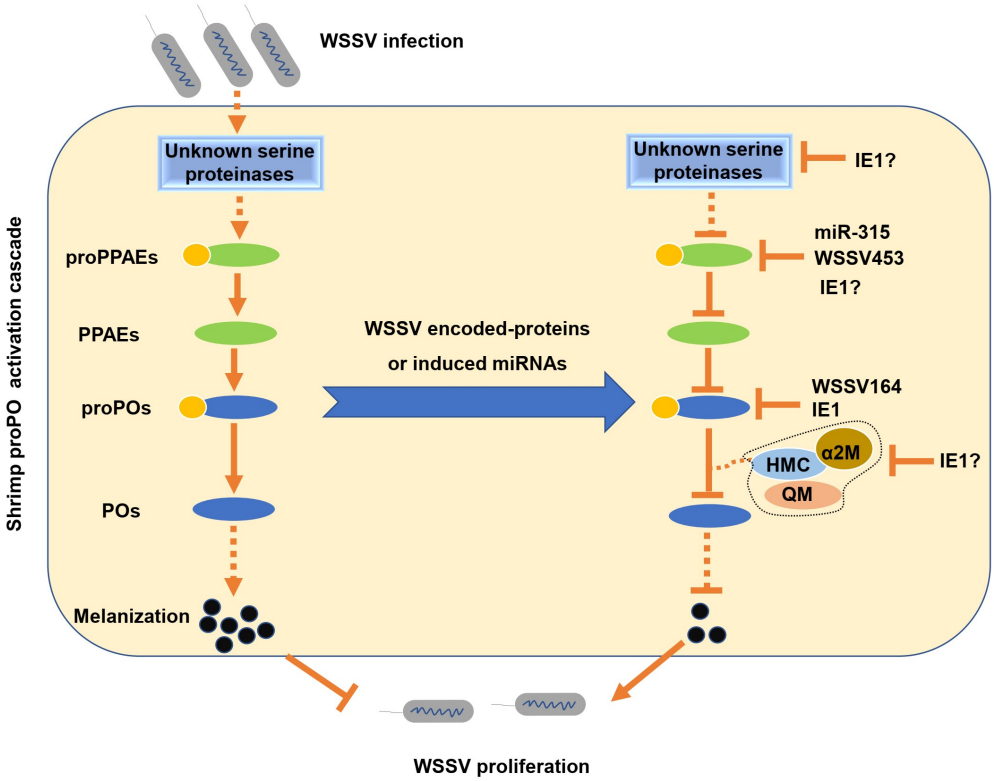

Figure 6. The proposed schematic diagram for the WSSV IE1-mediated inhibition of the proPO system in penaeid shrimp.

timepoints of infection by WSSV (i.e. 3, 6, 12 and 24 h). The result revealed that WSSV infection suppressed the PO activity at as early as 3 h after infection, and this inhibition effect was enhanced as the infection continued (Figure 5 a and b). These reports suggest that WSSV, presumably at the early stage, adopts various mechanisms to negatively modulate the host proPO system for aiding its own replication.

In invertebrates, the proPO system can be tightly regulated at multi-steps which involves various enzymes/modulators such as serine proteinases, serine proteinase inhibitors, PPAEs and proPOs. Recently, an increasing number of cell proteins have been shown to participate in control of the proPO activation in shrimp. For example, HMC is a proPO-like protein that not only has PO activity but also can regulate the proPO activation via binding of QM protein [46,47]. In addition, alpha 2 macroglobulin (α2 M) is a broad-spectrum proteinase inhibitor in plasma that functions in the inhibition of proPO activation [48]. Our recent study showed that HMC could interact with the receptor binding domain of the shrimp α2 M, thereby affecting the α2 M's inhibitory effect on PO activity [49]. In this study, it is noteworthy that IE1 was found to potentially interact with many proPO system proteins including serine proteinases, PPAEs, QM protein and alpha 2 macroglobulin apart from the validated *Pv*proPO1/2 and *Pv*HMC (Table 2). The data suggest that IE1 might utilize multi-mechanisms to inhibit the shrimp proPO system, which is probably different with WSSV453 and WSSV164. Of course, the underlying molecular mechanisms by which IE1 controls the shrimp proPO system require further investigation in the future.

In conclusion, this study for the first time explored the IE1-mediated interaction networks in penaeid shrimp, which led to identification of 361 potential IE1-interacting proteins with implications in many signaling pathways. Furthermore, we demonstrated that IE1 is a novel regulator encoded by WSSV that suppresses the host's melanization via binding and inhibition of the proPO system proteins such as *Pv*proPO and *Pv*HMC (Figure 6). The current finding provides new insights into the struggle between WSSV and host, which may contribute to virus prevention and control in shrimp aquaculture.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability statement

All the data supporting the findings of this study are available within the article and its supplementary materials.

Funding

This work was funded by the National Natural Science Foundation of China [Nos. 31702387 and 32173017], China Postdoctoral Science Foundation [No. 2018M630973], Science and Technology Planning Project of Guangdong Province [STKJ2021188] and Shantou University Scientific Research Foundation for Talents [No. NTF20008].

ORCID

Jude Juventus Aweya http://orcid.org/0000-0003-2554-9369

References

[1] Escobedo-Bonilla CM, Alday-Sanz V, Wille M, et al. A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus. J Fish Dis. 2008;31:1–18.

[2] Dey BK, Dugassa GH, Hinzano SM, et al. Causative agent, diagnosis and management of white spot disease in shrimp: a review. Rew Aquacult. 2020;12(2):822–865.

[3] Yang F, He J, Lin XH, et al. Complete genome sequence of the shrimp white spot bacilliform virus. J Virol. 2001;75(23):11811–11820. DOI:10.1128/JVI.75.23.11811-11820.2001

[4] Tsai JM, Wang HC, Leu JH, et al. Genomic and proteomic analysis of thirty-nine structural proteins of shrimp white spot syndrome virus. J Virol. 2004;78(20):11360–11370. DOI:10.1128/JVI.78.20.11360-11370.2004

[5] van Hulten MC, Witteveldt J, Peters S, et al. The white spot syndrome virus DNA genome sequence. Virology. 2001;286(1):7–22. DOI:10.1006/viro.2001.1002

[6] Marks H, Vorst O, van Houwelingen A, et al. Gene-Expression profiling of white spot syndrome virus in vivo. J Gen Virol. 2005;86(Pt 7):2081–2100. DOI:10.1099/vir.0.80895-0

[7] Fox HL, Dembowski JA, DeLuca NA. A herpesviral immediate early protein promotes transcription elongation of viral transcripts. Mbio. 2017;8(3):e00745-17. DOI:10.1128/mBio.00745-17.

[8] Guan XM, Zhang MD, Fu M, et al. Herpes simplex virus type 2 immediate early protein ICP27 inhibits IFN-β production in mucosal epithelial cells by antagonizing IRF3 activation. Front Immunol. 2019;10:290.

[9] Long XB, Yang ZW, Li Y, et al. BRLF1-Dependent viral and cellular transcriptomes and transcriptional regulation during EBV primary infection in B lymphoma cells. Genomics. 2021;113(4):2591–2604. DOI:10.1016/j.ygeno.2021.05.039

[10] Torres L, Tang QY. Immediate-Early (IE) gene regulation of cytomegalovirus: IE1- and pp71-mediated viral strategies against cellular defenses. Virol Sin. 2014;29 (6):343–352.
[11] Liu WJ, Chang YS, Wang CH, et al. Microarray and RT-PCR screening for white spot syndrome virus immediate-early genes in cycloheximide-treated shrimp. Virology. 2005;334(2):327–341. DOI:10.1016/j.virol.2005.01.047
[12] Li F, Li MY, Ke W, et al. Identification of the immediate-early genes of white spot syndrome virus. Virology. 2009;385(1):267–274. DOI:10.1016/j.virol.2008.12.007
[13] Lin FY, Huang H, Xu LM, et al. Identification of three immediate-early genes of white spot syndrome virus. Arch Virol. 2011;156(9):1611–1614. DOI:10.1007/s00705-011-1004-1
[14] He F, Kwang J. Identification and characterization of a new E3 ubiquitin ligase in white spot syndrome virus involved in virus latency. Virol J. 2008;5:151.
[15] Chen AJ, Gao L, Wang XW, et al. SUMO-Conjugating enzyme E2 UBC9 mediates viral immediate-early protein SUMOylation in crayfish to facilitate reproduction of white spot syndrome virus. J Virol. 2013;87(1):636–647. DOI:10.1128/JVI.01671-12
[16] Lu HS, Ruan LW, Xu X. An immediate-early protein of white spot syndrome virus modulates the phosphorylation of focal adhesion kinase of shrimp. Virology. 2011;419(2):84–89.
[17] Wang CQ, Ruan LW, Shi H, et al. Phosphorylation of shrimp tcf by a viral protein kinase WSV083 suppresses its antiviral effect. Front Immunol. 2021;12:698697.
[18] Liu WJ, Chang YS, Wang HC, et al. Transactivation, dimerization, and DNA-binding activity of white spot syndrome virus immediate-early protein IE1. J Virol. 2008;82(22):11362–11373. DOI:10.1128/JVI.01244-08
[19] Liu WJ, Chang YS, Huang WT, et al. *Penaeus monodon* TATA box-binding protein interacts with the white spot syndrome virus transactivator IE1 and promotes its transcriptional activity. J Virol. 2011;85(13):6535–6547. DOI:10.1128/JVI.02433-10
[20] Huang JY, Liu WJ, Wang HC, et al. *Penaeus monodon* thioredoxin restores the DNA binding activity of oxidized white spot syndrome virus IE1. Antioxid Redox Sign. 2012;17(6):914–926. DOI:10.1089/ars.2011.4264
[21] Ran XZ, Bian XF, Ji YC, et al. White spot syndrome virus IE1 and WSV056 modulate the G1/S transition by binding to the host retinoblastoma protein. J Virol. 2013;87(23):12576–12582. DOI:10.1128/JVI.01551-13
[22] Yao DF, Ruan LW, Lu HS, et al. Shrimp STAT was hijacked by white spot syndrome virus immediate-early protein IE1 involved in modulation of viral genes. Fish Shelfish Immunol. 2016;59:268–275.
[23] Wang S, Li HY, Weng SP, et al. White spot syndrome virus establishes a novel IE1/JNK/c-Jun positive feedback loop to drive replication. iScience. 2020;23 (1):100752. DOI:10.1016/j.isci.2019.100752
[24] Clevers H, Nusse R. Wnt/β-Catenin signaling and disease. Cell. 2012;149(6):1192–1205.
[25] Sun JZ, Ruan LW, Zhou CZ, et al. Characterization and function of a beta-catenin homolog from *Litopenaeus vannamei* in WSSV infection. Dev Comp Immunol. 2017;76:412–419.
[26] Ruan LW, Sun JZ, Zhou CZ, et al. Cloning, identification and function analysis of a Chibby homolog from *Litopenaeus vannamei*. Fish Shelfish Immunol. 2018;78:114–120.
[27] Xie XX, Li HY, Xu LM, et al. A simple and efficient method for purification of intact white spot syndrome virus (WSSV) viral particles. Virus Res. 2005;108(1–2):63–67. DOI:10.1016/j.virusres.2004.08.002
[28] Zhou Q, Qi YP, Yang F. Application of spectrophotometry to evaluate the concentration of purified White Spot Syndrome Virus. J Virol Methods. 2007;146(1–2):288–292.
[29] Zhao WL, Zheng ZH, Aweya JJ, et al. *Litopenaeus vannamei* Notch interacts with COP9 signalosome complex subunit 1 (CNS1) to negatively regulate the NF-kappaB pathway. J Proteomics. 2021;232:104074.
[30] Zheng ZH, Wang F, Aweya JJ, et al. Comparative transcriptomic analysis of shrimp hemocytes in response to acute hepatopancreas necrosis disease (AHPND) causing *Vibrio parahemolyticus* infection. Fish Shelfish Immunol. 2018;74:10–18.
[31] Amparyup P, Charoensapsri W, Tassanakajon A. Two prophenoloxidases are important for the survival of *Vibrio harveyi* challenged shrimp *Penaeus monodon*. Dev Comp Immunol. 2009;33(2):247–256.
[32] Zhang X, Zhu YT, Li XJ, et al. Lipopolysaccharide and beta-1, 3-glucan binding protein (LGBP) stimulates prophenoloxidase activating system in Chinese mitten crab (*Eriocheir sinensis*). Dev Comp Immunol. 2016;61:70–79.
[33] Zhan SX, Aweya JJ, Wang F, et al. *Litopenaeus vannamei* attenuates white spot syndrome virus replication by specific antiviral peptides generated from hemocyanin. Dev Comp Immunol. 2019;91:50–61.
[34] Cerenius L, Soderhall K. The prophenoloxidase-activating system in invertebrates. Immunol Rev. 2004;198:116–126.
[35] Cerenius L, Lee BL, Soderhall K. The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol. 2008;29(6):263–271.
[36] Amparyup P, Charoensapsri W, Tassanakajon A. Prophenoloxidase system and its role in shrimp immune responses against major pathogens. Fish Shelfish Immunol. 2013;34(4):990–1001.
[37] Charoensapsri W, Amparyup P, Hirono I, et al. Gene silencing of a prophenoloxidase activating enzyme in the shrimp, *Penaeus monodon*, increases susceptibility to *Vibrio harveyi* infection. Dev Comp Immunol. 2009;33(7):811–820. DOI:10.1016/j.dci.2009.01.006
[38] Pang Z, Kim SK, Yu J, et al. Distinct regulation patterns of the two prophenoloxidase activating enzymes corresponding to bacteria challenge and their compensatory over expression feature in white shrimp (*Litopenaeus vannamei*). Fish Shelfish Immunol. 2014;39(2):158–167. DOI:10.1016/j.fsi.2014.04.026
[39] Sutthangkul J, Amparyup P, Charoensapsri W, et al. Suppression of shrimp melanization during white spot syndrome virus infection. J Bio Chem. 2015;290 (10):6470–6481. DOI:10.1074/jbc.M114.605568
[40] Wang W, Luo P, Pan CK, et al. LvPPAE2 induced by WSV056 confers host defense against WSSV in *Litopenaeus vannamei*. Fish Shelfish Immunol. 2020;96:319–329.

[41] Jaree P, Wongdontri C, Somboonwiwat K. White spot syndrome virus-induced shrimp miR-315 attenuates prophenoloxidase activation via PPAE3 gene suppression. Front Immunol. 2018;9:2184.
[42] Sutthangkul J, Amparyup P, Eum JH, et al. Anti-Melanization mechanism of the white spot syndrome viral protein, WSSV453, via interaction with shrimp proPO-activating enzyme, PmproPPAE2. J Gen Virol. 2017;98(4):769–778. DOI:10.1099/jgv.0.000729
[43] Sangsuriya P, Charoensapsri W, Sutthangkul J, et al. A novel white spot syndrome virus protein WSSV164 controls prophenoloxidases, PmproPos in shrimp melanization cascade. Dev Comp Immunol. 2018;86:109–117.
[44] Ai HS, Huang YC, Li SD, et al. Characterization of a prophenoloxidase from hemocytes of the shrimp *Litopenaeus vannamei* that is down-regulated by white spot syndrome virus. Fish Shelfish Immunol. 2008;25(1–2):28–39. DOI:10.1016/j.fsi.2007.12.002
[45] Ai HS, Liao JX, Huang XD, et al. A novel prophenoloxidase 2 exists in shrimp hemocytes. Dev Comp Immunol. 2009;33(1):59–68. DOI:10.1016/j.dci.2008.07.017
[46] Garcia-Carreno FL, Cota K, Del Toro MAN. Phenoloxidase activity of hemocyanin in whiteleg shrimp *Penaeus vannamei*: Conversion, characterization of catalytic properties, and role in postmortem melanosis. J Agric Food Chem. 2008;56(15):6454–6459.
[47] Xu JY, Wu SJ, Zhang XB. Novel function of QM protein of shrimp (*Penaeus japonicus*) in regulation of phenol oxidase activity by interaction with hemocyanin. Cell Physiol Biochem. 2008;21(5–6):473–480.
[48] Ponprateep S, Vatanavicharn T, Lo CF, et al. Alpha-2-Macroglobulin is a modulator of prophenoloxidase system in pacific white shrimp *Litopenaeus vannamai*. Fish Shelfish Immunol. 2017;62:68–74.
[49] Zhou H, Chen XB, Aweya JJ, et al. Interaction of *Penaeus vannamei* hemocyanin and α2-macroglobulin modulates the phenoloxidase activity. Mol Immunol. 2021;138:181–187.