Developmental and Comparative Immunology 111 (2020) 103749

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/devcompimm

A cytokine receptor domeless promotes white spot syndrome virus infection via JAK/STAT signaling pathway in red claw crayfish *Cherax quadricarinatus*

Ling-ke Liu[a,1], Xiao-xiao Chen[a,b,1], Rui-lin Gao[a], Ke-jian Wang[a], Wen-yun Zheng[b,*], Hai-peng Liu[a,**]

[a] State Key Laboratory of Marine Environmental Science, College of Ocean and Earth Sciences, Xiamen University, State-Province Joint Engineering Laboratory of Marine Bioproducts and Technology, Xiamen, 361102, Fujian, PR China
[b] Shanghai Key Laboratory of New Drug Design, School of Pharmacy, East China University of Science and Technology, Shanghai, 200237, PR China

ARTICLE INFO

Keywords:
Domeless
White spot syndrome virus
JAK/STAT pathway
Cherax quadricarinatus
Phosphorylation

ABSTRACT

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway is pivotal in immune responses for a variety of pathogens in both vertebrates and invertebrates. Domeless (Dome), as a unique cytokine receptor, involves in the upstream JAK/STAT pathway in invertebrates. In this study, the full-length cDNA sequence of a cytokine receptor Dome was identified from red claw crayfish *Cherax quadricarinatus* (named as *CqDome*), which contained an open reading frame of 4251 bp, encoding 1416 amino acids. The *Cq*Dome contained extracellular conservative domains of a signal peptide, two cytokine binding modules (CBM), three fibronectin-type–III–like (FN3) domains and a transmembrane region. Tissue distribution analysis showed that *CqDome* generally expressed in all the tissues selected with a high expression in hemocyte. The gene expression of both the viral immediately early gene (*IE1*) and a late gene envelope protein *VP28* of white spot syndrome virus (WSSV) were significantly decreased after gene silencing of *CqDome* in crayfish haematopoietic tissue (Hpt) cells, indicating a key role of *Cq*Dome in promoting WSSV infection. Furthermore, the phosphorylation level of *Cq*STAT was significantly inhibited by gene silencing of *CqDome* in Hpt cells, indicating that *Cq*Dome participated in signal transduction of JAK/STAT pathway in red claw crayfish. These data together suggest that *Cq*Dome is likely to promote WSSV infection via JAK/STAT pathway, which sheds new light on further elucidation of the pathogenesis of WSSV.

1. Introduction

The Janus kinase/Signal transducer and activator of transcription (JAK/STAT) signaling pathway is pivotal in immune responses in both vertebrates and invertebrates, which is stimulated by a variety of cytokines, including interleukins, interferons, tumor necrosis factors and growth factors in mammals (Rawlings et al., 2004). The activated JAK/STA signal pathway is mainly involved in cell-cell communication essential for the growth and apoptosis of cells and the regulation of immune responses (Agaisse and Perrimon, 2004; Ozaki and Leonard, 2002). Typically, complete JAK/STAT pathway is comprised of four components, including cytokine, cytokine receptor, kinase JAK and transcription factor STAT (O'Shea et al., 2002). Cytokines are secreted polypeptides released from monocyte or lymphocyte in vertebrates (Oppenheim, 2001), which must interact with specific cytokine receptor complexes expressed on the surface of target cells. The cytokine receptor superfamily is a tyrosine kinase-linked receptor with no enzymatic activity (Rawlings et al., 2004). JAK/STAT signaling is initiated by recognition of multiple cytokines, then causing a spatial conformational changes of membrane receptor that produces intracellular signaling via activated JAK and STAT family (Rane and Reddy, 2002; Seubert et al., 2003), finally inducing the intra-nuclear specific genes expression to generate appropriate cellular responses (O'Sullivan et al., 2007). In mammals, JAK is a classical non-receptor tyrosine kinase and contains four family members: JAK1, JAK2, JAK3 and TYK2 (Song et al., 2015), and STAT is a typical transcription factor with seven major members of STAT1, STAT2, STAT3, STAT4, STAT5A/5B and STAT6 (Vainchenker and Constantinescu, 2013).

In invertebrates, the JAK/STAT pathway is relatively simple with certain components (Gilbert et al., 2005; Harrison et al., 1998). For

* Corresponding author.
** Corresponding author.
E-mail addresses: zwy@ecust.edu.cn (W.-y. Zheng), Haipengliu@xmu.edu.cn (H.-p. Liu).
[1] Contribute equally to this work.

https://doi.org/10.1016/j.dci.2020.103749
Received 29 April 2020; Received in revised form 18 May 2020; Accepted 18 May 2020
Available online 04 June 2020
0145-305X/

instance, the *Drosophila* JAK/STAT pathway comprises of a cytokine receptor called Dome (Brown et al., 2001) as well as three homogenous ligands Unpaired1, 2 and 3 (Upd1, 2 and 3) (Hombría et al., 2005; Wright et al., 2011), a tyrosine kinase named Hopscotch (HOP) (Binari and Perrimon, 1994) and a STAT known as STAT92E (Yan et al., 1996). As a key cytokine receptor mediating JAK/STAT pathway signal transduction, studies have proved that the *Drosophila* Dome and *Litopenaeus vannamei* Dome are both transmembrane protein similar to the interleukin 6 (IL-6) receptor-type family in vertebrates (Brown et al., 2001; Yan et al., 2015), which possesses similar extracellular structure of two CBM and several FN3 (Bazan, 1990a). In crustaceans, it has been reported that shrimp JAK/STAT pathway contained a Dome, a JAK and a STAT (Song et al., 2015; Sun et al., 2011; Yan et al., 2015), and previous studies have demonstrated that shrimp STAT was relatively close to human STAT5A/5B and human STAT6 (Chen et al., 2008). Importantly, invertebrate JAK/STAT pathway possesses complete signal transduction route which is analogous to that of vertebrates (Liongue et al., 2012).

White spot syndrome virus (WSSV) is one of the most serious pathogens with a wide range of hosts in crustaceans, which can cause up to 90–100% cumulative mortality in cultured shrimp and crayfish within 3–7 days (Escobedo-Bonilla et al., 2008). After delivery of the WSSV genome into host cell nucleus, the virus expresses immediate early (IE), early, and late genes to start viral replication (Marks et al., 2005). As a conserved innate immune signaling pathway, Dome-JAK/STAT pathway has been shown to regulate WSSV infection in crustaceans. The gene silencing of *LvJAK* led to increased mortality rate and viral load in *L. vannamei* after WSSV infection (Song et al., 2015). In addition, gene knockdown of *LvSOCS2* reduced the viral load and the susceptibility to WSSV of shrimp, demonstrating that the JAK/STAT pathway performed an anti-viral role in shrimp (Wang et al., 2016). On the other hand, WSSV has evolved several strategies to subvert host JAK/STAT pathway to facilitate viral gene expression (Li et al., 2019). The gene knockdown of JAK/STAT pathway receptor *Dome* could inhibit the proliferation of WSSV, and the survival rate of shrimp has been improved in *L. vannamei* (Yan et al., 2015). STAT was originally identified to be hijacked by WSSV to promote viral gene expression (*WSV051* and *IE1*) in shrimp (Liu et al., 2007; Yao et al., 2016). Notably, immediately early gene 1 (IE1) of WSSV, the first identified immediate early gene from WSSV (Liu et al., 2008), was regulated by STAT and could interact with STAT to promote its phosphorylation (Yao et al., 2016). Additionally, the successful infection of WSSV is proposed to need the subversion of the host JAK/STAT pathway by this virus to promote the expression of viral genes in the early stage of infection (Li et al., 2019). However, the molecular regulation of Dome, as the unique cytokine receptor of JAK/STAT pathway in invertebrates, has been poorly understood in case of its regulation on viral infection.

In this study, a cytokine receptor Dome in *C. quadricarinatus* was identified. We analyzed the molecular characteristics of *CqDome* and its expression in different tissues in red claw crayfish. In addition, RNA interference assay was used for studying the function mechanisms of *Cq*Dome in WSSV infection and JAK/STAT pathway activation. Our data provide the evidence that *Cq*Dome may play an important role in viral infection in a crustacean, which provides a theoretical basis for the formulation of relevant strategy in WSSV disease control.

2. Materials and methods

2.1. Animals, tissues collection and cell culture

Red claw crayfish *C. quadricarinatus* (average weight is 60 ± 5g) were obtained from Source Sentai Agricultural Science and Technology Co., Ltd of Zhangzhou, Fujian Province, China. Crayfish were kept in oxygenated freshwater at 26 °C for one week prior to experiments. Different tissues such as gill, muscle, hemocytes, haematopoietic tissue (Hpt), epithelium, heart, hepatopancreas, gonad, stomach, nerve, intestine and eyestalk were collected for total RNA isolation. Hemocytes were obtained with a sterile syringe in equal volume anticoagulant buffer and centrifuged for 10 min with 1000 × *g* at 4 °C. Hpt cells were separated from crayfish haematopoietic tissue and cultured with L-15 medium at 20 °C as described by Söderhäll et al. (Söderhäll, 2013). Hpt cells were cultured with 1.0×10^5 per well in 96-well plates and with 5.0×10^5 per well in 24-well plates.

2.2. Total RNA extraction and first strand cDNA synthesis

Twelve tissues from healthy red claw crayfish were collected as described in Section 2.1 and total RNA were extracted with Trizol reagent (Roche, Mannheim, Germany) according to the manufacturer's protocols. Then genomic DNA in the extracted RNA was eliminated using DNase I (Ambion, Austin, Texas, USA). The concentration of total RNA was detected by NanoDrop (2000) spectrophotometer (Thermo Scientific, USA). Hpt cells cultured in 24-well plates were harvested with lysis buffer and total RNA were extracted using GenElute™ Total RNA Miniprep Kit (Sigma, USA) following the instructions. Furthermore, the first strand cDNA were synthesized using the PrimeScript™ RT Reagent Kit (TaKaRa, Japan). And total RNA extracted from Hpt was reversely transcribed using the SMARTer™ RACE cDNA Amplification Kit (Clontech, Madison, Wisconsin, USA) for full-length cDNA obtained.

2.3. Gene cloning of CqDome full-length cDNA sequence

Previously, we obtained a partial *CqDome* cDNA sequence of *C. quadricarinatus* from the transcriptome library of Hpt cells after WSSV infection in our lab (Liu et al., 2011). To clone the full-length *CqDome* cDNA sequence, 5′ and 3′ Rapid Amplification of cDNA Ends (RACE)-PCR was carried out using SMART RACE cDNA Amplification kit (Clontech, USA) according to manufacturer's instruction. PCR primers required for experiments were shown in Table 1. RACE-PCR amplification condition was shown as follows: 5 min at 94 °C; 30 cycles of 94 °C for 30 s, 65 °C for 30 s (decrease 0.3 °C per cycle) and 72 °C for 2 min; and 72 °C for 10 min. The PCR productions were recovered using a Gel Extraction Kit (Sangon Biotech, China), and the expected DNA fragments were ligated into pMD18-T vectors (TaKaRa, Japan). Then the vectors were transformed into *Escherichia coli* DH5α cells and positive clones containing the expected size of the insert were selected, then the vectors were extracted using a SanPrep Column Plasmid Mini-Preps Kit (Sangon Biotech, China) and sequenced at Xiamen Borui Biotechnology Company, China.

2.4. Bioinformatics analysis of CqDome

The *CqDome* open reading frame (ORF) was predicted using program ORF Finder in NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). Signal peptide was identified by SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The homologous conserved domains were identified by SMART online website (http://smart.embl-heidelberg.de/). The alignment of CBM1 and CBM2 in *CqDome* was carried out by DNAMAN6 using the CBM amino acid sequence.

2.5. Tissue distribution analysis of CqDome in red claw crayfish

The twelve tissues of three randomly selected healthy red claw crayfish cDNA templates were prepared as mentioned above and the transcript levels of *CqDome* were detected by quantitative real-time PCR (qRT-PCR) using an ABI PCR machine (Applied Biosystems 7500, UK). The specific primers of *CqDome* gene (*CqDome*-qPCR-F (*CqDome*-qPCR-R; Table 1), were designed by Primer Premier 5.0, and the crayfish 16S ribosomal gene (Genbank: AF135975.1; Table 1) was used as the internal reference. The reaction was comprised of 10 μL of SYBR® Green qPCR ReadyMix, 1 μl L of cDNA, 7 μL of nuclease-free water, 1 μL of positive and reverse primers. And the conditions of qRT-PCR were as

Table 1
Primer sequences used in this study.

Primers	Sequence(5′-3′)
cDNA amplification	
CqDome-F	CACGGTTGTCAATGAATCTGCC
CqDome-R	GTAGCTGGTGTATGGACGCAAA
CqDome 5′-GSP	CCTCCAACTGGTCTCCGTCTTGATGC
CqDome 3′-GSP	TGGCAGTCGTCCTCCACCACTTGAA
UPM	CTAATACGACTCACTATAGGGC
CqDome-ORF-F	ATGGTGTTGACGTGGAGAGAAGGC
CqDome-ORF-R	TCACACCATTGATGGTTTATGGTTAGG
RNAi	
ds*CqDome*-F	TAATACGACTCACTATAGGGGCTGAAATGTCTTACGGGCAAT
ds*CqDome*-R	TAATACGACTCACTATAGGGATCCTCCAACTGGTCTCCGTCT
ds*GFP*-F	TAATACGACTCACTATAGGGCGACGTAAACGGCCACAAGT
ds*GFP*-R	TAATACGACTCACTATAGGGTTCTTGTACAGCTCGTCCATGC
qRT-PCR	
16S–F	AATGGTTGGACGAGAAGGAA
16S-R	CCAACTAAACACCCTGCTGATA
CqDome-qPCR-F	ACACGGTTGTCAATGAATCTGCC
CqDome-qPCR-R	TCTAAGAAGCCCAGCACAACATAC
IE1-F	CTGGCACAACAACAGACCCTACC
IE1-R	GGCTAGCGAAGTAAAATATCCCCC
VP28–F	AAACCTCCGCATTCCTGT
VP28-R	GTGCCAACTTCATCCTCATC
Dual luciferase reporter gene assay	
CqSTAT-ORF-F	CCCAAGCTTATGTCATTATGGAATAGAGCGCAGC
CqSTAT-ORF-R	CGCGGATCCTCCTGAGGCTTCATGAAGTTGGTT
pGL3-pIE1-F	CGGGTACCCCTTGTTACTCATTTATTCCTAGAAATGG
pGL3-pIE1-R	CCGCTCGAGCTTGAGTGGAGAGAGAGAGC
pGL3-pWSV051-F	CGGGTACCCAGTTCCAGGAAGAATGC
pGL3-pWSV051- R	CCGCTCGAGTTTCTCCCAACTTTG

Primer sequences used in this study.

follows: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curve analysis was used to confirm the specificity of qRT-PCR amplification and relative transcript expression of *CqDome* were calculated using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001).

2.6. Western blotting analysis

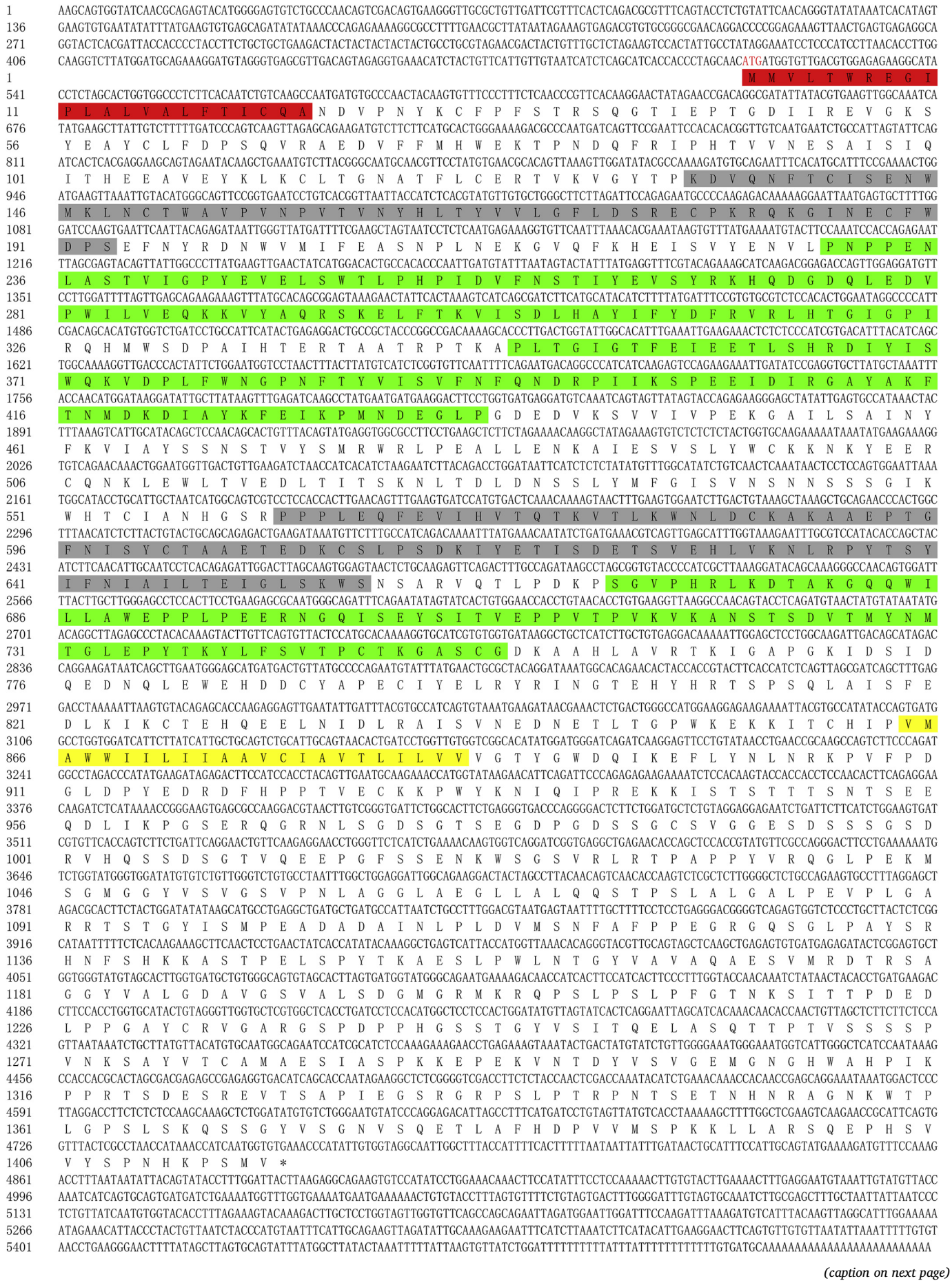
The protein samples with 1 × SDS loading buffer were resolved by 12% SDS-PAGE gel electrophoresis and transferred to a PVDF membrane (Bio-Rad, USA). The membrane was blocked with 1% bovine serum albumin (BSA) (Solarbio, Beijing, China) in TBST buffer for 1 h at room temperature, and subsequently incubated with mouse anti-VP28 antisera (1:3000), anti-β-actin antibody (TransGene Biotech, Beijing, China), or anti-STAT5A phosphorylation antibody (1:1000, Abcam, UK) used in the study of STAT from *Marsupenaeus japonicas* (Sun et al., 2017) and *L. vannamei* (Chen et al., 2008), for 1 h at room temperature followed by washing three times with TBST buffer, and then incubated for 1 h at room temperature with HRP-conjugated goat anti-mouse or anti-rabbit secondary antisera (1:5000). The unbounded IgG was washed away and then followed by an enhanced chemiluminescence system was used for detection.

2.7. RNA interference assay

To silence the *CqDome* gene in Hpt cells, the double-strand RNA (dsRNA) of *CqDome* was synthesized using the MegaScript kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions and the Hpt cell cultures from *C. quadricarinatus* were prepared in a 24-well plate as previously described (Liu et al., 2011). The *Cq*Dome dsRNA was transfected into Hpt cell cultures using Cellfectin II Reagent (Life Technologies, USA) according to the methods reported by Chen (Chen et al., 2016). Generally, 100 ng of dsRNA/well for 96-well plates or 400 ng of dsRNA/well for 24-well plates were prepared, respectively. Green fluorescent protein (GFP) dsRNA treatment was used as a control. The primers of *CqDome* and *GFP* were shown in Table 1. Then WSSV infection was performed at 12 h after the second transfection. WSSV infection was performed with MOI = 1 (multiple infection rate of 1) for detecting the replication of WSSV, and MOI = 10 for detecting the entry of WSSV in Hpt cells. The total RNA was extracted using the GenElute™ Total RNA miniprep kit (Sigma, USA) according to the protocol and cDNA synthesis was described in Section 2.2. The relative gene expression of *CqDome* and WSSV *IE1* and *VP28* were analyzed by qRT-PCR as mentioned above. The 16S ribosomal RNA was used as an internal standard. For the cells in 96-well plates, the samples were collected after WSSV infection to determine WSSV and *Cq*STAT phosphorylation (p*Cq*STAT) by Western blotting which was described in Section 2.6, and *Cq*β-actin was used as the internal reference. The experiments were repeated three times and the band intensities of VP28 were analyzed by Gel Image System ID 4.2 program. The experiments were biologically repeated at least for three times. All the primers required for these experiments were shown in Table 1.

2.8. Dual luciferase reporter gene assay

Sf9 cells were cultured in Sf-900™ II SFM (Gibco, USA) supplemented at 28 °C, and then Sf9 cells were seeded into 48-well plates at a density of 2×10^5 per well. The plasmid was transfected with Cellfectin II Reagent (Life Technologies, USA) according to the manufacturer's instructions. The related primers for vector constructions were shown in Table 1. For the dual luciferase reporter assay, Sf9 cells were transfected with 50 ng of the reporter plasmid (firefly luciferase), 25 ng of pRL-TK (Promega, USA) which was an internal control for expression of Renilla luciferase, and 200 ng of expression plasmid. In addition, the firefly luciferase reporter plasmids were driven by the WSV051, IE1 promoters, respectively. The empty pGL3-basic and PIZ-V5-His plasmids were used as controls. At 48 h post-transfection, a dual luciferase reporter assay was performed according to the manufacturer's instructions to calculate the relative proportions of firefly and Renilla luciferase activity, and each experiment was repeated for three times.



(caption on next page)

Fig. 1. The full-length cDNA sequence and deduced amino acid sequence of *CqDome* from the red claw crayfish *Cherax quadricarinatus*. The start codon (ATG) is marked in red, and the stop codon (TAA) is denoted with "*". The signal peptide is indicated by red shadow. The CBM is shown in grey shadow. The FN3 domain is shown in green shadow, and the transmembrane peptide is marked with yellow shadow. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.9. Statistical analysis

The data were analyzed by Student's *t*-test and presented as the mean ± standard deviation (SD) from more than three independent assays using the Statistical Product and Service Solutions (SPASS) package. Differences with $p < 0.05$ were considered as significant difference.

3. Results and discussion

3.1. Gene cloning and bioinformatics analysis of CqDome in red claw crayfish

To study the functional mechanism of *CqDome* in WSSV infection, the full-length cDNA sequence of *CqDome* was obtained by using RACE technology (Genbank No: MT103421). As shown in Fig. 1, the full-length cDNA of *CqDome* was 5534 bp, including an ORF of 4251 bp that encoded 1416 amino acids, a 5′-untranslated region (UTR) of 510 bp, and a 3′-UTR with a poly (A) tail of 773 bp. Furthermore, the SMART analysis showed that the deduced *Cq*Dome protein (Fig. 2A) contained a signal peptide at the N-terminus, two cytokine binding modules (CBM), three fibronectin-type–III–like (FN3) domains and a transmembrane domain. The transmembrane region of *Cq*Dome contained 23 hydrophobic amino acids and the intracellular region contained 530 amino acids. Similarly, Dome from *Penaeus vannamei* (*Pv*Dome) and *Drosophila melanogaster* (*Dm*Dome) contained two CBM domains named as CBM1 and CBM2, three FN3 (Brown et al., 2001; Yan et al., 2015). It has been reported that CBM was the typical domain of type I cytokine receptor, which was critical for ligand binding such as cytokines (Yan et al., 2015). Generally, CBM1 contains a set of four conserved cysteine residues in the N-terminal domain, and CBM2 contains a five-amino acid Trp-Ser-Xaa-Trp-Ser (WSXWS) motif which is conserved in various vertebrate type I cytokine receptors and functions as a molecular switch involved in receptor activation (Bazan, 1990b; Robert et al., 2012). To better characterize the two CBM visually, multiple sequences alignment of two CBM were carried out at *Cq*Dome and other species. We found that CBM1 of *Cq*Dome had obviously four conserved cysteine residues (Fig. 2B) and shared 45% identity with CBM1 from *P. vannamei* Dome (AGY46351.1) and *M. japonicas* Dome (APA16577.1), around 19% identity with CBM1 from *D. melanogaster* Dome (NP_523412.1), and 12% identity with CBM1 from *Homo sapiens* leukemia inhibitory factor receptor precursor (LIFR) (EAW55972.1). In addition, CBM2 of *Cq*Dome had an incomplete WSXWS motif (LSKWS) (Fig. 2C) with 43% identity of CBM2 of *P. vannamei* Dome and *M. japonicas* Dome, around 12% with *D. melanogaster* Dome, and 25% with *H. sapiens* LIFR. Previous studies have proved that *Dm*Dome and *Pv*Dome were similar to the mammal interleukin 6 (IL-6) receptor-type family (Brown et al., 2001; Yan et al., 2015). Considering the high similarity of the structure, we consider that *Cq*Dome is a member of the IL-6 receptor family that binds to extracellular cytokines and transmits intracellular signals, which is likely to possess similar biological functions with Dome from the other crustaceans.

3.2. Tissue distribution analysis of CqDome transcript in red claw crayfish

To reveal the possible role of *Cq*Dome related to innate immunity, we used qRT-PCR to quantify the mRNA transcript of *CqDome* in the twelve tissues selected. The results showed that *CqDome* had a broad expression profile and it was found to be expressed in all tested tissues of red claw crayfish with a relatively high expression in hemocyte and the lowest expression in the eyestalk (Fig. 3). Similarly, the *Eriocheir sinensis* Dome was reported to be highly expressed in hemocyte (Ruan et al., 2019). It is well-known that the crustacean replies solely on innate immune response, and hemocyte plays an important role in immune response against microbial infection, including recognition, release of antimicrobial substances, phagocytosis, encapsulation and cytotoxicity (Johansson et al., 2000). Therefore, many researches concerning on hemocyte response to WSSV have been studied. For instance, hemocyte was the target for the WSSV infection in shrimp (van de Braak et al., 2002; Wang et al., 2002) and circulating hemocyte showed a dramatic decrease in the red claw crayfish after WSSV infection (Gao et al., 2014). Meanwhile, WSSV infection could also induce apoptosis of hemocyte in signal crayfish *Pacifastacus. leniusculus* (Jiravanichpaisal et al., 2006). Hence, the highest expression of *CqDome* in the hemocyte was likely to play an important role in crayfish immune system.

3.3. The WSSV replication is inhibited by gene silencing of CqDome in Hpt cells from red claw crayfish

It has been reported that *Dome* gene was up-regulated after Poly (I:C) or WSSV stimulation in *L. vannamei* (Yan et al., 2015), indicating that Dome was responsive to pathogen infection. To determine the immune function of *Cq*Dome after WSSV infection, the *CqDome* gene was silenced followed by WSSV infection in red claw crayfish Hpt cell cultures. As shown in Fig. 4A, the gene silencing efficiency of *CqDome* was over 90% in comparison to that of control cells. Importantly, the expression of viral immediate early genes *IE1* and envelope protein

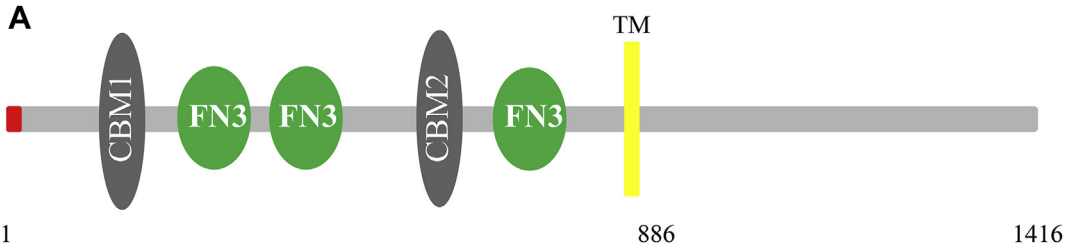


Fig. 2. The bioinformatics analysis of CqDome. (A) Predicted protein domain structure of CqDome. CqDome protein contains a signal peptide (red box), two CBM (CBM1 and CBM2), three FN3 and a transmembrane (TM) domain. (B) Multiple sequences alignment of CqDome CBM1 domain. The four conserved cysteine residues are shown with red arrow. (C) Multiple sequences alignment of CqDome CBM2 domain. The motif similar to "WSXWS" is marked with a red box. The amino acids sequences of CBM1 and CBM2 are from *C. quadricarinatus* Dome and other species, including *H. sapiens* LIFR (EAW55972.1), *Mus musculus* cytokine receptor GP130 (CAA44515.1), *D. melanogaster* Dome (NP_523412.1), *P. vannamei* Dome (AGY46351.1) and *M. japonicas* Dome (APA16577.1). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

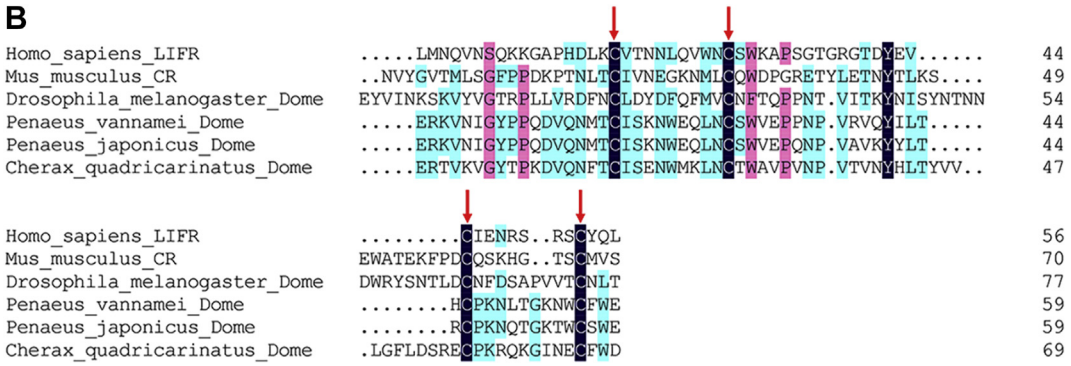


Fig. 2. (*continued*)

gene *VP28* both showed significant decrease in *CqDome* silenced Hpt cells (Fig. 4B). In addition, we also detected the expression of VP28 protein after WSSV infection 6 hpi in Hpt cells by Western blotting, which showed that the viral VP28 protein was significantly decreased after gene silencing of *CqDome* (Fig. 4C). These results clearly implied that the presence of *Cq*Dome was necessary for WSSV replication in crayfish Hpt cells. It has been supported that *Lv*Dome could be observed on the cell membrane, and belongs to a membrane protein (Yan et al., 2015). Similarly, *Cq*Dome contained a transmembrane domain, so we speculated that *Cq*Dome might recognize foreign pathogens and lead to a downstream immune response to resist pathogen invasion. To prove this hypothesis, the entry of WSSV into Hpt cells was determined after gene silencing of *CqDome* post WSSV infection 1 hpi. However, the result suggested that there was no significant difference of WSSV entry after gene silencing of *CqDome* when compared to GFP treated control cells (Fig. 4D), indicating that *Cq*Dome didn't affect the WSSV entry. Thus, all these data demonstrated that gene silencing of *CqDome* significantly inhibited the replication of WSSV, implying that WSSV could take advantage of *Cq*Dome for benefiting its replication. Similarly, it has been reported that the proliferation of WSSV was inhibited, and the survival rate of shrimp was improved in Dome silenced shrimp in *L. vannamei* (Yan et al., 2015). Previous studies have demonstrated Dome transmembrane protein has been identified as an important receptor in the JAK/STAT pathway in *Drosophila* (Brown et al., 2001). In crustaceans, Dome can function as a receptor to activate the JAK/STAT pathway in *L. vannamei* (Yan et al., 2015), and a C-lectin interacts with MjDome at hemocyte surface, thus activating the JAK/STAT pathway in *M. japonicas* (Sun et al., 2017). We speculated that *Cq*Dome promoted WSSV replication in a way of activating JAK/STAT, which was annexed by WSSV for successful infection in shrimp (Liu et al., 2007; Yao et al., 2016). This was consistent with a report that silence of STAT significantly decreased the WSSV load in shrimp (Wen et al., 2014). However, the function of *Cq*Dome in activating JAK/STAT pathway needed further confirmation.

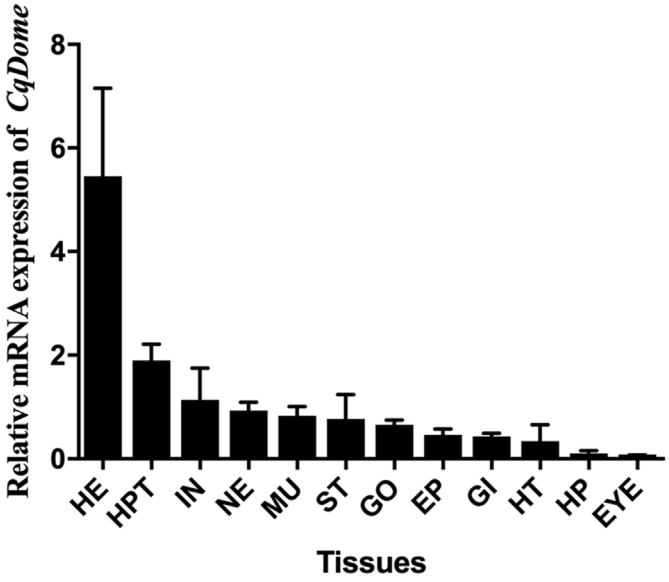


Fig. 3. Tissue distribution analysis of *CqDome* in red claw crayfish. The mRNA expression profile of *CqDome* in different tissues from *C. quadricarinatus*. HE: hemocyte; Hpt: haematopoietic tissue; IN: intestine; GI: gill; ST: stomach; GO: gonad; NE: nerve; EP: epithelial tissue; MU: muscle; HP: hepatopancreas; HT: heart; EYE: eyestalk. The relative transcript level in MU was employed as the calibrator (value set as 1). The 16S rRNA was used as an internal control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. CqDome participated in JAK/STAT signaling pathway to enhance WSSV infection

It has been reported that JAK/STAT activation has similar canonical signaling model in vertebrates and invertebrates. The binding of a

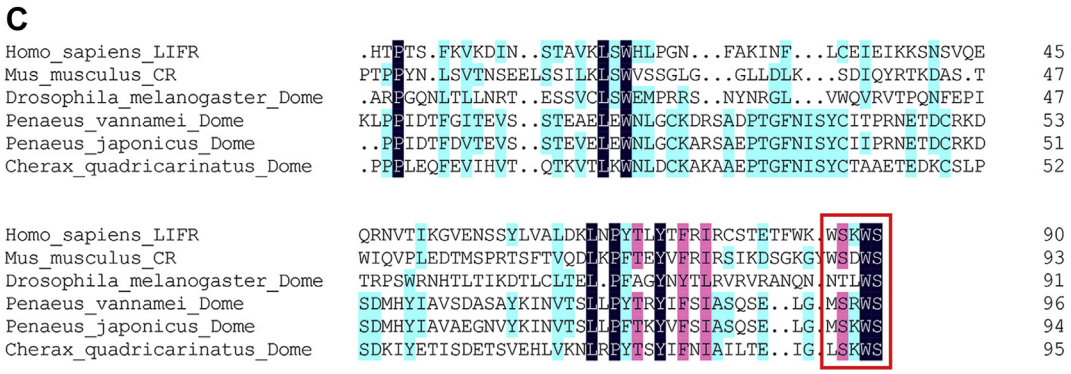


Fig. 2. (*continued*)

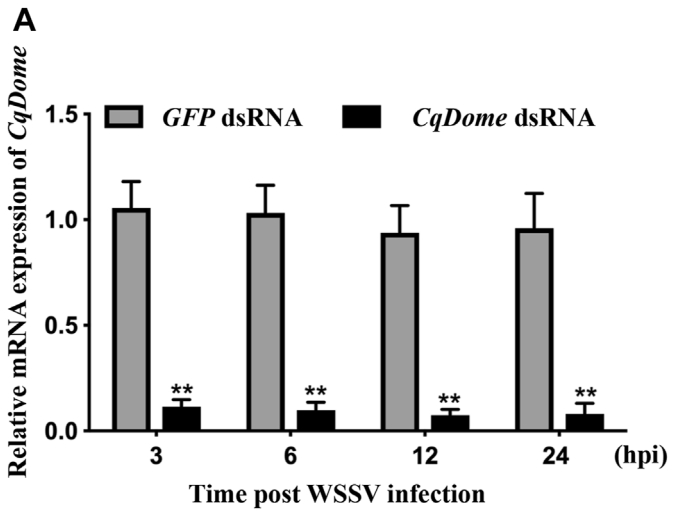


Fig. 4. WSSV replication is inhibited by gene silencing of *CqDome* in crayfish Hpt cells. (A) The mRNA expression of *CqDome* is significantly decreased after gene silencing of *CqDome*. The mRNA expression of *CqDome* during WSSV infection was determined by qRT-PCR. GFP dsRNA treated cells was used as the control groups. (B) Decreased gene expression of immediate early gene IE1 (a) and late gene VP28 (b) of WSSV in *CqDome* silenced Hpt cells. The expression of IE1 and VP28 were determined by qRT-PCR in *CqDome* silenced Hpt cells post WSSV infection. The relative transcript levels in control cells at 3 h post WSSV infection were employed as the calibrator (value set as 1). (C) Decreased expression of WSSV envelope protein VP28 in *CqDome* silenced Hpt cells. The viral envelope proteins VP28 was immunoblotted post WSSV infection 6 hpi. (D) No significant effect in WSSV entry was observed in *CqDome* silenced Hpt cells. The viral envelope protein VP28 were immunoblotted post WSSV infection 1 hpi. *GFP* dsRNA treated cells were used as the control groups. The band intensities of three independent experiments were calculated by using Gel Image SystemID 4.2 program. The asterisk indicates significant difference compared with those of controls (*$p < 0.05$, **$p < 0.01$).

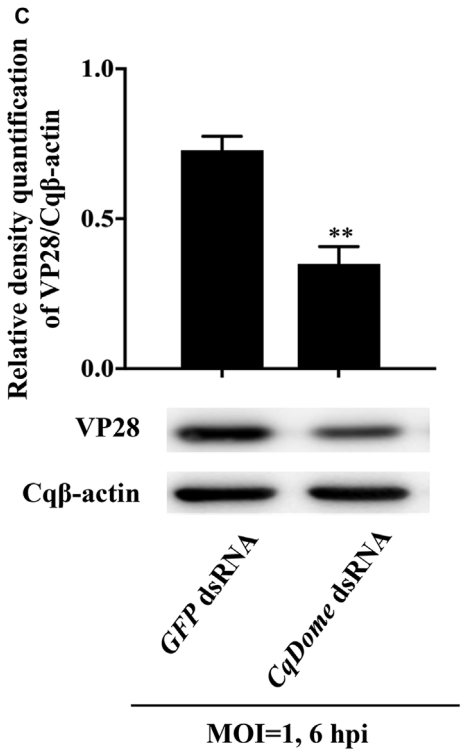


Fig. 4. (*continued*)

in crayfish. In addition, the overexpression of *Cq*STAT significantly induced the promoter activity of WSV051 and IE1 genes in Sf9 cells (Fig. 5B), suggesting that transcription factors *Cq*STAT could enhance the promoter activity of WSSV IE genes. It has been reported that WSSV can induce the phosphorylation of STAT and annexes STAT to enhance

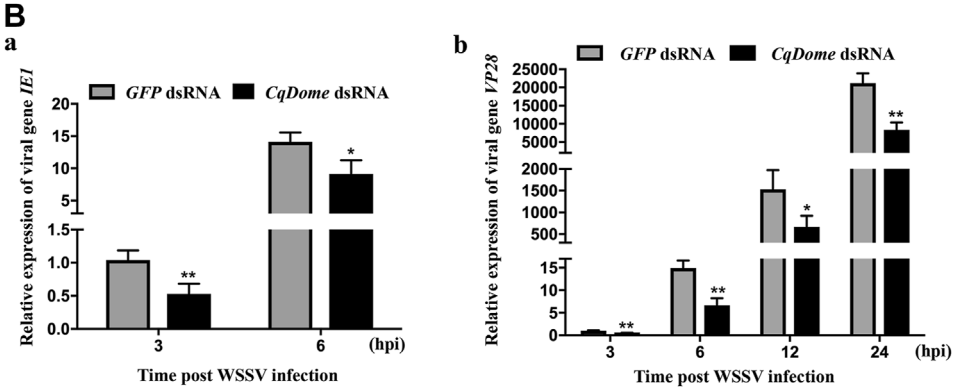


Fig. 4. (*continued*)

cytokine to its receptor induces receptor dimerization and activation of the JAK that is constitutively associated with the cytoplasmic tail of the receptor. Activated JAK phosphorylate each other and specific tyrosine residues on the cytoplasmic part of the receptor, which act as docking sites for the Src homology 2 domains of STAT molecules. Also, the STAT is tyrosine phosphorylated by JAK, which allows to form dimers and translocate into the nucleus, where they bind the promoters of their target gene (Hiu and Sandra E, 2012; Myllymaki and Ramet, 2014). Therefore, the phosphorylation of STAT is an important marker to identify whether JAK/STAT is activated. Furthermore, we determined the phosphorylation of *Cq*STAT (p*Cq*STAT) protein post WSSV infection in Hpt cells. The results showed that the p*Cq*STAT was clearly increased 3 and 6 hpi in WSSV infected Hpt cells (Fig. 5A), indicating that JAK/STAT signaling pathways participated in the process of WSSV infection the expression of WSSV IE1 and WSV051 genes in *L. vannamei* (Chen et al., 2008; Liu et al., 2007; Yao et al., 2016). To identify whether *Cq*Dome promoted WSSV replication via JAK/STAT signaling pathway, we detected the phosphorylation level of *Cq*STAT protein after gene knocking down of *CqDome*. As shown in Fig. 5C, the phosphorylation level of *Cq*STAT was obviously decreased at each time point of WSSV infection after gene silencing of *CqDome*, indicating that *Cq*Dome was closely correlated to *Cq*STAT activity and was important to signal transduction of the JAK/STAT pathway. Similarly, STAT phosphorylation was significantly decreased in *Dome*-silenced shrimp, and STAT translocation into the nucleus was inhibited in shrimp hemocytes in *M. japonicas* (Sun et al., 2017). Therefore, these results further confirm the function of Dome in activating JAK/STAT pathway and signal transduction in invertebrates, which can promote the infection of WSSV in

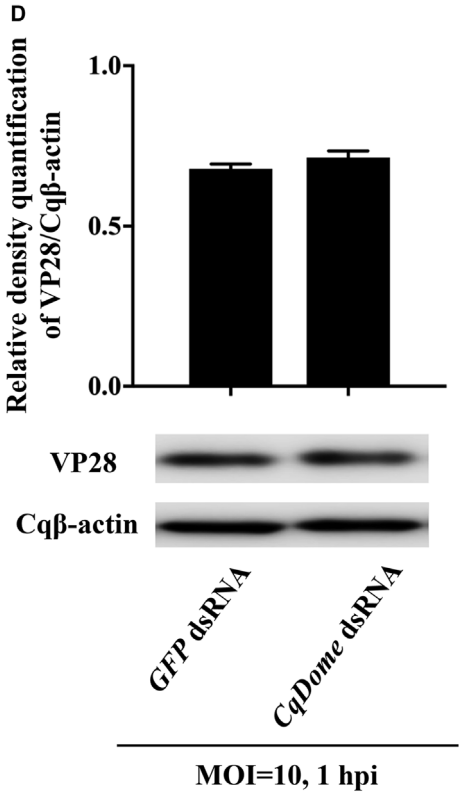


Fig. 4. (*continued*)

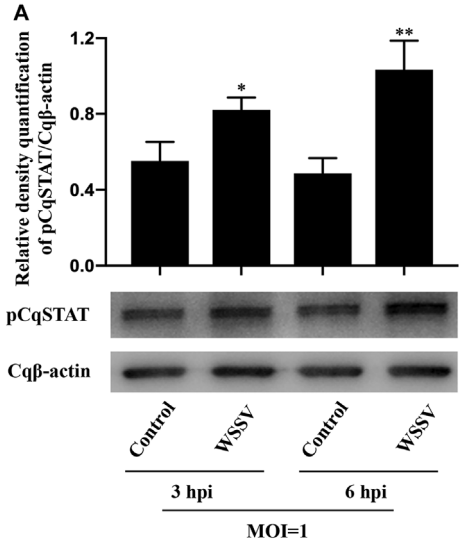


Fig. 5. The phosphorylation of CqSTAT was decreased by gene silencing of *CqDome* in crayfish Hpt cells. (A) Increased phosphorylation level of CqSTAT (pCqSTAT) post WSSV infection in Hpt cells. The pCqSTAT was detected after WSSV infection 3 and 6 hpi by immunoblotting with STAT phosphorylation antibody. (B) Increased promoter activity of WSSV IE genes by over-expression of CqSTAT in Sf9 cells. The Sf9 cells were co-transfected with the effector plasmids (PIZ-V5-His or PIZ-CqSTAT), the reporter plasmids (pGL3-basic, pGL3-WSV051 or pGL3-IE1), and the internal control plasmid pRL-TK. The relative luciferase activity was obtained by dividing firefly luciferase activity with the internal Renilla luciferase activity. (C) Decreased phosphorylation level of CqSTAT post WSSV infection in *CqDome* silenced Hpt cells. The pCqSTAT was detected by Western blotting with STAT phosphorylation antibody. The band intensities of three independent experiments were calculated by using Gel Image SystemID 4.2 program. The asterisk indicates significant difference compared with those of controls (*$p < 0.05$, **$p < 0.01$).

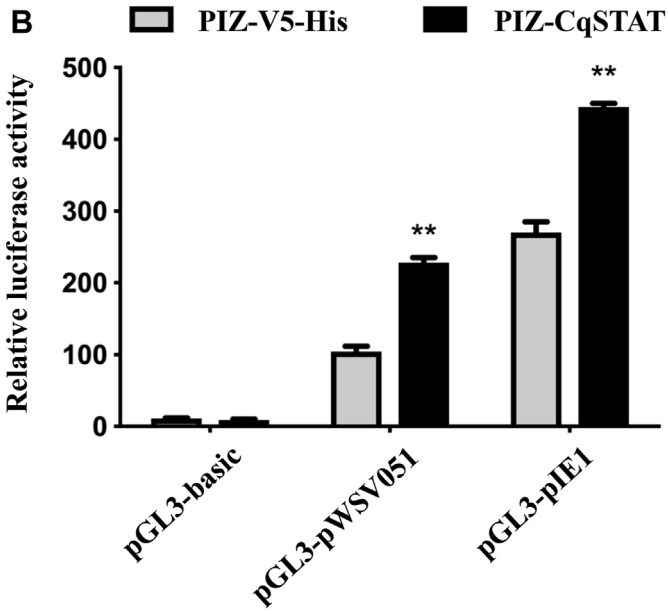


Fig. 5. (*continued*)

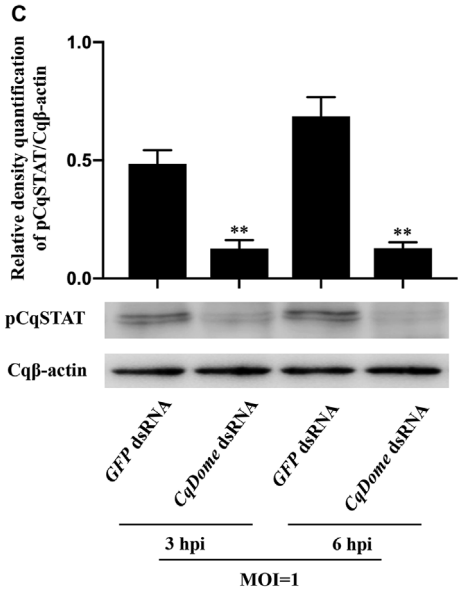


Fig. 5. (*continued*)

crayfish. Unfortunately, similar to the JAK/STAT activation signal model in mammals, it hasn't been proved whether the phosphorylated JAK can bind to the Dome receptor and phosphorylate specific tyrosine residues in the cytoplasmic part of the receptor, thus acting as docking sites for the Src homology 2 domains of STAT molecules. Hence, the precise interaction mechanism between Dome and JAK/STAT needs to be further studied.

4. Conclusion

In summary, our study shows that *Cq*Dome participates in the transduction of JAK/STAT signaling pathway and then promotes WSSV infection by activating the phosphorylation level of *Cq*STAT. Therefore, these data will be helpful for the further study of the molecular mechanism between WSSV and Dome-JAK-STAT pathway in host cells, which will benefit the control of WSSV disease in aquaculture.

Acknowledgement

This study was supported by the Natural Science Foundation of China (Nos. U1605214, 41676135), Fundamental Research Funds for the Central Universities of China (No. 20720180123) and Entrepreneurship Training Programs (20720162010, 20720170083, 2017X0622) and science and technology innovation action plan of Shanghai, China (17431904600).

References

Agaisse, H., Perrimon, N., 2004. The roles of JAK/STAT signaling in *Drosophila* immune responses. Immunol. Rev. 198, 72–82.
Bazan, J.F., 1990b. Structural design and molecular evolution of a cytokine receptor superfamily. Proc. Natl. Acad. Sci. U.S.A. 87, 6934–6938.
Bazan, J.F., 1990a. Haemopoietic receptors and helical cytokines. Immunol. Today 11, 350–354.
Binari, R., Perrimon, N., 1994. Stripe-specific regulation of pair-rule genes by hopscotch, a putative Jak family tyrosine kinase in *Drosophila*. Genes Dev. 8, 300.
Brown, S., Hu, N., Hombria, J.C., 2001. Identification of the first invertebrate interleukin JAK/STAT receptor, the *Drosophila* gene domeless. Curr. Biol. 11, 1700–1705.
Chen, R.Y., Shen, K.L., Chen, Z., Fan, W.W., Xie, X.L., Meng, C., Chang, X.J., Zheng, L.B., Jeswin, J., Li, C.H., Wang, K.J., Liu, H.P., 2016. White spot syndrome virus entry is dependent on multiple endocytic routes and strongly facilitated by Cq-GABARAP in a CME-dependent manner. Sci. Rep. 6, 28694.
Chen, W.Y., Ho, K.C., Leu, J.H., Liu, K.F., Wang, H.C., Kou, G.H., Lo, C.F., 2008. WSSV infection activates STAT in shrimp. Dev. Comp. Immunol. 32, 1142–1150.
Escobedo-Bonilla, C.M., Alday-Sanz, V., Wille, M., Sorgeloos, P., Pensaert, M.B., Nauwynck, H.J., 2008. A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus. J. Fish. Dis. 31, 1–18.
Gao, M., Li, F., Xu, L., Zhu, X., 2014. White spot syndrome virus strains of different virulence induce distinct immune response in *Cherax quadricarinatus*. Fish Shellfish Immunol. 39, 17–23.
Gilbert, M.M., Weaver, B.K., Gergen, J.P., Reich, N.C., 2005. A novel functional activator of the *Drosophila* JAK/STAT pathway, unpaired2, is revealed by an in vivo reporter of pathway activation. Mech. Dev. 122, 939.
Harrison, D.A., Mccoon, P.E., Binari, R., Gilman, M., Perrimon, N., 1998. *Drosophila* unpaired encodes a secreted protein that activates the JAK signaling pathway. Genes Dev. 12, 3252.
Hiu, K., Sandra E, N., 2012. Biology and significance of the JAK:STAT signalling pathway. Growth Factors 30, 88–106.
Hombría, J.C., Brown, S., Häder, S., Zeidler, M.P., 2005. Characterisation of Upd2, a *Drosophila* JAK/STAT pathway ligand. Dev. Biol. 288, 420–433.
Jiravanichpaisal, P., Sricharoen, S., Söderhäll, I., Söderhäll, K., 2006. White spot syndrome virus (WSSV) interaction with crayfish haemocytes. Fish Shellfish Immunol. 20, 718–727.
Johansson, M.W., Keyser, P., Sritunyalucksana, K., Söderhäll, K., 2000. Crustacean haemocytes and haematopoiesis. Aquaculture 191, 45–52.
Li, C., Weng, S., He, J., 2019. WSSV–host interaction: host response and immune evasion. Fish Shellfish Immunol. 84, 558–571.
Liongue, C., O'Sullivan, L.A., Trengove, M.C., Ward, A.C., 2012. Evolution of JAK-STAT pathway components: mechanisms and role in immune system development. PloS One 7, e32777.
Liu, H.P., Chen, R.Y., Zhang, Q.X., Peng, H., Wang, K.J., 2011. Differential gene expression profile from haematopoietic tissue stem cells of red claw crayfish, *Cherax quadricarinatus*, in response to WSSV infection. Dev. Comp. Immunol. 35, 716–724.
Liu, W.J., Chang, Y.S., Wang, A.H., Kou, G.H., Lo, C.F., 2007. White spot syndrome virus annexes a shrimp STAT to enhance expression of the immediate-early gene ie1. J. Virol. 81, 1461–1471.
Liu, W.J., Chang, Y.S., Wang, H.C., Leu, J.H., Kou, G.H., Lo, C.F., 2008. Transactivation, dimerization, and DNA-binding activity of white spot syndrome virus immediate-early protein IE1. J. Virol. 82, 11362–11373.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\triangle\triangle Ct}$ method. Methods 25, 402–408.
Marks, H., Vorst, O., van Houwelingen, A.M., van Hulten, M.C., Vlak, J.M., 2005. Gene-expression profiling of White spot syndrome virus in vivo. J. Gen. Virol. 86, 2081–2100.
Myllymaki, H., Ramet, M., 2014. JAK/STAT pathway in *Drosophila* immunity. Scand. J. Immunol. 79, 377–385.
O'Shea, J.J., Gadina, M., Schreiber, R.D., 2002. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. Cell 109 (Suppl. l), S121–S131.
O'Sullivan, L.A., Liongue, C., Lewis, R.S., Stephenson, S.E., Ward, A.C., 2007. Cytokine receptor signaling through the Jak-Stat-Socs pathway in disease. Mol. Immunol. 44, 2497–2506.
Oppenheim, J.J., 2001. Cytokines: past, present, and future. Int. J. Hematol. 74, 3–8.
Ozaki, K., Leonard, W.J., 2002. Cytokine and cytokine receptor pleiotropy and redundancy. J. Biol. Chem. 277, 29355–29358.
Rane, S.G., Reddy, E.P., 2002. JAKs, STATs and Src kinases in hematopoiesis. Oncogene 21, 3334.
Rawlings, J.S., Rosler, K.M., Harrison, D.A., 2004. The JAK/STAT signaling pathway. J. Cell Sci. 117, 1281–1283.
Robert, D., Maiken, J.K., Johan, G.O., Charlotte, O.S., Magnus, F., Vincent, G., Kaare, T., Jens, B., Birthe, B.K., 2012. The WSXWS motif in cytokine receptors is a molecular switch involved in receptor activation: insight from structures of the prolactin receptor. Structure 20, 270–282.
Ruan, Z., Wan, Z., Yang, L., Li, W., Wang, Q., 2019. JAK/STAT signalling regulates antimicrobial activities in *Eriocheir sinensis*. Fish Shellfish Immunol. 84, 491–501.
Seubert, N., Royer, Y., Staerk, J., Kubatzky, K.F., Moucadel, V., Krishnakumar, S., Smith, S.O., Constantinescu, S.N., 2003. Active and inactive orientations of the transmembrane and cytosolic domains of the erythropoietin receptor dimer. Mol. Cell. 12, 1239–1250.
Söderhäll, I., 2013. Recent advances in crayfish hematopoietic stem cell culture: a model for studies of hemocyte differentiation and immunity. Cytotechnology 65, 691–695.
Song, X., Zhang, Z., Wang, S., Li, H., Zuo, H., Xu, X., Weng, S., He, J., Li, C., 2015. A Janus Kinase in the JAK/STAT signaling pathway from *Litopenaeus vannamei* is involved in antiviral immune response. Fish Shellfish Immunol. 44, 662–673.
Sun, C., Shao, H.L., Zhang, X.W., Zhao, X.F., Wang, J.X., 2011. Molecular cloning and expression analysis of signal transducer and activator of transcription (STAT) from the Chinese white shrimp *Fenneropenaeus chinensis*. Mol. Biol. Rep. 38, 5313–5319.
Sun, J.J., Lan, J.F., Zhao, X.F., Vasta, G.R., Wang, J.X., 2017. Binding of a C-type lectin's coiled-coil domain to the Domeless receptor directly activates the JAK/STAT pathway in the shrimp immune response to bacterial infection. PLoS Pathog. 13, e1006626.
Vainchenker, W., Constantinescu, S.N., 2013. JAK/STAT signaling in hematological malignancies. Oncogene 32, 2601–2613.
van de Braak, C.B., Botterblom, M.H., Huisman, E.A., Rombout, J.H., van der Knaap, W.P., 2002. Preliminary study on haemocyte response to white spot syndrome virus infection in black tiger shrimp *Penaeus monodon*. Dis. Aquat. Org. 51, 149–155.
Wang, S., Song, X., Zhang, Z., Li, H., Lu, K., Yin, B., He, J., Li, C., 2016. Shrimp with knockdown of LvSOCS2, a negative feedback loop regulator of JAK/STAT pathway in *Litopenaeus vannamei*, exhibit enhanced resistance against WSSV. Dev. Comp. Immunol. 65, 289–298.
Wang, Y.T., Liu, W., Seah, J.N., Lam, C.S., Xiang, J.H., Korzh, V., Kwang, J., 2002. White spot syndrome virus (WSSV) infects specific hemocytes of the shrimp *Penaeus merguiensis*. Dis. Aquat. Org. 52, 249–259.
Wen, R., Li, F., Li, S., Xiang, J., 2014. Function of shrimp STAT during WSSV infection. Fish Shellfish Immunol. 38, 354–360.
Wright, V.M., Vogt, K.L., Smythe, E., Zeidler, M.P., 2011. Differential activities of the *Drosophila* JAK/STAT pathway ligands Upd, Upd2 and Upd3. Cell. Signal. 23, 920.
Yan, M., Li, C., Su, Z., Liang, Q., Li, H., Liang, S., Weng, S., He, J., Xu, X., 2015. Identification of a JAK/STAT pathway receptor domeless from Pacific white shrimp *Litopenaeus vannamei*. Fish Shellfish Immunol. 44, 26–32.
Yan, R., Small, S., Desplan, C., Dearolf, C.R., Jr, D.J., 1996. Identification of a Stat gene that functions in *Drosophila* development. Cell 84, 421–430.
Yao, D., Ruan, L., Lu, H., Shi, H., Xu, X., 2016. Shrimp STAT was hijacked by white spot syndrome virus immediate-early protein IE1 involved in modulation of viral genes. Fish Shellfish Immunol. 59, 268–275.