C-type lectin binds envelope protein of white spot syndrome virus and induces antiviral peptides in red swamp crayfish

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A B S T R A C T

Previously a pattern recognition receptor (PRR) from kuruma shrimp was found able to recognize bacterial glycans by the C-type lectin domain (CTLD) and to interact with Jak/Stat receptor Domeless by the interleukin-like coiled coil (cc) region. In the current study, its homolog, namely Pc-ccCL, was found important in the antiviral response in red swamp crayfish Procambarus clarkii. This PRR plays a role by inhibiting white spot syndrome virus (WSSV) infection in a Jak/Stat dependent manner. The CTLD can bind the viral envelope protein VP28, while the cc region determines the dependence on Jak/Stat pathway. Two anti-lipopolysaccharides factors were identified as the downstream antiviral peptides. This study provides new insights into the antiviral signaling in invertebrates. Furthermore, the mechanism that a PRR recognizes virus and directly activates Jak/Stat pathway and antiviral-effector expression represents a simple but fast antiviral strategy in crustaceans.

Introduction

Invertebrates possess efficient antiviral immunity to combat viral infections. RNA interference (RNAi) is regarded as a main antiviral mechanism in invertebrates by sensing and degrading the virus-derived nucleic acids [1,2]. Host Dicer-2 cleaves virus-derived dsRNAs into small interfering RNAs which are subsequently loaded in the RNAi-induced silencing complex. The complex then targets complementary sequences and leads to degradation of virus mRNA [3]. This antiviral immunity is not only effective towards RNA viruses, but also involved in the defense against DNA viruses [4]. In addition to RNAi, other innate immune strategies have been found related to antiviral defense, including phagocytosis of virions and infected cells, apoptosis, heat shock response, and phenoloxidase-mediated melanization [5].

Increasing studies in recent years show that innate antiviral signaling pathways, such as the Toll, Imd and Jak/Stat pathways which were investigated in detail because of the involvement in antibacterial and antifungal immunity, also represent important antiviral mechanisms in invertebrates [5]. For example, our previous study demonstrated that the unique lipid component from the envelope of white spot syndrome virus (WSSV) could be sensed by shrimp myeloid differentiation factor 2 homolog and could stimulate the NF-κB-mediated expression of antiviral proteins [6]. Besides, the glycoprotein G of vesicular stomatitis virus could be recognized by Drosophila Toll-7 with a consequence of triggering the antiviral autophagy [7]. However, whether the viral protein component can be sensed by host and lead to the antiviral transcriptional response lacks direct evidence.

As an abundant and diverse family of immune recognition receptors in crustacean, C-type lectins (CTLs) played important roles, regardless of beneficial for host or virus, during WSSV infection [8]. Many CTLDs possess the binding ability to viral proteins of WSSV, and this is the basis for the involvement of these CTLs in the host-WSSV interaction. For example, a CTL containing a solely CTLD from L. vannamei, LvCTL1, neutralizes WSSV infection by binding several viral proteins [9]. Our previous study showed that M. japonicus MjsvCL, which relies CTLD to recognize VP28, bridges the virions to shrimp cells by interacting with cell surface receptor calreticulin through its Q/N rich region and facilitate virus entry [10]. Whether there is any CTL which can activate downstream molecular response is unknown.

Previously a CTL, namely MjCC-CL, displaying both CTLD which was responsible to recognize bacterial glycans and an additional coiled coil (cc) region, was found able to activate Jak/Stat pathway by interacting with the cell surface receptor Domeless in kuruma shrimp Marsupenaeus japonicus [11]. In this study we investigated the significance its homolog, Pc-ccCL, in the antiviral immunity against WSSV in red swamp crayfish Procambarus clarkii. Pc-ccCL was found to recognize the WSSV envelope protein VP28, and stimulate the expression of several direct
antiviral effectors. Therefore, the results support that the protein components of viral particles can be sensed by PRRs to induce an innate antiviral signaling pathway in invertebrates. Moreover, the mechanism that a PRR with both recognition and cytokine activity directly activates Jak/Stat pathway and antiviral effector expression may represent a simple but fast, even though not as accurately regulated as in mammals, antiviral strategy in crustacean.

Materials and methods

Animals

Healthy red swamp crayfish (*P. clarkii*, 5–10 g) were collected from an aquaculture farm in Xuyi, Jiangsu, China. The animals were cultured in aerated water at 25 °C in the laboratory at least 1 week before the experiments, and fed commercial diets daily. All animals were randomly selected for study.

Three-dimensional model analysis

The identity between the cc region of Pc-ccCL and human interleukin 10 was predicted by DNAMAN. The structure of the cc region of Pc-ccCL was predicted by SWISS-MODEL by using the structure of human interleukin 10 (PDB: 2H24) as a model. The structure of the interleukin receptor region (ILR) of PcDomeless was automatically predicted also using SWISS-MODEL. The model of the interaction between the cc region of Pc-ccCL and the ILR of PcDomeless was predicted using ZDOCK Server. The top-ranked model was presented.

Viral inoculums preparation

The viral inoculums were prepared as follows. Moribund shrimp artificially infected with WSSV were collected and stored at 80 °C before use. Gills (1 g) were homogenized in 10 ml of sterile phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4). After two rounds of freeze-thaw, the homogenate was centrifuged at 3000 × g for 10 min at 4 °C, and the supernatant was centrifuged at 10,000 × g for 10 min at 4 °C.

| Table 1 | Primers used for this study |
|---|---|
| **Primers** | **Sequence (5’-3’)** |
| (q)RT-PCR | |
| Pc-ccCLRTF | GCCGGGATGGAAGATCTGCT |
| Pc-ccCLRTF | TCTTGGCTGGTGATGGGTA |
| Pcj-actinRTF | AAACCTCTCAATCCCGGCTATG |
| Pcp-actinRTF | CGAAGATTTCGTGGCTTCG |
| PcDomelessRTF | CCTTCCACTGAGTATGTC |
| PcDomelessRTF | TTGGTAGCGACACTCTT |
| PcjJakRTF | TGATGATGGGATCTGTC |
| PcjJakRTF | CGATGTGAAAGTGTGGTC |
| Alf7052RTF | CGGAAGTCGAGGGA |
| Alf7052RTF | TAAAGAGGAGGTTGAC |
| Alf7073RTF | CTCAGCGCAGTGAGTAC |
| Alf7073RTF | CACATTTCTCTTCTGAGT |
| VP28RTF | AGTCGACACTCTCGTCAGAG |
| VP28RTF | TTACTCGGCGTACTGAGG |
| RNAi | |
| PccCRNAiF | GCCGAAGGTCAGGAG |
| PccCRNAiF | CGAAGATTTCGTGGCTTCG |
| PcdomelessRNAiF | CCTTCCACTGAGTATGTC |
| PcdomelessRNAiF | TTGGTAGCGACACTCTT |
| PcjJakRNAiF | TGATGATGGGATCTGTC |
| PcjJakRNAiF | CGATGTGAAAGTGTGGTC |
| Alf7052Si1-1 | GATATAGGAGGATATTATG |
| Alf7052Si1-1 | CGAAGATTTCGTGGCTTCG |
| Alf7052Si2-1 | AATGATGGGATATTATG |
| Alf7052Si2-1 | CGAAGATTTCGTGGCTTCG |
| Alf7073Si1-1 | GGTATGATGGGATATTATG |
| Alf7073Si1-1 | CGAAGATTTCGTGGCTTCG |
| Alf7073Si2-1 | AATGATGGGATATTATG |
| Alf7073Si2-1 | CGAAGATTTCGTGGCTTCG |
| GFPRNAiF | GCCGAAGGTCAGGAG |
| GFPRNAiF | CGAAGATTTCGTGGCTTCG |
| GFPRNAiR | GCCGAAGGTCAGGAG |
| GFPRNAiR | CGAAGATTTCGTGGCTTCG |
| GFPSi1-1 | GCCGAAGGTCAGGAG |
| GFPSi1-1 | CGAAGATTTCGTGGCTTCG |
| GFPSi1-2 | AATGATGGGATATTATG |
| GFPSi1-2 | CGAAGATTTCGTGGCTTCG |
| GFPSi2-1 | AATGATGGGATATTATG |
| GFPSi2-1 | CGAAGATTTCGTGGCTTCG |
| Recombinant expression | |
| PccCL1 | CGCGGACCTAGCTGAGGGA |
| PccCL1 | CGCGGACCTAGCTGAGGGA |
| PccCLF3 | CGCGGACCTAGCTGAGGGA |
| PccCLF3 | CGCGGACCTAGCTGAGGGA |
| Alf7052EF | CGCGGACCTAGCTGAGGGA |
| Alf7052EF | CGCGGACCTAGCTGAGGGA |
| Alf7073EF | CGCGGACCTAGCTGAGGGA |
| Alf7073EF | CGCGGACCTAGCTGAGGGA |
| ChIP | |
| Alf7073chipF | GCAGGCAAGGGCGAGAGAAGG |
| Alf7073chipF | GCAGGCAAGGGCGAGAGAAGG |
| Alf7073chipP | GCAGGCAAGGGCGAGAGAAGG |
| Alf7073chipP | GCAGGCAAGGGCGAGAGAAGG |
collected and filtered through a 0.45 μm filter. The genomic DNA was extracted from 100 μl of the filtrate using the MagExtractor Genome (Toyobo, Shanghai, China) to qualify the virus titer by quantitative real-time PCR (qRT-PCR). Generally, a recombinant pBlueScript plasmid containing the VP28 fragment (Table 1) was generated. Serially diluted plasmid samples with known concentrations and copies were used to do qRT-PCR to generate a standard curve which can be used to determine the viral copies in the inoculum. The remaining filtrate was stored at −80 °C, used as the viral inoculum and diluted to the appropriate titer with PBS before use. The virus copy in tissues was determined similarly.

Expression profiles analysis

WSSV challenge was performed by intramuscular injection at the abdominal segment of animals with the inoculum containing 1 × 10^6 virions. Equal volume of PBS was injected as a mock challenge. At different times after challenge, the crayfish hemolymph was collected into pre-cooled anticoagulant (0.14 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6) [12] and centrifuged to isolate the hemocytes pellet at 800 g for 5 min. The hemocytes were resuspended in pre-cooled anticoagulant (0.14 M NaCl, 0.1 M glucose, 30 mM tri- nescent protein (GFP) was produced as the control. dsRNA was injected into the hemocel of animals with a dose of 5 μg per g body weight. The recombinant vector was introduced into Escherichia coli BL21 for recombinant protein expression. The expression was induced with 0.2 mM of isopropyl-B-D-thiogalactopyranoside (IPTG) at 28 °C for 6–8 h after the OD600 of the culture reached 0.8. The recombinant proteins were purified by affinity chromatography using GST-resin (GenScript, Nanjing, China). For proteins prepared to administrate in vivo, the endotoxins contamination was removed by an additional thorough washing with cold 0.1% Triton X-114 before the final elution of the proteins from the resin [13]. The proteins were dialyzed in PBS trice at 4 °C, and concentrated using PEG20000. The protein concentration was determined with the Bradford assay using bovine serum albumin BSA as the standard. The protein solution containing 5% glycerol was stored at −80 °C before use. The recombinant Alfs were expressed and processed similarly, using the primers listed in Table 1, with the help of pET32a(+) vector.

RNA interference

Partial DNA fragments of Pc-ccCL were produced by PCR using the specific primers linked with T7 promoter (Table 1), and ligated into the pGEX-4T-1 vector (GE Healthcare, Piscataway, NJ, USA) with conventional molecular cloning techniques. The recombinant vectors were introduced into Luria-Bertani (LB) plates and grown overnight. The recombinant Alfs were expressed and processed similarly using the primers listed in Table 1.

Western blot

Tissues were homogenized in PBS and centrifuged at 12,000 × g for 20 min to obtain the supernatant. The protein concentration was determined using the Bradford assay. Protein Loading Dye (Sangon, Shanghai, China) was added in the protein solution before boiling 100 °C for 5 min and centrifugation at 8,000 × g for 3 min. The protein samples were separated by 12.5% SDS-PAGE with a loading of 10 μg per well. The proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with 3% nonfat milk dissolved in Tris-buffered saline (TBS), incubated with specific antiserum (1:1,000 dilution) for 2 h at 37 °C, washed trice with TBS (TBS with 0.05% Tween 20), incubated with horseradish peroxidase (HRP)-labeled secondary antibody (1:10,000 dilution, Zhongshan, Beijing, China) for 1 h at 37 °C, and washed trice with TBS. The bands were visualized using the High-signal ECL western blotting substrate (Tanon, Shanghai, China), and detected using a 5200 Chemiluminescence Imaging System (Tanon). The anti-serum specific to ccCL, VP28 and β-actin were prepared previously by immunizing New Zealand rabbits with the recombinant proteins.

Recombinant protein production

The fragments encoding the mature Pc-ccCL, CTLD and cc region were generated by PCR using specific primers listed in Table 1, and ligated into the pGEX-4T-1 vector (GE Healthcare, Piscataway, NJ, USA) with conventional molecular cloning techniques. The recombinant vectors were introduced into Escherichia coli BL21 for recombinant protein expression. The expression was induced with 0.2 mM of isopropyl-B-D-thiogalactopyranoside (IPTG) at 28 °C for 6–8 h after the OD600 of the culture reached 0.8. The recombinant proteins were purified by affinity chromatography using GST-resin (GenScript, Nanjing, China). For proteins prepared to administrate in vivo, the endotoxins contamination was removed by an additional thorough washing with cold 0.1% Triton X-114 before the final elution of the proteins from the resin [13]. The proteins were dialyzed in PBS trice at 4 °C, and concentrated using PEG20000. The protein concentration was determined with the Bradford assay using bovine serum albumin BSA as the standard. The protein solution containing 5% glycerol was stored at −80 °C before use. The recombinant Alfs were expressed and processed similarly, using the primers listed in Table 1, with the help of pET32a(+) vector.

In vivo administration of recombinant proteins

To determine the role of Pc-ccCL, CTLD (2 μg) was injected together with the viral inoculum (1 × 10^6 virions) intramuscularly into crayfish to detect the virus infection, while the control animals were injected with rGST tag with the inoculum. The gills were sampled for genomic DNA determine the virus titer 48 h later. Each sample was derived from at least 10 crayfish. The injection of protein and viral inoculum was performed 24 h after PcDomeless dsRNA injection, or 6 h after Stat inhibitor application. The inhibitor application was performed by injecting the inhibitor (SH-4-54, Selleck, Shanghai, China) intramuscularly into crayfish with a dose of 10 μg per g body weight.

Pull down assay

Pull down assay was used to study the possible interaction between rccCL and viral protein. The virus-infected gills were homogenized in PBS. The homogenate was centrifuged at 12,000 × g for 15 min to isolate the supernatant which was used as the pool of viral proteins. The mixture containing 2 μg of recombinant proteins and 3 ml of the viral proteins pool was gently mixed by agitation at 4 °C for 3 h. Subsequently, 20 μl of the GST resin was added and the agitation continued for another 1 h. The resin was washed trice with PBS by alternative centrifugation and suspension. Finally, the resin was re-suspended in 30 μl of the SDS-PAGE sample buffer, boiled and analyzed by Western blot using the
antibodies against the viral proteins generated previously.

**Immunocytochemistry**

Immunocytochemistry was used to analyze whether Pc-ccCL could or not bind to the surface of crayfish hemocytes. Firstly, the expression of PcDomeless was silenced by RNAi, as described above. The hemolymph was collected in equal volume anticoagulant, and centrifuged at 800 × g for 5 min to isolate the hemocytes. The pellet was suspended in Leibovitz L-15 medium (Sangon) containing 15% fetal bovine serum (Sigma) and 100 μg/ml of streptomycin sulfate. The suspension was inoculated into a 24-well plate with 10^5 cells per well for 1 h for attachment to the slides pre-placed in the wells. Afterwards, recombinant protein (5 μg) was added into the wells and the incubation lasted for 30 min. The slides were washed trice with PBS, fixed with 4% paraformaldehyde dissolved in PBS for 1 h at 4°C. The slides were washed trice with PBS, blocked with 2% BSA dissolved in PBS for 30 min at 37°C. The CCCL antiserum (1: 100 diluted) was added onto the slides, and the incubation lasted overnight at 4°C. After washing with PBS trice, the slides were incubated with goat anti-rabbit-Alexa 488 (Molecular Probes, 1:1,000 diluted) for 1 h at 37°C. The slides were washed trice with PBS. Afterwards, the cell membrane was stained with Alexa Fluor 594-conjugated wheat germ agglutinin (WGA, Invitrogen), which could target the glycoproteins on plasma membrane, for 15 min, and the nuclei was stained with DAPI for 10 min at 25°C. After the final trice wash with PBS, the slides were observed using the Zeiss LSM 700 laser confocal microscope (Zeiss, Thornwood, NY, USA).

**Statistical analysis**

The results in this study was analyzed using Student’s t test using Microsoft Excel, and significance was accepted with p < 0.05.

**Results**

**Pc-ccCL responds to WSSV infection**

Similar to its homolog from kuruma shrimp *M. japonicus*, Pc-ccCL displays an architecture with a single CTLD and a cc region (Fig. 1A). The identity between the cc regions and the human IL10 was 21%. Using the structure of human IL10 as a model to do the homologous modeling, the cc region of both ccCLs from *P. clarkii* and *M. japonicus* were found showing an overall similar structure to IL10 (Fig. 1B). Moreover, docking analysis showed that the molecular interaction between the cc region of Pc-ccCL and the ILR of Domeless (Fig. 1C). This was consistent with the previous finding.

To check whether or not Pc-ccCL is involved in the host-WSSV interaction, its expression profiles were firstly studied. As shown in Fig. 2, WSSV infection significantly induced the expression of Pc-ccCL in the gills, hemocytes and hepatopancreas. Particularly in crayfish gills, the expression of Pc-ccCL started to increase at the very beginning (6 h) of the intramuscular infection. The induction peaked as high as 6-fold in the mid-term (12–24 h) and persisted until the late stage (48 h) of the infection. The expression profiles suggested that Pc-ccCL might play a role during WSSV infection.

**Pc-ccCL inhibits WSSV infection**

To study the function of Pc-ccCL in viral infection, the expression of Pc-ccCL was silenced by RNAi. As revealed by qRT-PCR and shown in Fig. 3A, the Pc-ccCL expression could be suppressed in gills by exogenous dsRNA, and the RNAi efficiency lasted until 48 h after dsRNA application. The viral inoculum was then introduced through intramuscular injection into Pc-ccCL pre-silenced crayfish to determine whether the virus infection was influenced. Results showed that WSSV replication was increased in the Pc-ccCL silenced crayfish. The virus copies in the gills and stomach were both higher than those in control crayfish at both 24 h and 48 h after WSSV infection (Fig. 3B). The expression of VP28, which was the most abundant structural protein of WSSV, in the experimental samples were stronger than that in the corresponding controls, confirming that the virus replication was enhanced.
strengthened after Pc-ccCL expression silencing (Fig. 3C). These data suggested an antiviral role of Pc-ccCL in red swamp crayfish.

Pc-ccCL binds to WSSV envelope protein VP28

To reveal the mechanism how Pc-ccCL inhibits WSSV infection, whether Pc-ccCL could bind to viral proteins was studied since shrimp ccCL was proved as a PRR for bacterial surface components and many CTLs were demonstrated as viral receptors [14,15]. rccCL was used as the prey for the pull down analysis to capture possible target from the pool of viral proteins. As shown in Fig. 4, rccCL could interact with VP28 which was the major envelope protein of WSSV. However, no interaction was observed between rccCL and VP19, another viral envelope protein. These results suggested that Pc-ccCL might exert the antiviral role as an extracellular receptor by recognizing the WSSV virions through interacting with the viral envelope protein VP28.

Antiviral function of Pc-ccCL is dependent on JAK/Stat signaling

Because previous study showed that recognition of the bacterial glycans by the CTLD of shrimp ccCL directly activated Jak/Stat signaling through interacting with the cell surface receptor Domeless by the cc region, whether the antiviral function of Pc-ccCL was related with Jak/Stat signaling was investigated. Pull down assay showed that the CTLD of Pc-ccCL was responsible for the binding of VP28, while the cc region did not participate in the recognition of virus (Fig. 5A). To determine whether Pc-ccCL could or not interact with PcDomeless, rccCL or rCTLD was applied to crayfish hemocytes which were pre-treated with the PcDomeless dsRNA or the control dsRNA. As shown in Fig. 5B, rccCL, but not the rCTLD, was found attached to the surface of crayfish hemocytes, suggesting that the cc region was critical for the cell surface
Binding of Pc-ccCL. Moreover, the cell surface binding was not observed when PcDomeless expression was pre-silenced, indicating that target of cc region of Pc-ccCL was PcDomeless.

Subsequently whether the antiviral role of Pc-ccCL was dependent on PcDomeless and Jak/Stat signaling was determined. As shown in Fig. 5C, the inhibitory effect of Pc-ccCL was greatly weakened upon the knockdown of PcDomeless expression. The viral amount in the group administrated with rccCL was only 16% of the group administrated with control tag, while the number was 75% when the experiments were performed using the PcDomeless pre-silenced animals. Significant impairment of the virus inhibitory ability of Pc-ccCL was also observed when using a Stat inhibitor to suppress Stat activity in crayfish. These results showed that the antiviral role of Pc-ccCL was indeed dependent on JAK/Stat signaling.

Alfs are key antiviral effectors downstream of WSSV-Pc-ccCL-Jak/Stat signaling

Because Alfs in other species had been proven important in antiviral response by directly acting on the WSSV virions [16,17], we detected whether WSSV-Pc-ccCL-Jak/Stat signaling would lead to the expression of Alfs. The expression of possible candidates should be significantly induced by WSSV infection, and suppressed by Pc-ccCL knockdown or PcJak knockdown upon WSSV infection. We found the expression of two Alfs was indeed obviously induced by WSSV infection, and the induction was indeed suppressed when Pc-ccCL or PcJak expression was silenced by RNAi (Fig. 6A). To clarify the roles of two Alfs, RNAi were performed to knockdown their expression before WSSV infection (Fig. 6B). The results showed that silencing the expression of either Alf led to higher amounts of WSSV virions than the control test, suggesting the protective role of both Alfs in crayfish antiviral immunity (Fig. 6C). This data provided evidence that two Alfs were downstream antiviral effectors.

Above results suggested a signal transduction from WSSV/VP28 to Pc-ccCL, Jak/Stat and finally two Alfs. However, whether Stat could directly or indirectly regulate the transcription of two Alfs upon WSSV infection remained uncertain. The promoter region of two Alfs were cloned, and two perfect γ-interferon activation site (GAS)-related DNA elements which were the binding sites of mammalian STAT were identified in the promoters of both Alfs, suggesting the possible regulation of two Alfs by Stat in crayfish (Fig. 7A). A ChIP assay was next performed to detect whether Stat could bind the DNA fragments containing the GAS motif. As shown in Fig. 7B, positive signals for each Alf was detected only in the immunoprecipitates of Stat antibody when the WSSV infected cells were used as the pool for ChIP (Fig. 7B). This suggested that Stat could directly regulate the expression of two Alfs by binding the GAS motif. Therefore, these results supported the antiviral signal transduction from the recognition of WSSV by Pc-ccCL to the activation of Jak/Stat signaling and finally the expression of direct antiviral effectors.

Discussion

Though RNAi is the major antiviral strategy in invertebrates, increasing studies showed that the induced-responses also play a role in antiviral defense [18]. These induced responses involve both cellular mechanisms including apoptosis and autophagy, and the induction of antiviral effectors which contribute to the inhibition of virus infection in multiple ways [5]. However, how the viruses are sensed and how the downstream responses are activated in the induced antiviral immunity remains largely unknown. This study reported that a PRR was involved in the induced antiviral immunity of crustaceans by recognizing the viral protein and activating the expression of antiviral effectors. Thus, these
findings provide evidence for the presence of induced antiviral immunity in invertebrates. Besides, this study also provides new insight into the evolution of induced antiviral signaling. The interferon (IFN) production in mammals is mainly induced by the viral DNA or RNAs. These recognition events are generally occurred within host cells. However, some cell surface or extracellular PRRs had been found to recognize virus surface components to activate the induced innate antiviral signaling in invertebrates [6]. Together with these reports, this study shows that sensing the materials on the surface of virions, including proteins, lipids and glycans, is an effective alternative to initiate the induced antiviral signaling.

Moreover, this study revealed a different antiviral manner in decapod crustacean from that in mammals. Mammalian IFNs are produced upon the activation of the antiviral signaling transduction from the immune sensors to the transcription factors. The newly synthesized IFNs would target the receptors, cause the initiation of JAK/Stat pathway and finally lead to the expression of multiple IFN-stimulated genes, which are the real executors to resist and control virus infection [21]. Differently, in this study, it was found that Pc-ccCL firstly recognized virus as a PRR, and secondly initiated JAK/Stat pathway to induce the expression of direct antiviral factors. The binary function of ccCL was originated from its arrangement with both the receptor domain and the signaling domain. This arrangement enables crustaceans to start an immediate response against virus infection using only a single protein to accomplish the jobs of both immune recognition and effector stimulation. This may represent an effective strategy for crustaceans which primarily live in an aquatic environment full of pathogen threats. However, compared to the indirect manner in mammals, such direct activation may be not so exercise since the intermediate immune signal modulation was skipped, and may generate an insufficient or excessive antiviral response.

Jak/Stat signaling was important in the antiviral immunity of invertebrates. Studies in Drosophila showed that Jak/Stat pathway was involved in the control of drosophila C virus in infected flies, and was required for the induced expression of some antiviral genes [22]. This pathway was also activated by the reactive oxygen species induced by DNA virus Invertebrate iridescent Virus 6 to regulate the robust expression of many antiviral factors [23]. This study emphasized the significance of Jak/Stat signaling in the defense against WSSV in crayfish, suggesting that Jak/Stat-mediated defense is a conserved antiviral strategy in invertebrates.

Many CTLs contain additional region besides of the CTLD. The additional region is critical for the function of a CTL, and the diversity of the arrangement may be a determinant of the functional diversity of CTL family. This kind of arrangement is frequently observed in the genome of invertebrates. For example, Caenorhabditis elegans genome encodes as much as 278 CTLs, and most of them are of unique arrangement with CTLD and additional regions including complement Uegf Bmp1 domain, von Willebrand factor, and so on [24]. Among the 31 CTLs in D. melanogaster genome, some contained fibronectin type 3 or Sushi domain [25]. These additional domains have been proven as important effector modules for signal transduction [26–28]. Therefore, concluding from these reports and this study, the frequent structure with both recognition module and effector module indicates that the direct activation of downstream signaling after immune recognition may be general and important in invertebrates.

**Statement**

All authors declare there are no conflicts of interests. The research on live animals meets the guidelines approved by the Animal Ethical Committee of the School of Life Sciences, Shandong University.

**Declaration of Competing Interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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