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Short communication

Regulation of shrimp *PjCaspase* promoter activity by WSSV VP38 and VP41B

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ABSTRACT

Members of the *Caspase* family play essential roles in apoptosis. In kuruma shrimp *Marsupenaeus japonicus* the *caspase* gene (*PjCaspase*) was previously found dramatically up-regulated in viral-challenged and -resistant shrimp, suggesting that *PjCaspase* plays an important role in protecting host from viral infection. In order to further delineate the transcriptional regulation of *PjCaspase* in response to viral infection, the promoter activity was confirmed by fusing the 5′-flanking promoter region of the *PjCaspase* gene to the enhanced green fluorescence protein (EGFP) gene and transformed to *Trichoplusia ni* High Five™ cell line. With streptavidin-bead pulldown assay, two envelope proteins VP38 and VP41B of white spot syndrome virus (WSSV) were found to bind to *PjCaspase* promoter *in vitro*. Luciferase reporter assay by cotransfection of *PjCaspace* promoter with VP38 or VP41B revealed that the proteins act as repressor and activator of *PjCaspase* transcription respectively. Our study suggested a potential role for the two WSSV proteins on shrimp *PjCaspase* regulation in response to WSSV challenge. To our knowledge this is the first report on WSSV envelope proteins found to be involved in gene regulation. These results provide insights into the molecular regulation of *PjCaspase* gene expression, which will be helpful for shrimp viral disease control.

White spot syndrome virus (WSSV) is one of the most devastating pathogens of shrimps and other crustaceans [1]. In the past decade, studies of WSSV mainly focused on the viral structural proteins and infection pathogenesis [2–4], the mechanism by which the shrimp defend themselves against viral invasion is still elusive.

Apoptosis or programmed cell death plays important role in the antiviral immunity of many organisms including shrimp [5]. It is a tightly regulated process to removes unwanted and potentially dangerous cells such as virus-infected cells [6,7]. Viruses can induce apoptosis as an innate cellular response to limit virus production and also decrease or eliminate spread of progeny virus in the host [8]. Previous study had shown that the *PjCaspase* gene was up-regulated in the hemocytes of viral-resistant and -challenged shrimp *Marsupenaeus japonicus* [7,9]. Furthermore, RNAi assays revealed that when *Pjcaspase* gene was silenced, the WSSV-induced apoptosis was significantly inhibited and resulted in increase of viral copies. These results suggest that *PjCaspase* was essential effector in the apoptotic pathway against virus infection [7].

The transcriptional regulation of *PjCaspase* is critical since the expression of this gene is regulated in response to viral stimuli. In this study, we report studies using DNA pull down assays showing that VP38 and VP41B protein of WSSV can bind to *PjCaspase* promoter. Transient transfection assays revealed VP38 and VP41B functions as transcriptional repressor and activator respectively. These results improve our understanding of the still poorly understood molecular events involved in innate immune response against virus infection of invertebrates, which will be helpful for shrimp viral disease control.

Transient reporter assay was performed to analyse the promoter activity in the 5′-flanking region of *PjCaspase* gene. The reporter pIZΔIE/EGFP and pIZ/EGFP plasmids were constructed as described by Luo et al. [10]. The upstream region of *PjCaspase* gene (−678 to 141 bp from the transcription initiation site) was amplified from the marine shrimp *M. japonicus* DNA with primers Cas-EGFP-F: 5′-GCAAGCTTAGAGGGAACGGGTAGACT-3′ and Cas-EGFP-R: 5′-AACTCGAGCAAGTAATTATTGCGATTACAT-3′. The upstream region was then inserted into the *Hin*d III and *Xho* I restriction sites in front of the EGFP gene of the promoter-absent vector pIZΔIE/EGFP. The plasmids pIZ/EGFP (constructed by inserting the EGFP gene between *Bam*H I and *Eco*R I sites of pIZ/V5-His) and pIZΔIE/EGFP were used as the positive and negative controls, respectively.

Abbreviations: WSSV, white spot syndrome virus; EGFP, enhanced green fluorescence protein; NP-40, Nonidet P-40.

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Since there is no stable shrimp cell line available worldwide, *Trichoplusia ni* High Five™ cell (Invitrogen) was used for the promoter activity assay. Cells were seeded onto the 6-well plate (3×10^5 cells/well) and grown in Express Five SFM media (Invitrogen) overnight at 27 °C. When the cells reached 70% confluence, 1 ml of serum-free medium, 1 μg of plasmid DNA and 2 μl of Cell-Fectin reagent (Life Technologies) were added to transfect the cells according to the manufacturer's instructions. EGFP fluorescence signal was imaged with Nikon Eclipse TE2000-S (Nikon, Melville, NY) inverted fluorescence microscope 72 h post-transfection. Green EGFP fluorescent signals were observed in cells transfected with the plasmids containing the 5′-flanking region of *PjCaspase* promoter, though the fluorescent signal was not as strong as in positive control group driven by the OpMNPV IE2 promoter. In contrast no fluorescence was detected in the negative group where the promoter was absent (Fig. 1).

In an attempt to investigate the mechanism by which *PjCaspase* is up regulated in response to WSSV infection, we employed the cloned promoter region of *PjCaspase* as the DNA target for affinity binding assays to identify potential *PjCaspase* regulatory proteins in WSSV. Double stranded biotinylated *PjCaspase* promoter sequence −30 to −508 was amplified by the following biotinylated primers. Cas-Bio-F:Bio-5′-AGCTGCATTCTTGCTTCCCTC-3′; Cas-Bio-R: Bio-5′-GGGGCAATACTGACACAAGAT-3′. About 100 pmol of biotin-labeled PCR product was coupled to 3 mg of Streptavidin Agarose Resin (Thermo Scientific) with incubation buffer (20 mM Tris pH 7.9, 150 mM NaCl, 5% glycerol, 0.5 mM EDTA, 1 mM $MgCl_2$, 1% Triton X-100).

White spot syndrome virus (WSSV) virions were purified following the method of Xie et al. [11]. Briefly, the muscle tissue of crayfish *Procambarus clarkii*, which had succumbed to the WSSV infection, were homogenized in TNE buffer (50 mM Tris−HCl, 400 mM NaCl, 1 mM PMSF, 5 mM EDTA, pH 8.5), and then centrifuged at 3500 × *g* for 5 min at 4 °C. The supernatant was filtered by nylon net (400 mesh) and then centrifuged at 30,000 × *g* for 30 min at 4 °C. The upper loose pellet was rinsed out carefully, and the lower white pellet was suspended in 10 ml TM buffer (50 mM Tris−HCl, 10 mM $MgCl_2$, pH 7.5). After centrifugation at 3500 g for 5 min, the virus particles were sedimented by centrifugation at 30,000 g for 20 min at 4 °C and resuspended and kept in 1 ml TN buffer. Then equal volume of 2% Nonidet P-40 (NP-40) was added to the virons. After gentle agitation at room temperature for 1 h, the extract was centrifugated at 30,000 g for 20 min at 4 °C. Subsequently, the supernatant was incubated with DNA free resin at 4 °C overnight to preclear nonspecific binding. Precleared sample was bound for 2 h at room temperature (25 °C) to the biotinylated DNA sequences. The protein binding resins were washed 3 times with 1 mL of incubation buffer and then 1 time with 5 M NaCl. Finally, resins were combined with 20 μL of SDS-PAGE loading buffer and heated at 100 °C in order to liberate very tightly-bound proteins. DNA-binding proteins were resolved by SDS-PAGE and visualized by Coomassie Blue staining (Fig. 2).

The binding assays allowed reproducibly detection of 2 proteins (apparent molecular masses of 38 and 41 kDa) interact with *PjCaspase* promoter (Fig. 2). The bands were excised and subjected to protein identification by MALDI-TOF/TOF mass spectrometry. The two proteins were identified as WSSV envelope proteins VP38 and VP41B. These proteins appeared to bind tightly to the promoter regions, since they were not eluted even at high concentrations of NaCl (5 M), and were liberated from the beads only by heating in SDS-PAGE loading buffer.

Luciferase reporter assays was performed to examine the effect of VP38 and VP41B expression on promoter activities of *PjCaspase* genes. The pGL3-ProCas reporter construct was created by subcloning the *PjCaspase* promoter fragment encompassing −678 to 141 bp into the pGL3-Basic firefly luciferase reporter vector (Promega) using primers Cas-Luc-F: (5′-C*GAGCTC*GAGAGGGAACG GGTAGACT-3′) and Cas-Luc-R: (5′-AACTCGAGCAAGTAATTATTGC-GATTACAT-3′), where the underlined nucleotides are *Sac* I and *Xho* I recognition sequences, respectively. The VP38 and VP41B expressing plasmid (pIZ-VP38 and pIZ-VP41B) were generated by cloning the full length viral gene into the *Hin*d III-*Eco*R I sites of the pIZ/V5-His plasmid (Invitrogen). The primers used are pIZ-VP38-F: 5′-CCC*AAGCTT*GGGATGTCTTCTTCGTCTTCTG-3′ pIZ-VP38-R: 5′-CCG *GAATTC*CGGTTATGAACATGTTACAATTATTC-3′; pIZ-VP41B-F: 5′-CCC *AAGCTT*GGGATGGGAGATAAGCAAAAG-3′ and pIZ-VP41B-R: 5′-CCG *GAATTC*CGGCTAGGAGCATGTGCATG-3′.

High Five™ cells were grown in 24-well plate and transfected using Cellfection (Invitrogen) with 1 μg of reporter construct, 1 μg of expressing plasmid (pIZ-VP38 or pIZ-VP41B) or empty pIZ/His-V5

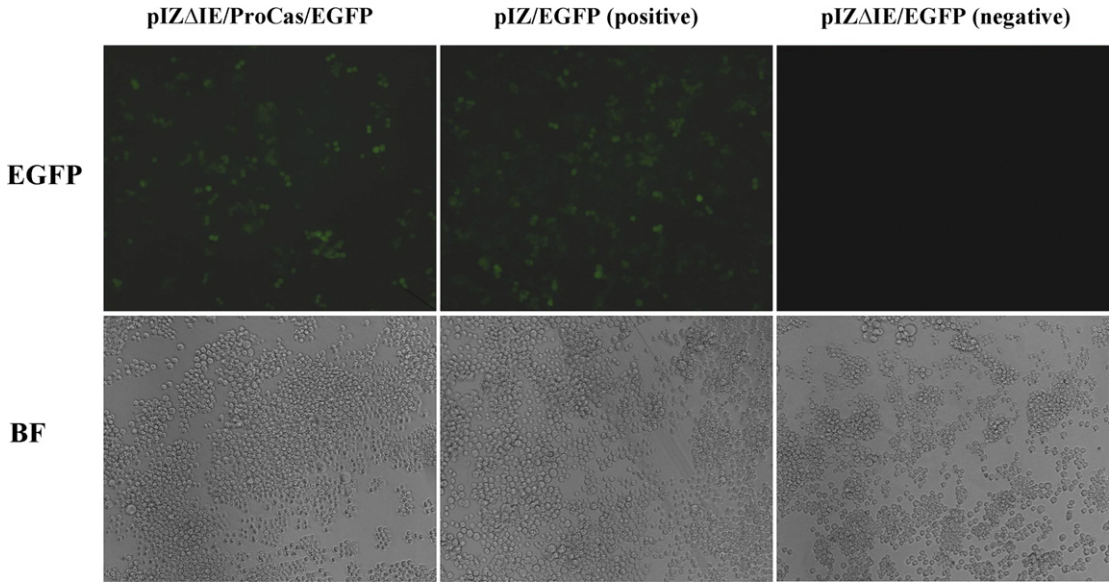

Fig. 1. Promoter activity assay for *PjCaspase* gene. Brightfield (BF) and EGFP green fluorescence signals in High Five™ cells transfected with pIZΔIE/ProCas/EGFP which used *PjCaspase* promoter (Left), pIZ/EGFP which used OpMNPV IE2 Promoter as a positive control (Middle) and promoter-less pIZΔIE/EGFP as a negative control (Right).