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Identification of a c-Jun homolog from *Litopenaeus vannamei* as a downstream substrate of JNK in response to WSSV infection

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ABSTRACT

c-Jun, a major substrate of c-Jun N-terminal kinase (JNK), participates in regulating gene transcription in response to various stimuli, including cytokines, stress signals, bacterial and viral infection. Results from our previous studies suggested that *Litopenaeus vannamei* JNK (LvJNK) could be utilized by white spot syndrome virus (WSSV) to facilitate viral replication and gene expression. In this article, a c-Jun homolog from *Litopenaeus vannamei* (designated as Lvc-Jun) was cloned and its role in WSSV infection was studied. Sequence analysis displayed that Lvc-Jun was a novel homolog of c-Jun family, which contained characteristic Jun and basic leucine zipper (bZIP) domains, and two conserved serine phosphorylation sites (Ser49/59). Semi-quantitative RT-PCR analysis showed that Lvc-Jun mRNAs were expressed in all examined tissues. Further investigation determined that Lvc-Jun was located in the nucleus through self-interaction and its phosphorylation levels could be reduced by JNK inhibitor, suggesting that Lvc-Jun could be regulated by LvJNK through phosphorylation and function as a transcription regulator in a homodimer. During the process of WSSV infection, the transcription levels of Lvc-Jun were up-regulated associating with the raising expression and phosphorylation levels of its protein. Moreover, TPA (12-*O*-tetradecanoylphorbol-13-acetate), a potent inducer of c-Jun, could remarkably promote viral immediate-early gene wsv069 transcription in crayfish hemocytes. Conclusively, our results provided experimental evidences that Lvc-Jun was engaged in WSSV infection and further implied that JNK-c-Jun signaling pathway might be important for WSSV replication and viral gene expression.

1. Introduction

White spot syndrome virus (WSSV), a large double-stranded DNA virus of the *Nimaviridae* family, is an extremely lethal shrimp pathogen worldwide (Leu et al., 2009). Since its discovery 20 years ago, WSSV has caused serious economic losses in shrimp aquaculture. Viral infections are known to activate and manipulate host cellular signaling networks to create a more hospitable cellular context for proliferation (Davey et al., 2011). In shrimp, it has been documented that cellular signaling pathways, such as NF-κB and JAK-STAT, were annexed by WSSV for its replication and gene expression (Huang et al., 2010; Liu et al., 2007). Moreover, previous evidences in our group have established that *Litopenaeus vannamei* c-Jun N-terminal kinase (LvJNK), one well characterized subfamily of mitogen-activated protein kinases (MAPKs) (Johnson and Lapadat, 2002), was triggered at the early phase of WSSV infection to achieve the same effects (Shi et al., 2012). However, the mechanisms by which LvJNK mediates WSSV infection remain unknown.

c-Jun, a major downstream target of JNK, is a member of the Jun family containing c-Jun, JunB and JunD. It is also a central component of activator protein-1 (AP-1) complex which is a dimeric transcription factor composed of Jun, Fos and activating transcription factor (ATF) family members in mammals (Karin et al., 1997). Normally, the low expression of c-Jun in the quiescent cells can be rapidly induced under various extracellular stimulators (Wagner, 2001). Upon stimulation in human, c-Jun can be specifically phosphorylated and activated by the upstream protein kinase JNK at Ser63 and Ser73 residues, resulting in remarkable increase of its transactivation activity (Dunn et al., 2002). Then the activated c-Jun either homodimerizes or heterodimerizes with other components of AP-1 complex to regulate target genes transcription through binding to the phorbol 12-*O*-tetradecanoylphorbol-13-acetate (TPA) responsive element (TRE; TGACTCA) or the cAMP responsive element (CRE; TGACGTCA) (Karin et al., 1997). Since its identification in 1987 (Maki et al., 1987), c-Jun has been demonstrated to participate in the control of numerous cell processes, including proliferation, apoptosis, survival, tumorigenesis and immune response (Foletta et al., 1998; Shaulian and Karin, 2001; van Dam and Castellazzi, 2001).

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In addition, increasing numbers of literature also reported the observations of c-Jun activation in various viral invasion such as hepatitis B virus (Benn et al., 1996), human immunodeficiency virus type 1 (Kumar et al., 1998), herpes simplex virus type 1 (Zachos et al., 1999) and reovirus (Clarke et al., 2001), suggesting its importance for viral infections.

To further investigate the potential downstream targets that mediate the possible biological effects of LvJNK activation during WSSV infection, we isolated a c-Jun homolog (designated as Lvc-Jun) from *L. vannamei* for the first time and analyzed its transcription and expression levels after WSSV challenge. Our results demonstrated that Lvc-Jun was activated after WSSV infection. Furthermore, induction of c-Jun with TPA could obviously enhance the transcription of viral gene. These results revealed that Lvc-Jun was involved in WSSV infection and might positively regulate WSSV replication.

2. Materials and methods

2.1. Reagents and antibodies

JNK inhibitor (SP600125) was purchased from Calbiochem and dissolved in dimethyl sulfoxide (DMSO) at 10 mM stock solution. TPA was bought from Cell Signaling Technology and dissolved in DMSO to provide a 1.6 mM stock solution.

Rabbit monoclonal phospho-c-Jun (Ser73) antibody (Cell Signaling Technology) and rabbit polyclonal c-Jun antibody (ExCell Biology) were used to identify the levels of expression and phosphorylation of c-Jun in shrimp, respectively. Antibody against wsv069 was prepared in our lab using purified recombinant protein to immunize rabbit. Mouse and rabbit polyclonal antibodies against V5 epitope tag (anti-V5) were obtained from Invitrogen and Millipore, respectively. Mouse polyclonal antibodies against β-tubulin and FLAG epitope tag (anti-FLAG) were purchased from Sigma. Horseradish peroxidase (HRP)-linked goat anti-mouse and anti-rabbit secondary antibodies were bought from Thermo Scientific. Rhodamine-labeled goat anti-mouse secondary antibody was acquired from KPL.

2.2. Total RNA isolation and cDNA synthesis

Total RNA was isolated using TRIzol reagent (Molecular Research Center) according to the manufacturer's instruction. After digestion with RNase-free recombinant DNaseI (Takara), an aliquot of 1–2 μg total RNA mixed with oligo(dT)$_{18}$ primer (Fermentas) was denatured by heating at 70 °C for 10 min. Then the first-strand cDNA was synthesized by reverse transcriptase M-MLV (Takara) according to the instruction. The obtained cDNA was employed for PCR analysis.

2.3. cDNA isolation and sequence analysis of Lvc-Jun

The cDNA sequence of Lvc-Jun was originated from partial sequences encoding c-Jun in *Marsupenaeus japonicus* obtained from hepatopancreas and hemocytes mixed cDNA library constructed in our lab. Firstly, the 5′-region of *M. japonicus* c-Jun transcript was acquired by rapid amplification of cDNA ends (RACE) using a SMARTer™ RACE cDNA amplification kit (Clontech) with the 5′-race-c-Jun-GSP primer (Table 1). Then the full-length cDNA of Lvc-Jun was cloned based on the *M. japonicus* c-Jun sequence with specific primers c-Jun-F and c-Jun-R (Table 1). The RACE was performed using the following program: one cycle at 94 °C for 3 min, 30 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min and 72 °C for 3 min for a final extension. The cDNA sequence was deposited in the NCBI database.

The BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST/) were used to analyze the Lvc-Jun nucleotide sequences and search similar protein sequences from other species. Characteristic domains were predicted through Pfam program (http://pfam.xfam.org/). Multiple protein sequence alignment was performed using the DNA Software (Lynnon BioSoft). A neighbor-joining (NJ) phylogenic tree was constructed using Mega5.1 beta software (http://www.megasoftware.net/) based on amino acid sequences of c-Jun and bootstrap sampling was reiterated for 1000 times.

Table 1
Primers used in this study.

Name	Sequence (5′-3′)
For Lvc-Jun isolation	
5′-race -c-Jun-GSP	TGTTCCAAGGTATCCTCAAATCC
c-Jun-F	GTGTGAGTCCGTCCGCTAGTG
c-Jun-R	TCGGTCTGGCTGGCTGTG
For semi-quantitative RT-PCR	
Lvc-Jun-F	GCCCTCCCAGTTCTTCTTCC
Lvc-Jun-R	CACCTTCTCCTCCAGACGGC
Lvactin-F	GACGGTCAGGTGATCACCAT
Lvactin-R	CGATTGATGGTCCAGACTCG
Cfactin-F	GACATGGAGAAGATCTGG
Cfactin-R	GGGAAGCTCGTAGGACTT
For real-time quantitative PCR[a]	
Lvc-Jun-q-F	CCATCCAGAACAGCAACACGG
Lvc-Jun-q-R	GTTCCAAAGTGTCCTCAAATCC
Lvtubulin-q-F	GCCTCGTGCCATCCTTGTTG
Lvtubulin-q-R	CCCTTAGCCCAGTTGTTTCCAG
wsv069-q-F	GCACAACAACAGACCCTACCC
wsv069-q-R	GAAATACGACATAGCACCTCCAC
For plasmid construction[b]	
V5-Lvc-Jun-F	CGG**GGTACC**ATGGAGGCAACCATGTAC
V5-Lvc-Jun-R	CCG**CTCGAG**CGCTGGTGCGTTACGAAG
FlAG-Lvc-Jun-F	CGG**GGTACC**ATGGACTACAAGGACGA CGATGACAAGATGGAGGCAACCATGTA
FLAG-Lvc-Jun-R	CCG**CTCGAG**CGTCACTGGTGCGTTACGAAG

[a] Primers wsv069-q-F/R were also used in semi-quantitative RT-PCR for detecting wsv069 expression.
[b] Restriction sites for cloning were highlighted in bold and the nucleotide sequences of FLAG tag were underlined.

2.4. Tissue distribution of Lvc-Jun

Six tissues, including hemocyte, gill, intestine, hepatopancreas, muscle and heart, were sampled from *L. vannamei* for RNA isolation and cDNA synthesis. The transcriptional levels of Lvc-Jun were measured by semi-quantitative RT-PCR using primers Lvc-Jun-F and Lvc-Jun-R (Table 1). PCR reactions were performed as follows: one cycle at 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and 72 °C for 5 min for a final extension. *Lvactin* was used to normalize PCR reactions.

2.5. Shrimps and WSSV challenge

L. vannamei (about 10–15 g), free of WSSV, were purchased from a supermarket in Xiamen, China, and stored at 25 °C in 500 L tanks filled with air-pumped sea water. WSSV stock used in this study was isolated and prepared from viral infected crayfish and measured as described previously (Xie et al., 2005). In the challenge experiment, each shrimp was intramuscularly injected with 100 μL virions diluted in PBS (approximately 1×10^6 virions) at the third abdominal segment. Shrimps injected with PBS were used as negative controls.

2.6. Real-time quantitative PCR

Real-time quantitative PCR was applied to measure the transcriptional levels of Lvc-Jun after WSSV infection. At 0, 6, 12, 24, 48 h post injection with WSSV, hemocytes of four individuals were randomly collected by using a 2 mL sterile syringe preloaded with 0.5 mL shrimp Elsevier solution (0.8% sodium citrate, 0.05% citric