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c-Jun N-terminal kinases 3 (JNK3) from orange-spotted grouper, *Epinephelus coioides*, inhibiting the replication of Singapore grouper iridovirus (SGIV) and SGIV-induced apoptosis

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ABSTRACT

C-Jun N-terminal kinases (JNKs), a subgroup of serine-threonine protein kinases that activated by phosphorylation, are involve in physiological and pathophysiological processes. JNK3 is one of JNK proteins involved in JNK3 signaling transduction. In the present study, two JNK3 isoforms, Ec-JNK3 X1 and Ec-JNK3 X2, were cloned from orange-spotted grouper, *Epinephelus coioides*. Both Ec-JNK3 X1 and Ec-JNK3 X2 were mainly expressed in liver, gill, skin, brain and muscle of juvenile grouper. The relative expression of Ec-JNK3 X2 mRNA was much higher in muscle and gill than that of Ec-JNK3 X1. Isoform-specific immune response to challenges was revealed by the expression profiles in *vivo*. Immunofluorescence staining indicated that JNK3 was localized in the cytoplasm of grouper spleen (GS) cells and shown immune response to SGIV infection in *vitro*. Over-expressing Ec-JNK3 X1 and/or Ec-JNK3 X2 inhibited the SGIV infection and replication and the SGIV-induced apoptosis. To achieve the antiviral and anti-apoptosis activities, JNK3 promoted the activation of genes ISRE and type I IFN in the antiviral IFN signaling pathway, and inhibited the activation of transcription factors NF-κB and p53 relating to apoptosis, respectively. Ec-JNK3 X2 showed stronger activities in antivirus and anti-apoptosis than that of Ec-JNK3 X1. Our results not only define the characterization of JNK3 but also reveal new immune functions and the molecular mechanisms of JNK3 on iridoviruses infection and the virus-induced apoptosis.

1. Introduction

Iridoviruses are a group of the nucleo cytoplasmic large DNA viruses (NCLDV) that infect a broad range of hosts (Piaskoski and Plumb, 1999; Hyatt et al., 2000; Williams, 2008). Iridoviruses have caused huge economic losses (Whittington et al., 2010; Lesbarreres et al., 2012) and shown great threat to global biodiversity (Chinchar et al., 2011; Rothermel et al., 2013). Singapore grouper iridovirus (SGIV), a novel member of Iridoviruses, often induces a highly lethal and serious systemic disease (Qin et al., 2001; Gibson-Kueh et al., 2003). The molecular mechanism of SGIV pathogensis has been investigated in viral life cycle and assembly, including genomics (Song et al., 2004), transcriptomics (Teng et al., 2008), proteomics (Song et al., 2006), lipids (Wu et al., 2010) and behavior (Wang et al., 2014). Virus specifically modulates apoptosis for efficient evasion and viral propagation (Clarke and Tyler, 2009; Sun et al., 2015). Multiple factors and/or cellular signaling pathways of host are involved in SGIV infection (Huang et al., 2011a, 2011b). The c-Jun NH2-terminal kinase 1 (JNK1) and JNK2 molecules in JNK signaling pathway have been involved in the evasion and replication of SGIV and/or the SGIV-induced apoptosis (Guo et al., 2016a, b).

Three genes, JNK1, JNK2, and JNK3 are contained in the JNK subfamily involving in multiplicity of physiological and

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pathophysiological processes to extracellular stimuli (Ip and Davis, 1998; Davis, 2000; Raman et al., 2007). JNK1 and/or JNK2 has shown multiple roles in virus infection and replication (Lee et al., 2011; Stahl et al., 2012; Rau et al., 2013) and host signal transduction (Arbour et al., 2002; Holloway and Coulson, 2006; Li et al., 2009). JNK1 has been modulated by SGIV to inactivate antiviral signaling, enhance SGIV-induced apoptosis, and activate transcription factors for efficient infection and replication (Guo et al., 2016a). JNK2 has been contributed to the evasion and infection of SGIV (Guo et al., 2016b). JNK3 is gaining interest due to its involvement in the pathology of neurodegenerative diseases (Hunot et al., 2004; Quigley et al., 2011), neuronal apoptosis (Yang et al., 1997, 2012; Kuan et al., 2003), cell migration (Pi et al., 2009) and differentiation (Waetzig and Herdegen, 2003). However, JNK3 is extremely poor for other functions compared with JNK1 and/or JNK2. Little information is known about the immune roles of JNK3 in pathogen infection. The molecular mechanism of JNK3 has not yet been elucidated in virus infection.

Due to the high sequence identity among JNK isoforms, chemical agents of JNK inhibitors are too general to lack selective inhibition of single JNK isoforms (Scapin et al., 2003; Antoniou et al., 2011; Park et al., 2015). Gene-targeting strategies have been used to reveal distinct and overlapping functions of JNK isoforms (Yang et al., 1997; Davis, 2000; Kyriakis and Avruch, 2001). Therefore, ectopic expression of target gene (over-expressing JNK3) was performed to analyze its unique roles in SGIV infection.

In this study, two JNK3 isoforms, Ec-JNK3 X1 and Ec-JNK3 X2, were identified from orange-spotted grouper, *Epinephelus coioides*. The molecular characterizations and tissue distributions were analyzed. The immune responses of both JNK3 isoforms were investigated after injecting challenges in *vivo*. Immunofluorescence staining indicated that JNK3 was localized in the cytoplasm and changed to nucleus of grouper spleen (GS) cells after infecting with SGIV in *vitro*. Over-expressing Ec-JNK3 X1 and Ec-JNK3 X2 in FHM cells showed similar roles in inhibiting the SGIV infection and SGIV-induced apoptosis. Transcriptional activations of pathway-specific reporter genes were regulated by both JNK3 isoforms during SGIV infection. All results emphasized the immune response of JNK3 in *vivo* and revealed the roles and molecular mechanism of JNK3 in virus infection and virus-induced apoptosis in *vitro*.

2. Materials and methods

2.1. Fish, cells and virus

To collect tissue samples for total RNA extraction, juvenile orange-spotted grouper, *E. coioides* (length 6–10 cm, weight 15–30 g) were purchased and treated as described previously (Guo et al., 2016a). Briefly, anesthetized fishes were used for the tissue distribution and challenge experiments. Immune-related tissues, including liver, spleen, kidney, brain, intestine, heart, skin, muscle, gill, stomach and head kidney were collected for distribution analysis. In challenging experiments, tissue samples of spleen were obtained from fishes injected with Lipopolysaccharide (LPS), SGIV and Polyriboinosinic Polyribocytidylic Acid (poly I:C) at time points of 1, 2, 6, 12, 24, 48, 72 and 96 h post-injection. Each group contained five individuals. The group untreated with anything was used as the blank (0 h).

Fish cell lines of GS (Qin et al., 2006) and fathead minnow (FHM) epithelial cells (Gravell and Malsberger, 1965) were cultured with Leibovitz's L15 medium and M199 medium containing 10% fetal bovine serum (Invitrogen, USA) at 25 °C, respectively. Propagation of SGIV (strain A3/12/98 PPD) was performed as described previously (Qin et al., 2001).

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instruction. RQ1 RNase-Free DNase (Promega, USA) was used to remove the contaminated DNA. After detecting quality by electrophoresis on 1% agarose gel, total RNAs were reverse transcribed to cDNA by ReverTra Ace Kit (TOYOBO, Japan). Liver total RNA was used to synthesize the first-strand cDNAs for 3′RACE and 5′RACE with SMART™ RACE cDNA amplification kit (Clontech, USA).

2.3. Cloning of the full-length JNK3 cDNA

Based on JNK3 homologues from fishes *Danio rerio* (NM_001037701), *Carassius auratus* (FJ183484) and *Poecilia formosa* (XM_007558893), primers Ec-JNK3F1 (5′-ATGAGCAAAAG-CAAAGTGGACAACC-3′) and Ec-JNK3R1 (5′-TCACGGCTG-CACCTSCGCTGARGGA-3′) were designed to amplify the partial sequence of JNK3. According to the manufacturer's protocol of SMART™ RACE cDNA amplification kit, first-strand cDNAs of 3′RACE and 5′RACE were used as template to amplify the full-length cDNA of JNK3 with gene-specific primers of 3′EcJNK3F1 (5′-AGCCAGAGACCTGCTGTCTAAGATG-3′) and 3′EcJNK3F2 (5′-AGCCTTACAGCACCCCTACATCA-3′), 5′EcJNK3R1 (5′-CTGAAT-CACCTGGCACAAGTTGGCAT-3′) and 5′EcJNK3R2 (5′-ACAGCATCA-TAGCCCGCACAGACAAT-3′), respectively.

2.4. Sequence analysis and amino acid alignment

Sequence similarities were analyzed using the BLAST algorithm at NCBI web site (www.ncbi.nlm.nih.gov/BLAST). SMART (Simple modular architecture research tool, http://smart.embl-heidelberg.de/) was used to analyze the deduced amino acid sequences of Ec-JNK3 X1 and Ec-JNK3 X2. Based on amino acid sequences, the comparison and phylogenetic analyses were performed with software Clustalx 1.83 (http://www.ebi.ac.uk/clustalW/) and MEGA 4.0 (http://megasoftware.net).

2.5. Real-time quantitative PCR analysis

Real-time quantitative PCR (RT-qPCR) was employed for expression profiles analyses. Two gene-specific primer pairs, RT-EcJNK3F (5′-CCCCGACTGCCTTTTCCCTG-3′) and RT-EcJNK3X1R (5′-GGTGGAGGCATGGATATTTGGA-3′), RT-EcJNK3F and RT-EcJNK3X2R (5′-GATCTGAGGTGGAGGTGCCT-3′), were designed to amplify Ec-JNK3 X1 and Ec-JNK3 X2, respectively. And β-actin gene amplified with primers ActinF (5′-TACGAGCTGCCTGACGGACA-3′) and ActinR (5′- GGCTGTGATCTCCTTTTGCA-3′), was selected to normalize relative gene expression for correspond samples. cDNAs reverse transcribed from total RNA were used as templates. RT-qPCR was carried out on Roche LightCycler® 480 Real-time PCR system (Roche, Switzerland) and obtained data was analyzed as previously described (Guo et al., 2012). Relative gene expression was analyzed by the comparative Ct method ($2^{-\Delta\Delta C_T}$ method) (Schmittgen and Zakrajsek, 2000; Livak and Schmittgen, 2001). Target C_T values were normalized to that of β–actin. Results for each treated sample were represented as N-fold changes relative to the same gene target in the calibrator sample, both normalized to the β–actin.

2.6. Production of recombinant protein and antiserum

Primers pET28a-EcJNK3F (5′-CGGGATCCATGGTATTTATGAGCA-GACA-3′) and pET28a-EcJNK3R (5′-CCCAAGCTTGTCACGGCTG-CACCTGTGCT-3′) were used to amplify the open reading frame (ORF) of Ec-JNK3 X1 and Ec-JNK3 X2. The target PCR product was