Molecular clone and characterization of c-Jun N-terminal kinases 2 from orange-spotted grouper, Epinephelus coioides

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Molecular clone and characterization of c-Jun N-terminal kinases 2 from orange-spotted grouper, *Epinephelus coioides*

Minglan Guo a, b, Jingguang Wei a, b, Yongcan Zhou c, Qiwei Qin a, b, *

Keywords: c-Jun N-terminal kinases 2, Epinephelus coioides, Molecular clone, Singapore grouper iridovirus (SGIV)

**Abstract**

c-Jun N-terminal kinase 2 (JNK2) is a multifunctional mitogen-activated protein kinases involving in cell differentiation and proliferation, apoptosis, immune response and inflammatory conditions. In this study, we reported a new JNK2 (Ec-JNK2) derived from orange-spotted grouper, *Epinephelus coioides*. The full-length cDNA of Ec-JNK2 was 1920 bp in size, containing a 174 bp 5'-untranslated region (UTR), 483 bp 3'-UTR, and a 1263 bp open reading frame (ORF), which encoded a putative protein of 420 amino acids. The deduced protein sequence of Ec-JNK2 contained a conserved Thr-Pro-Tyr (TPY) motif in the domain of serine/threonine protein kinase (S-TKc). Ec-JNK2 has been found to involve in the immune response to pathogen challenges in vivo, and the infection of Singapore grouper iridovirus (SGIV) in vitro. Immunofluorescence staining showed that Ec-JNK2 was localized in the cytoplasm of grouper spleen (GS) cells, and moved to the nucleus after infecting with SGIV. Ec-JNK2 distributed in all immune-related tissues examined. After challenging with lipopolysaccharide (LPS), SGIV and polyriboinosinic polyribocytidylic acid (poly I:C), the mRNA expression of Ec-JNK2 was significantly up-regulated in juvenile orange-spotted grouper. Over-expressing Ec-JNK2 in fathead minnow (FHM) cells increased the SGIV infection and replication, while over-expressing the dominant-negative Ec-JNK2Δ181–183 mutant decreased it. These results indicated that Ec-JNK2 could be an important molecule in the successful infection and evasion of SGIV.

**1. Introduction**

The c-Jun NH2-terminal kinase (JNK) belongs to the family of mitogen-activated protein kinases (MAPK), together with the p38 and extracellular regulated kinases (ERK). Within the JNK subfamily, three genes have been identified, JNK1, JNK2, and JNK3, which can altogether give rise to 10 splice isoforms [1–3]. Whereas JNK1 and JNK2 are expressed ubiquitously, JNK3 is primarily expressed in the heart, brain, and testis [1,2]. It seems that the different JNK isoforms may have evolved for specific biological functions, probably depending on the activating stimuli and responding cell type [1,4]. JNK2, for example, is involved in a multiplicity of physiological and pathophysiological processes like cell proliferation [4,5] and differentiation [6–8], immune response [9–11], inflammatory conditions [2,12], and apoptosis [13–15]. Recently, JNK2 was found to play roles in *Toxoplasma gondii*-induced immunopathology and promote susceptibility to this parasitic pathogen [16], and activation of the innate response to viral infection [11]. Although several studies have reported the resistant pathology and immune response of JNK2 to pathogen infection, the function of JNK2 in pathogen infection remains elusive.

Groupers (*Epinephelus* spp.) are favorite marine fishes widely cultured in Southeast Asian countries. However, with rapid development of the farming activities, outbreaks of viral disease Singapore grouper iridovirus (SGIV) have caused heavy economic losses in grouper aquaculture [17,18]. Viral infection induces innate immunity, which represent effective means for the host to restrict the spread of pathogens. The role of host signal transduction in immunity to infection is an area of intense interest. Our previous work found that the JNK signaling pathway has been involved in...
SGIV infection [19]. JNKs have both overlapping and distinct functions in different cell types, although the mechanisms that dictate isoform specificity are not well understood [6,20,21]. Individual JNK isoforms may serve different signaling functions [4,22]. Because JNK inhibitors are widely pursued for the treatment of metabolic as well as other inflammatory diseases, consideration of isoform-specific interventions should take into account the compensatory changes in JNK activity that may be caused by differential inhibition of JNK isoforms [12]. The chemical and genetic approaches have been successfully employed in studies of JNK signal pathway members in mammals [23–25]. Therefore, ectopic expression of dominant-negative and dominant-active JNK2 was used to analyze its roles involving in pathogen infection.

The difficulty of clearing SGIV infection makes host entry a key target for disease control. However, how SGIV first infect host remains unclear. Epithelial cells not only serve as the primary targets for many viral pathogens [26–28], but also are the site and source of a wide range of mediators that drive subsequent immune and physiological responses to virus [29–31]. Viral infection of epithelia can damage or disrupt the epithelial barrier that protects underlying tissues. The fishes infected with SGIV have enlarged spleen with hemorrhage and multifocal areas of splenic degeneration [17,32]. And our previous study showed that SGIV induced typical apoptosis in fish epithelial fathead minnow (FHM) cell [33]. Further analyses of host and/or SGIV genes [34,35] based on cell FHM have contributed to understanding the mechanisms of iridovirus pathogenesis. Therefore, the available cell FHM was used to study the function of JNK2 on SGIV infection in vitro.

In this study, a new JNK2 molecule (Ec-JNK2) from orange-spotted grouper, Epinephelus coioides, was cloned and characterized. The expression profiles of Ec-JNK2 were investigated in different immune-related tissues under normal conditions and in spleen after challenging with viral and bacterial pathogens in juvenile grouper. The recombinant protein and antiserum of Ec-JNK2 were obtained. The intercellular localization of Ec-JNK2 was detected in grouper spleen (GS) cells using immunofluorescence staining. In addition, role of Ec-JNK2 in SGIV infection was analyzed by over-expressing Ec-JNK2 and the dominant-negative Ec-JNK2Δ181–183 mutant in FHM cell.

2. Materials and methods

2.1. Fish, cells and virus

Juvenile orange-spotted grouper (length 6–10 cm, weight 15–30 g) were purchased from a marine-culture farm at Honghai bay, Shanwei City, Guangdong Province, China. Fishes were treated as described previously [36]. A series of tissues, including liver, spleen, kidney, brain, intestine, heart, skin, muscle, gill, stomach and head kidney, were collected for analysis of mRNA expression. For challenges assays in vivo, tissue samples of spleen were obtained at indicated hours (0 h, 1 h, 2 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h) post injection with SGIV (5 × 10⁷ TCID₅₀/ml, tissue culture infective dose), lipopolysaccharide (LPS, 15 mg/kg i.v., Sigma–Aldrich, #L2880) and polyribosininosinic polyribocytidylic acid (poly I:C, 10 mg/kg i.v., Sigma–Aldrich, #P9582), respectively. All samples were immediately frozen by liquid nitrogen and stored at −80 °C until used for total RNA extraction.

Fish cell lines of grouper spleen (GS) [37] and fathead minnow (FHM) epithelial cells [38] were grown in Leibovitz’s L15 medium and M199 medium containing 10% fetal bovine serum (Invitrogen, USA) at 25 °C, respectively. Virus strain A3/12/98 PDG of SGIV was used and propagated as described in the literature [39].

2.2. Extraction of total RNA and synthesis of cDNA

Total RNA of collected tissues was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. The quality of total RNA was assessed by electrophoresis on 1% agarose gel. Total RNA was reverse transcribed to cDNA by ReverTra Ace Kit (TOYOBO, Japan). First-strand cDNAs of 3’RACE and 5’RACE were obtained from total liver RNA with SMART™ RACE cDNA amplification kit (Clontech, USA).

2.3. Cloning and sequencing JNK2 from E. coioides (Ec-JNK2)

Based on the partial sequence amplified from liver cDNA using primers Ec-JNK2F and Ec-JNK2R (Table 1), full-length cDNA of Ec-JNK2 was obtained from first-strand cDNAs for 3’RACE and 5’RACE using primers 3’EcJNK2F1 and 3’EcJNK2F2, 5’EcJNK2R1 and 5’EcJNK2R2 (Table 1) according to the manufacturer’s protocol of SMART™ RACE cDNA amplification kit. The nucleotide and predicted amino acid sequences of Ec-JNK2 were analyzed using BioEdit and Exapy search program (http://au.exasy.org/tools/). The domain structure was predicted using SMART program (http://smart.embl-heidelberg.de/). The prediction of transmembrane helices in protein was using programs TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The similarity of Ec-JNK2 with other JNK2s was analyzed using the BLAST search program at the NCBI (http://www.ncbi.nlm.nih.gov/). Multiple-sequence alignment of the reported JNK2 amino acid sequences was performed using Clustalx 1.83 (http://www.ebi.ac.uk/clustalw/), and a phylogenetic tree was constructed using the MEGA 4.0 software (http://megasoftware.net/).

2.4. Expression profiles of Ec-JNK2 in vivo

Expression profiles of Ec-JNK2 in tissue distribution and challenge assays were determined by real-time quantitative PCR (RT-qPCR) using primers RT-EcJNK2F and RT-EcJNK2R (Table 1). β-actin was used as internal control and amplified with primers ActinF and ActinR (Table 1). RT-qPCR was performed as previously described [36]. Relative gene expression was determined by the comparative Ct method (2ΔΔCt method) [40,41]. Target Ct values were normalized to the endogenous gene β-actin. Results for each treated sample were expressed as N-fold changes in target gene expression relative to the same gene target in the calibrator sample, both normalized to the β-actin gene.

Table 1

<table>
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<th>Sequences of primers used in this study.</th>
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<tr>
<td>Primers</td>
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<td>Ec-JNK2F</td>
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<td>Ec-JNK2R</td>
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2.5. Recombinant protein expression and antisem preparation

The open reading frame (ORF) region of Ec-JNK2 was amplified from grouper liver cDNA with primers pET28a-EcJNK2F and pET28a-EcJNK2R (Table 1). It was cloned in vector pET-28a-c (+) (Novagen, Germany). The recombinant plasmid pET28a-EcJNK2 was transformed into Escherichia coli BL21 (DE3). The protein of Ec-JNK2 was expressed and purified as methods described previously [38]. The recombinant and purified protein of Ec-JNK2 was detected with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS—PAGE). Mouse anti-Ec-JNK2 serum was produced by immunizing BALB/c mice according to the conventional method [42] and collected for further study. The specificity of obtained Ec-JNK2 antibody was detected with purified proteins of Ec-JNK2 and GS cell lystate using western blot. Pre-immune mice serum was used as the negative control. In brief, the purified proteins and/or GS cell lystate were separated using 12% SDS—PAGE and transferred to a PVDF membrane (Hybond-p; Amersham Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer’s instructions. The membrane was blocked with 5% nonfat milk in TBST (1 × Tris-buffer saline (TBS), 0.1% Tween-20) for 1 h and washed in TBST for three times. The mouse anti-Ec-JNK2 serum at a dilution of 1:2000 was used as the primary antibody, and HRP-conjugated goat anti-mouse antibody (Pierce, USA) was used as the secondary antibody at a dilution of 1:2000.

2.6. Immunofluorescence staining

The intercellular localization of Ec-JNK2 was detected with mouse anti-EcJNK2 serum using immunofluorescence staining. At indicated time points of 0, 2, and 24 h post-infection (p.i.), GS cells (2 × 10⁵) infected with SGIV (0.5 MOI) were fixed with paraformaldehyde at 4 °C for 2 h. The fixed cells were permeabilized for 15 min and blocked with 2% bovine serum albumin (BSA) for 30 min. Ec-JNK2 in cells was fluorescently labeled using mouse anti-Ec-JNK2 serum (1:100) for 2 h and fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse IgG antibody (Pierce, USA) for 1 h. Nuclei or viral DNA were stained using 4’, 6-diamidino-2-phenylindole (DAPI, 1 μg/mL) (Sigma, USA) and followed by fluorescence microscopic observation and analysis.

2.7. Over-expression of Ec-JNK2 and dominant-negative Ec-JNK2Δ181–183 mutant

ORF region of Ec-JNK2 was cloned into vector pcDNA3.1 (+) (Invitrogen, USA) with primers pcDNA-EcJNK2F and pcDNA-EcJNK2R (Table 1). The plasmid pcDNA-Ec-JNK2 was used as template to construct the TPy mutant of Ec-JNK2 (Ec-JNK2Δ181–183). Briefly, overlap-PCR method [43] was performed to obtain the site-directed mutagenesis. Primers pcDNA-EcJNK2F and EcJNK2mutR, EcJNK2mutF and pcDNA-EcJNK2R (Table 1) were used to replace threonine 181 and tyrosine 183 with alanine and phenylalanine, respectively. The dominant-negative Ec-JNK2Δ181–183 mutant was confirmed by sequencing and then cloned into the pcDNA3.1 (+) vector.

Stable transfection was performed in FHM cells for plasmids of pcDNA-Ec-JNK2, pcDNA-Ec-JNK2Δ181–183 and pcDNA3.1 (+) (the control) using methods described previously [38]. Cells expressing plasmids above were isolated in medium containing 400 ng/mL G418 (Sigma–Aldrich, USA) for one month. Protein expression of JNK2 in screening cells was examined by western blot using the mouse anti-Ec-JNK2 serum as the primary antibody. HRP-conjugated goat anti-mouse antibody (Pierce, USA) at a dilution of 1:2000 was served as secondary antibody. The α-tubulin, which was detected with Rabbit anti-tubulin antibody (Cell Signaling), was used as the internal controls. HRP-labeled goat anti-Rabbit antibody (Pierce, USA) at a dilution of 1:1000 was used as the secondary antibody. FHM cells over-expressing Ec-JNK2, dominant-negative Ec-JNK2Δ181–183 mutant (partially inhibits the phosphorylation of Ec-JNK2) and empty vector pcDNA3.1 (+) were established for further analysis.

2.8. Roles of Ec-JNK2 and dominant-negative Ec-JNK2Δ181–183 mutant in SGIV infection

To examine the function of Ec-JNK2 on viral infection, three cells, FHM/pcDNA-Ec-JNK2, FHM/pcDNA-Ec-JNK2 Δ181–183 and FHM/pcDNA3.1 obtained above (5 × 10³), were incubated with PBS (mock) or SGIV (0.5 MOI). At 24 and 48 h p.i., the morphology of mock- or infected cells was observed. Mock- and infected (2, 6, 12, 24 and 48 h p.i.) cells were harvested. Equal amounts of cell lysate extracts were subjected to the SDS-PAGE and examined by western blot as described above. The phosphorylation of Ec-JNK2 was detected with primary antibody p-JNK/SAPK (Cell Signaling). To detect the replication of SGIV, viral protein productions of ORF 136 (early stage gene) and ORF 072 (late stage gene) were detected using primary antibody mouse anti-SGIV ORF136 and ORF072 serum at a 1:2000 dilution. The internal controls α-tubulin was detected using method above. Meanwhile, the cell viability was analyzed for mock- or infected cells using the trypan blue exclusion test. In brief, cells were collected by trypsinization, and incubated in 0.2% trypan solution (Sigma) for 5 min. Three independent hemocytometer counts were performed to determine the dead cells of each sample. The cell viability was calculated as the percentage of live cells over total number of cells. In parallel, supernatants and infected cell lysates were collected to test the viral titer 50% tissue culture infectious dose (TCID₅₀) [44].

2.9. Statistical analyses

All assays were repeated in triplicate. Data were presented as means ± standard deviation (S.D.). Software SPSS 16.0 was used for statistical analyses. Student’s t-test and One-Way ANOVA with post hoc tests were performed for data analysis. The values of P < 0.05 and P < 0.01 were denoted as statistically significant and highly significant.

3. Results

3.1. Sequence and phylogenetic analysis of Ec-JNK2

The obtained full-length cDNA of Ec-JNK2 (GenBank accession no. KR869885) contained a 174 bp 5’ terminal untranslated region (UTR), 483 bp 3’UTR, and a 1263 bp open reading frame (ORF), which encoded a putative protein of 420 amino acids residues with predicted molecular mass of 47.7 kDa. No signal peptide and transmembrane helices were found in the deduced amino acid of Ec-JNK2. A conserved dual phosphorylation motif Thr-Pro-Tyr (TPY) (at the positions of 181–183) was found in the predicted domain of serine/threonine protein kinase (S_TKc) (at the position of 24–319) (Fig. 1). Ec-JNK2 shared 95% identity to Stequates partitus. Phylogenetic tree was made, and Ec-JNK2 was clustered into the Osteichthyes branch (Fig. 2). Alignment of the JNK2 amino acid sequences in various species demonstrated that the JNK2 subfamily was conserved in Osteichthyes.

3.2. Expression profiles of Ec-JNK2 in vivo

In juvenile orange-spotted grouper, Ec-JNK2 was distributed in all examined tissues. It was expressed mainly in brain, liver, skin
and heart, but a few in spleen, kidney, intestine, muscle, gill, head kidney and stomach (Fig. 3A). To pathogenic challenges, the mRNA expression of Ec-JNK2 was significantly (P < 0.01) increased and spiked at different post-injection times for different pathogens (LPS at 6 h, SGIV at 24 h, and poly I:C at 2 h) in spleen (Fig. 3B).

3.3. Recombinant protein expression and antibody preparation for Ec-JNK2

The recombinant protein and antibody of Ec-JNK2 was detected by SDS-PAGE and western blot methods, respectively (Fig. 4). The E. coli BL21 (DE3) containing the plasmid pET28a-EcJNK2 expressed a unique protein at about 54 kDa, which contained the His tag and the recombinant Ec-JNK2 (Fig. 4, line 1). The protein was expressed in the supernatant (Fig. 4, line 2). The negative control of pET-28a-c (+) expressed nothing after being induced with IPTG (Fig. 4, line 3). The purified protein of Ec-JNK2 (line 4) was used to produce the polyclonal antibody (mouse anti-EcJNK2 serum) (Fig. 4, line 5). Mouse anti-EcJNK2 serum was positive (Fig. 4, line 5, 7) while pre-immune mice serum was negative (Fig. 4, line 6, 8).

3.4. Immunofluorescence staining

The intracellular localization of Ec-JNK2 was determined by immunofluorescence staining using mouse anti-EcJNK2 serum.
The protein Ec-JNK2 in GS cells were immunofluorescently labeled with FITC (green). Normally, Ec-JNK2 was localized in the cytoplasm of GS cells (mock). The nucleus (red arrows) was negative. After infecting with SGIV, it was moved to the nucleus (2 h p.i., 24 h p.i.). The nucleus was then changed to be positive. At the late stage (24 h p.i.) of SGIV infection, the inclusion body (viral factories, white arrows) was appeared. The detection of Ec-JNK2 was positive in nucleus but negative in inclusion body.

Fig. 2. Phylogenetic analysis of Ec-JNK2 with other reported JNK2 in vertebrates. The phylogenetic tree of the alignment amino acid sequences was constructed by the neighbor-joining method within MEGA 4.0. Numbers at branch nodes represent the bootstrap majority consensus values of 1000 replicates. NCBI RefSeq or GenBank accession number was listed on the right of the species name.

Fig. 3. Expression profiles of Ec-JNK2 in vivo. (A) Tissue distribution of Ec-JNK2 in juvenile orange-spotted grouper. (B) Immune responses of Ec-JNK2 in spleen after challenging with LPS, SGIV and poly I:C. All data were expressed as mean ± S.D. (n = 5). Statistic differences were indicated as *, p < 0.05; **, p < 0.01.

Fig. 4. Protein expression and antiserum preparation for Ec-JNK2. M: Protein marker, kDa; 1: pET-28a-EcJNK2 in BL21 (DE3), IPTG induced for 12 h; 2: Supernatant of cell lysates from BL21 (DE3) with pET-28a-EcJNK2 which was induced for 12 h; 3: pET-28a-c (+) expressed product; 4: Purified proteins of Ec-JNK2 from the supernatant; 5, 6: Purified protein of Ec-JNK2 detected with mouse anti-EcJNK2 serum and pre-immune mice serum, respectively; 7, 8: Cell lysates of GS detected with mouse anti-EcJNK2 serum and pre-immune mice serum, respectively. Mouse anti-EcJNK2 serum (line 5 and 7) and pre-immune mice serum (line 6 and 8) was used to verify the specificity of obtained antibody of Ec-JNK2.

Fig. 5. The protein Ec-JNK2 in GS cells were immunofluorescently labeled with FITC (green). Normally, Ec-JNK2 was localized in the cytoplasm of GS cells (mock). The nucleus (red arrows) was negative. After infecting with SGIV, it was moved to the nucleus (2 h p.i., 24 h p.i.). The nucleus was then changed to be positive. At the late stage (24 h p.i.) of SGIV infection, the inclusion body (viral factories, white arrows) was appeared. The detection of Ec-JNK2 was positive in nucleus but negative in inclusion body.
3.5. Role of Ec-JNK2 and dominant-negative Ec-JNK2Δ181–183 mutant on SGIV infection and replication in vitro

To examine the role of Ec-JNK2 on virus infection in vitro, three FHM cells were screened to stably expressing pcDNA-Ec-JNK2, pcDNA-Ec-JNK2Δ181–183 and pcDNA3.1 vector (control). Ec-JNK2 and Ec-JNK2Δ181–183 mutant were stably expressed in FHM cells by detecting with the mouse anti-Ec-JNK2 serum (Fig. 6A). The dominant-negative Ec-JNK2Δ181–183 mutant inhibited the cytopathic effect (CPE) induced by SGIV infection, while Ec-JNK2 promoted it. FHM/pcDNA-Ec-JNK2 showed more CPE than that of FHM/pcDNA3.1, while FHM/pcDNA-Ec-JNK2Δ181–183 was less than both above (Fig. 6B). Ec-JNK2 increased the phosphorylation of JNK2 and promoted the infection and replication of SGIV, while the dominant-negative Ec-JNK2Δ181–183 mutant showed opposite effects. Over-expression Ec-JNK2 promoted the phosphorylation of JNK2 at the early stage (2 h p.i.), and increased the infection and replication of SGIV. Ec-JNK2Δ181–183 mutant, partially inhibiting the phosphorylation of JNK2, acted as a dominant-negative inhibitor of endogenous JNK2 at the early stage of SGIV infection, inhibited the infection and replication of SGIV (Fig. 6C). The infection and replication of SGIV was indicated by proteins produced at the early stage (ORF 136) and at the late stage (ORF 072), respectively. The protein accumulation of SGIV ORF136 and ORF072 (major capsid protein, MCP) were significantly increased in FHM/pcDNA-Ec-JNK2, but decreased in FHM/pcDNA-Ec-JNK2Δ181–183 (Fig. 6C).

Ec-JNK2 increased the infectivity of SGIV, while dominant-negative Ec-JNK2Δ181–183 mutant inhibited it. Dominant-negative Ec-JNK2Δ181–183 mutant significantly increased the cell viability (69.40% at 24 h p.i. and 44.60% at 48 h p.i.) compared to that of the control (60.75% at 24 h p.i. and 30.81% at 48 h p.i.), while Ec-JNK2 (51.29% at 24 h p.i. and 22.35% at 48 h p.i.) highly decreased it after infecting with SGIV (Fig. 6D). At 24 and 48 h p.i., the virus titers TCID50 of Ec-JNK2 cell lysates were 3.99 and 4.52 times higher than that of the dominant-negative Ec-JNK2Δ181–183 mutant, which were 1.72 and 2.37 times lower than that of the control. At 24 h p.i., the viral infectivity of Ec-JNK2 cell lysates was significantly stronger than that of the control (p < 0.05), and highly significantly stronger than that of Ec-JNK2Δ181–183 mutant (p < 0.01). The differences of viral infectivity were highly significant (p < 0.01) at 48 h p.i (Fig. 6E).

4. Discussion

In this study, a new c-Jun N-terminal kinase 2 (Ec-JNK2) was cloned from orange-spotted grouper, E. coioides. The deduced Ec-JNK2 protein showed high similarity of the conserved S-TKc domain with that of other vertebrates. In mammals, JNK2 was activated and involved in the innate immunity[2,11,12]. We found that Ec-JNK2 mainly expressed in brain, liver, skin and heart, and pathogens significantly (p < 0.05) induced the expression of Ec-JNK2 in immune-related tissue spleen in vivo. The mRNA expression profiles of Ec-JNK2 dependent on the pathogens. Activation of JNK was time course to SGIV infection[19]. The immune responses of individual and/or cell mediated by Ec-JNK2 to SGIV infection seems to be related with infecting time. The mRNA level of Ec-JNK2 was spiked at 24 h p.i. to the SGIV infection in spleen. However, the phosphorylation of Ec-JNK2 was activation at the early stage of SGIV infection (2 h p.i.) in vitro.
Fig. 6. Roles of Ec-JNK2 and dominant-negative Ec-JNK2Δ181–183 mutant in SGIV infection in vitro. (A) The detection of FHM cells stably over-expressing Ec-JNK2, the control (pcDNA3.1) and dominant-negative Ec-JNK2Δ181–183 mutant. a, FHM/pcDNA-Ec-JNK2; b, FHM/pcDNA3.1; c, FHM/pcDNA-Ec-JNK2Δ181–183. (B) Microscopy observation of CPE induced by SGIV infection in screened FHM cells. (C) Protein accumulation of the host endogenous JNK phosphorylation and the viral protein ORF136 and ORF072. M, Protein Marker. a, FHM/pcDNA-Ec-JNK2; b, FHM/pcDNA3.1; c, FHM/pcDNA-Ec-JNK2Δ181–183. (D) Cell viability and (E) virus titers (viral infectivity) of three screened FHM cells after infecting with SGIV. (*p < 0.05, **p < 0.01).
Very litter is known about the function of JNK2 in innate and acquired immunity, and a few studies have addressed the roles of this molecule during the viral infection [11,45]. Recent observation pointed out, that it is the cellular context, localization and the composition of JNK molecules that determines JNK functions [46–48]. Here we observed that Ec-JNK2 distributed normally in cytoplasm, and moved to nucleus after infection with SGIV. The intercellular localization of Ec-JNK2 indicated that Ec-JNK2 involved in the immune response to SGIV infection in vitro. Phosphorylation and activation status of JNKs has crucial impacts on infection-induced immune response [49–51]. Our previous study showed that the phosphorylation of JNK was observed in nuclei at 2 h p.i., phosphorylated JNK could be detected from 1 to 6 h p.i. but not at the late stage of SGIV infection [19]. Therefore, the phosphorylation of Ec-JNK2 seems to play key roles during the SGIV infection.

The activation by dual phosphorylation of Thr and Tyr residues within a Thr-Pro-Tyr motif located in kinase subdomain VIII may contribute to cellular response to stress and many physiological processes [2,52,53]. Interactions between isoforms can determine compensatory total activity in isolated JNK-deficiency models [12]. Therefore, over-expression of Ec-JNK2 and dominant-negative Ec-JNK2 mutant, which partially inhibited the phosphorylation of TPY motif, were employed to analyze the function of Ec-JNK2 on the SGIV infection. Different to the roles of activating the innate response to viral infection [11], Ec-JNK2 was found to increase the viral infectivity. Over-expression of Ec-JNK2 and dominant-negative Ec-JNK2 mutant demonstrated that Ec-JNK2 could promote the viral infection and replication in vitro. Activation of JNK by virus infection plays crucial roles in virus replication and viral progeny release [50,54,55]. The over-expression of Ec-JNK2 promoted the phosphorylation of JNK, while the dominant-negative Ec-JNK2 mutant inhibited it. Therefore, the function of Ec-JNK2 could depend on its phosphorylation.

The principle of dominant-negative mutant method is a process of competition to achieve the purpose of partial inhibition [56–58]. Dominant-negative mutant inhibits the gene activity through multiple mechanisms and with different potencies [56,57]. Although the phosphorylation of JNK was decreased slightly, the accumulation of SGIV proteins was significant by western blot. In addition to the function in increasing the viral infectivity, other proteins and/or mechanisms mediated by Ec-JNK2 may be activated to promote the SGIV infection. Viruses can exploit various intracellular signaling pathways to induce cellular and/or viral gene expression for completion of their life cycle [55,59,60]. Therefore, further investigation needs to be done to reveal the multiple mechanisms mediated by Ec-JNK2 that contributes to the successful infection and evasion of SGIV.

In conclusion, a new c-Jun N-terminal kinase 2 (Ec-JNK2) was derived from grouper, *E. coioides*. Ec-JNK2 mRNA was expressed ubiquitously, and the expression of Ec-JNK2 was up-regulated by bacterial and viral challenges in vivo. The recombinant Ec-JNK2 protein and its antisemur were successfully obtained. The intercellular localization of Ec-JNK2 indicated that Ec-JNK2 involved in the immune response to SGIV infection in vitro. Ec-JNK2 increased the infection and replication of SGIV, while the dominant-negative Ec-JNK2A181–183 mutant decreased the viral infectivity in FHM cells. All results indicated that Ec-JNK2 involved in the immune response to pathogenic challenges, and could be an important molecule in the successful infection and evasion of SGIV.

Acknowledgments

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