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IKKε-like plays an important role in the innate immune signaling of the Pacific oyster (*Crassostrea gigas*)

Baoyu Huang[a,c,d,e,1], Xueying Tang[a,c,d,e,1], Linlin Zhang[a,c,e], Li Li[a,b,d,e,**], Wei Wang[a,b,d,e], Mingkun Liu[a,d,e], Guofan Zhang[a,c,d,e,*]

[a] Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, Shandong, 266071, China
[b] Laboratory for Marine Fisheries and Aquaculture, Qingdao National Laboratory for Marine Science and Technology, Qingdao, Shandong, 266071, China
[c] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, Shandong, 266071, China
[d] National & Local Joint Engineering Laboratory of Ecological Mariculture, Qingdao, Shandong, 266071, China
[e] Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao, 266071, China

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ABSTRACT

IκB-related kinase ε (IKKε) plays a crucial role in the activation of nuclear factor κB (NF-κB) by phosphorylating inhibitor of NF-κB (IκB) and in the regulation of interferon (IFN) gene expression by phosphorylating IFN regulatory factors (IRFs). In this study, we cloned an *IKKε* homologue cDNA (designated as *CgIKKε-like*) from the Pacific oyster, *Crassostrea gigas*. The full 2896-bp cDNA sequence comprised a 2163-bp open reading frame (ORF) encoding 720 amino acids. *CgIKKε-like* is ubiquitously expressed, and its mRNA levels in hemocytes after poly I:C, *V. alginolyticus*, or OsHV-1 μVar challenge were analyzed by real-time PCR. Compared to that in the control, *CgIKKε-like* mRNA expression levels were significantly increased at 3 h and peaked at 6 h after OsHV-1 μVar challenge; no obvious changes were observed in expression levels until 24 h after either *V. alginolyticus* or poly I:C challenge, reaching a maximum at 24 h ($p < 0.01$) and then rapidly decreasing. CgIKKε-like transfection into human cell lines induced NF-κB and ISRE activation, while transfection with CgIKKε-like deletion mutants abolished NF-κB and ISRE reporter gene activation. Additionally, CgIKKε-like could interact with CgTBK1 and could form homodimers strongly, which may be critical for the immune signaling transduction. Last but not least, we found that CgIKKε-like may increase CgIκBs phosphorylation and could interact with CgIRF8. Together, these results suggest that CgIKKε-like could respond to pathogenic infection, participate in the immune signal transduction and activate NF-κB and ISRE reporter genes. Thus, CgIKKε-like could play an important role in the oyster immune system.

1. Introduction

Innate immunity or non-specific immunity refers to an organism's natural ability to defend against pathogens, and it has a wide range of effects, which are not limited to a specific antigen [1]. Innate immunity is highly complicated, often rapidly induced, and is characterized by the expression of numerous pattern recognition receptors (PRRs) [2]. PRRs are capable of recognizing different pathogen-associated molecular patterns (PAMPs) and activating the immune responses of organisms via different signal pathways [3].

IκB-related kinase ε (IKKε) and TRAF [TNF (Tumor necrosis factor) receptor associated factor]-associated NF-κB kinase (TANK) binding kinase 1 (TBK1) are so-called IKK-related kinases that have a sequence similarity to canonical IκB kinases (IKKs), IKKα and IKKβ. IKKα and IKKβ have been purified and cloned based on their ability to phosphorylate IκB proteins in response to stimulation with tumor necrosis factor α (TNFα) and are present in cells as part of a high molecular weight complex that also possesses a regulatory subunit termed IKKγ or NF-κB essential modulator (NEMO) [4,5]. In contrast, IKK-related kinases activate two transcription factors, IRF3 and IRF7, which are critical for expression of type I IFN genes [6–8].

IKKε has been cloned as a gene that undergoes LPS-induced transcription and was also identified through a database search for proteins with similarity to IKKα and IKKβ [9]. On the other hand, TBK1 was cloned because of its interaction with TANK [10]. Despite the structural similarities of IKKε and TBK1 to IKKα and IKKβ, neither IKKε nor TBK1

* Corresponding author. Institute of Oceanology, Chinese Academy of Sciences, 7th Nanhai Rd., Qingdao, China.
** Corresponding author. Institute of Oceanology, Chinese Academy of Sciences, 7th Nanhai Rd., Qingdao, China.
E-mail addresses: lili@qdio.ac.cn (L. Li), gzhang@qdio.ac.cn (G. Zhang).
[1] These authors contributed equally to this work.

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are part of the classical IKK complex [11]. Nevertheless, both kinases were initially suggested to also control NF-κB activity. When overexpressed in human HEK293 cells, IKKε and TBK1 activate NF-κB and can induce phosphorylation of IκB-α [9]. Studies have firmly established a role for IKKε and TBK1 in activation of the IFN-I response through phosphorylation and activation of IRF3 and IRF7 [7]. As for IKK, the upstream events leading to activation of IKKε and TBK1 are only partially understood. Studies have revealed several distinct modes of virus recognition that lead to IFN-I production: (1) MyD88-independent Toll-like receptor (TLR) signaling pathway (TLR3-and TLR4-mediated) and (2) retinoic acid-inducible gene I (RIG-I)-dependent signaling pathway [12].

Oysters are among the most economically important mollusk species in the world [13], and their production has been greatly affected by pathogenic infections in recent years [14]. Innate immunity is the primary defense mechanism against invading pathogens in invertebrates, where adaptive immune systems have not fully been developed [15]. In the past decade, several key genes involved in TLR- and RLR-signaling pathways have been characterized in oysters [16–21]. In this study, to better understand the molecular mechanisms underlying immunity as well as the origin and evolution of immune systems in invertebrates, we identified a cDNA encoding a serine kinase that is structurally and functionally homologous to the mammalian IKKε in the Pacific oyster *Crassostrea gigas*. Our results suggest that CgIKKε-like could respond to pathogenic infection, participate in the immune signal transduction and activate NF-κB and ISRE reporter genes. These findings might lay the foundation for future studies to better understand the innate immune response mechanism of the oysters in addition to the evolution of the IKK family.

2. Materials and method

2.1. Oysters, pathogenic challenge and preparation of samples

Oysters used in this study were obtained from aquaculture zones of Jiaonan in Qingdao, Shandong province, China. These oysters were cultured under laboratory conditions at 18 ± 1 °C, receiving filtered seawater exchanges and feeding with marine algae (*Spirulina platensis*) once daily. Tissue samples of the mantle, adductor muscle, labial palp, gill, gonad, and digestive gland and hemocytes were collected from three oysters and snap-frozen in liquid nitrogen for tissue-specific expression analysis. Hemolymph was collected from the pericardial cavity using 1-mL sterile syringes, and the samples were immediately centrifuged at 1000 g for 10 min at 4 °C. The resulting cell pellet contained the hemocytes.

To examine changes in expression of *CgIKKε-like* induced by poly I:C stimulation or infection with either *V. alginolyticus* or OsHV-1 μVar, 200 oysters were randomly divided into four groups. Each group was injected with 100 μL phosphate buffered saline (PBS, pH 7.4) vehicle, live *V. alginolyticus* (10^8 cells), or poly I:C (0.5 μg). For OsHV-1 μVar infection, viral homogenate was prepared by thoroughly mincing tissues with a high viral load in PBS and then sequentially passing the resulting suspension through 20-μm, 5-μm, 0.45-μm, and 0.22-μm filters [22]. Viral DNA quantification by qPCR using C9/C10 primers (Supplementary Table 1) showed a viral load of 8.4×10^4 viral genomic units in the homogenate [23]. Hundred microliters of this homogenate was injected into the fourth group. All injections were made into the adductor muscle through a notch filed in the oyster shell. Subsequently, hemocytes samples were collected from six animals per group at 0, 6, 12, 24, 48, and 72 h post-injection.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted with TRIzol Reagent (Invitrogen, USA), according to the manufacturer's instructions. The $A_{260/280}$ and $A_{260/230}$ ratios of all the RNAs prepared were measured by a Nanodrop 3000 spectrophotometer (Nanodrop Technologies, USA), with the values being within 1.90–2.10 and 2.00–2.50, respectively. Total RNA (1 μg) was used to synthesize first-strand cDNA using the PrimeScript RT Reagent Kit (TaKaRa, Japan). Thereafter, the cDNA was used as a template in PCRs to analyze expression of *CgIKKε-like*. All primers used in this study are shown in Supplementary Table 1.

2.3. Cloning and sequence analysis strategies

CgIKKε-like gene sequence information was retrieved from OysterBase (http://www.oysterdb.com/FrontHomeAction.do?method=home), and primers were designed to verify the sequence of *CgIKKε-like*. To obtain a complete cDNA sequence, rapid amplification of cDNA ends (RACE) was performed using the 5′- and 3′-RACE method (Invitrogen). Nucleotide and protein sequence analyses were conducted in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and a multiple sequence alignment was generated using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Protein structure analysis was performed with Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) and InterPro (http://www.ebi.ac.uk/interpro/scan.html). Based on an amino acid sequence alignment of the full-length protein, a phylogenic tree was constructed with the MEGA 6.0 software using the neighbor-joining method. IKKε-like sequences of other species were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov).

2.4. qRT-PCR analysis of IKKε-like gene expression

CgIKKε-like mRNA expression in different tissues of oysters and in samples collected from challenged oysters was assessed by quantitative real-time PCR (qRT-PCR) using a 7500 Fast Real-Time PCR System (Applied Biosystems, USA) with a SYBR Green Real Time PCR Master Mix kit (TaKaRa). A 155-bp product was amplified from the oyster cDNA using CgIKKε-like-QF and CgIKKε-like-QR primers. *GAPDH* and *β-actin* genes were employed as internal control genes for cDNA normalization [24,25]. PCR conditions were as follows: one cycle of 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. A melting curve analysis was performed at the end of each qPCR to confirm specificity of the PCR products. Target gene expression profiles were calculated using the $2^{-\Delta\Delta CT}$ method [26]. Results were analyzed by one-way ANOVA, followed by an unpaired, two-tailed t-testing. $p < 0.05$ was considered statistically significant.

2.5. Plasmid construction, cell culture and transient transfection

For the expression of fused -myc and -flag protein, the corresponding gene sequences were amplified with specific primers (Supplementary Table 1) using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, USA). pCMV-Myc and pCMS-flag plasmids vector was digested with EcoRI or XhoI respectively, and subsequently purified amplification products were fused into the purified, digested pCMV-Myc or pCMS-flag plasmid using the Ligation-Free Cloning System (Applied Biological Materials, Inc., Canada).

HEK293T (ATCC, USA) cells were cultured in Dulbecco's Modified Eagle's Medium/High Glucose (HyClone, USA) containing 10% heat-inactivated fetal bovine serum and 1 × penicillin-streptomycin resistance solution under 5% CO_2 at 37 °C. Cells were subcultured at 2-d intervals. For transfection, the cells were seeded into plates and cultured for 24 h, after which they were transfected with the plasmids using Lipofectamine 3000 (Life Technologies, USA) according to the manufacturer's protocol.

2.6. Dual-luciferase reporter assays

For the dual-luciferase reporter assays, the cells were seeded in a 96-well plate and transfected with 230 ng of total plasmids containing either 200 ng CgIKKε-like, 200 ng CgIKKε-like-KD, or 200 ng empty

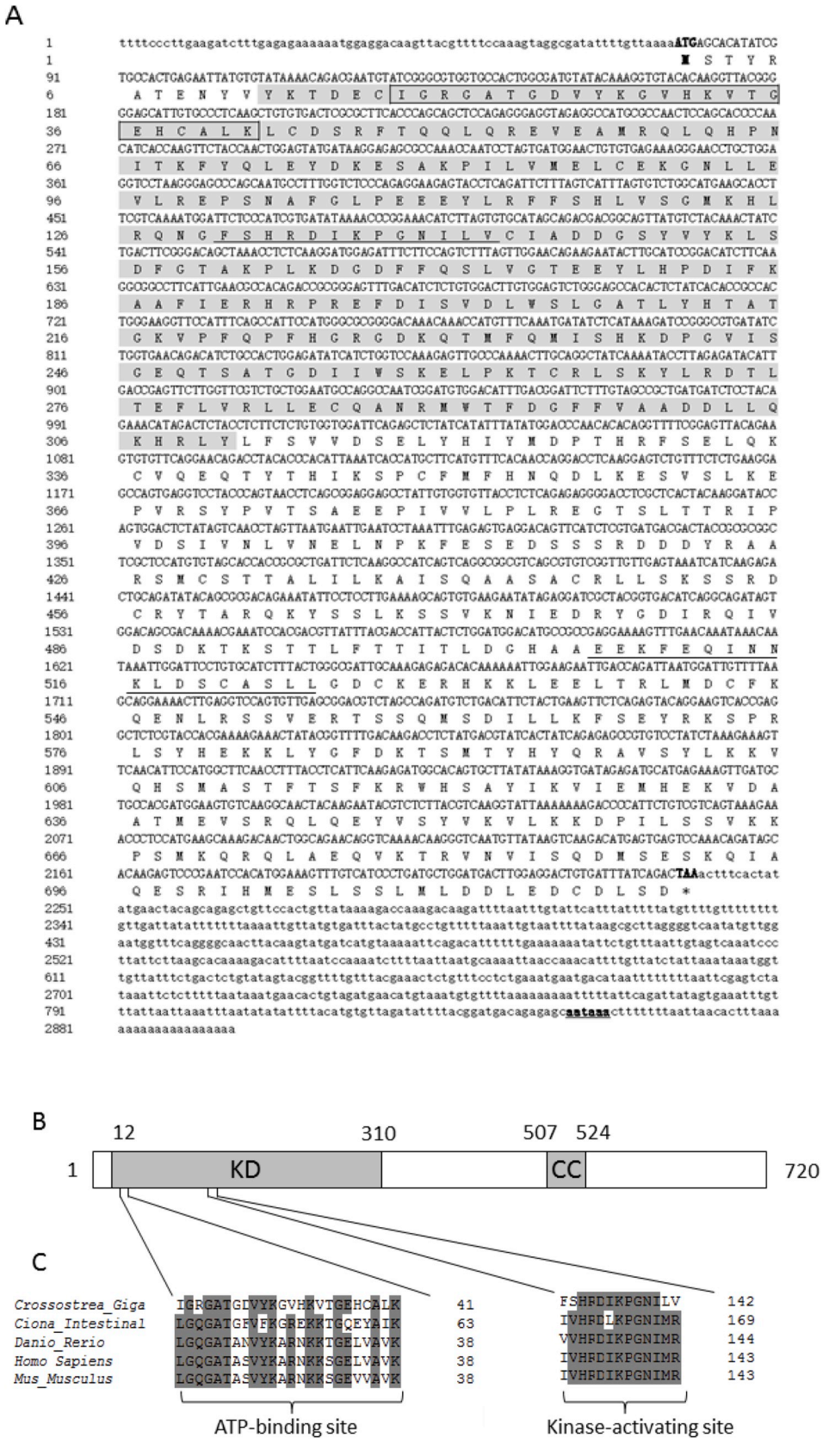

Fig. 1. Nucleotide and amino acid sequence of *CgIKKε-like*. **(A)** The numbers on the left indicate nucleotide and amino acid sequences. Start and stop codons are indicated in bold. The protein kinase domain is shaded. The ATP-binding site is shaded and bordered and the Serine/threonine-protein kinase active site is shaded and underlined. The coiled coil domain is underlined. The putative polyadenylation (aataaa) signal is indicated in bold and underlined. **(B)** Schematic domain structure of CgIKKε-like protein based on SMART program. **(C)** Comparison of the amino acid sequences of IKKε conserved motifs. *Ciona intestinalis* (NP_001072034.1); *Danio rerio* (NP_001002751); *Homo sapiens* (AF241789.1); *Mus musculus* (NM_019777.3).

plasmid with 25 ng of NF-κB or ISRE reporter plasmid and 5 ng of the pRL-TK internal reference plasmid per well. After 24 h, the culture medium was discarded, and the cells were washed with PBS. NF-κB or ISRE motif activity was determined by measuring the luciferase activity in cell extracts. Luciferase values were normalized for differences in transfection efficiency based on the pRL-TK activity in the same extract and expressed as fold induction values relative to the unstimulated empty vector control. Values were expressed as mean relative

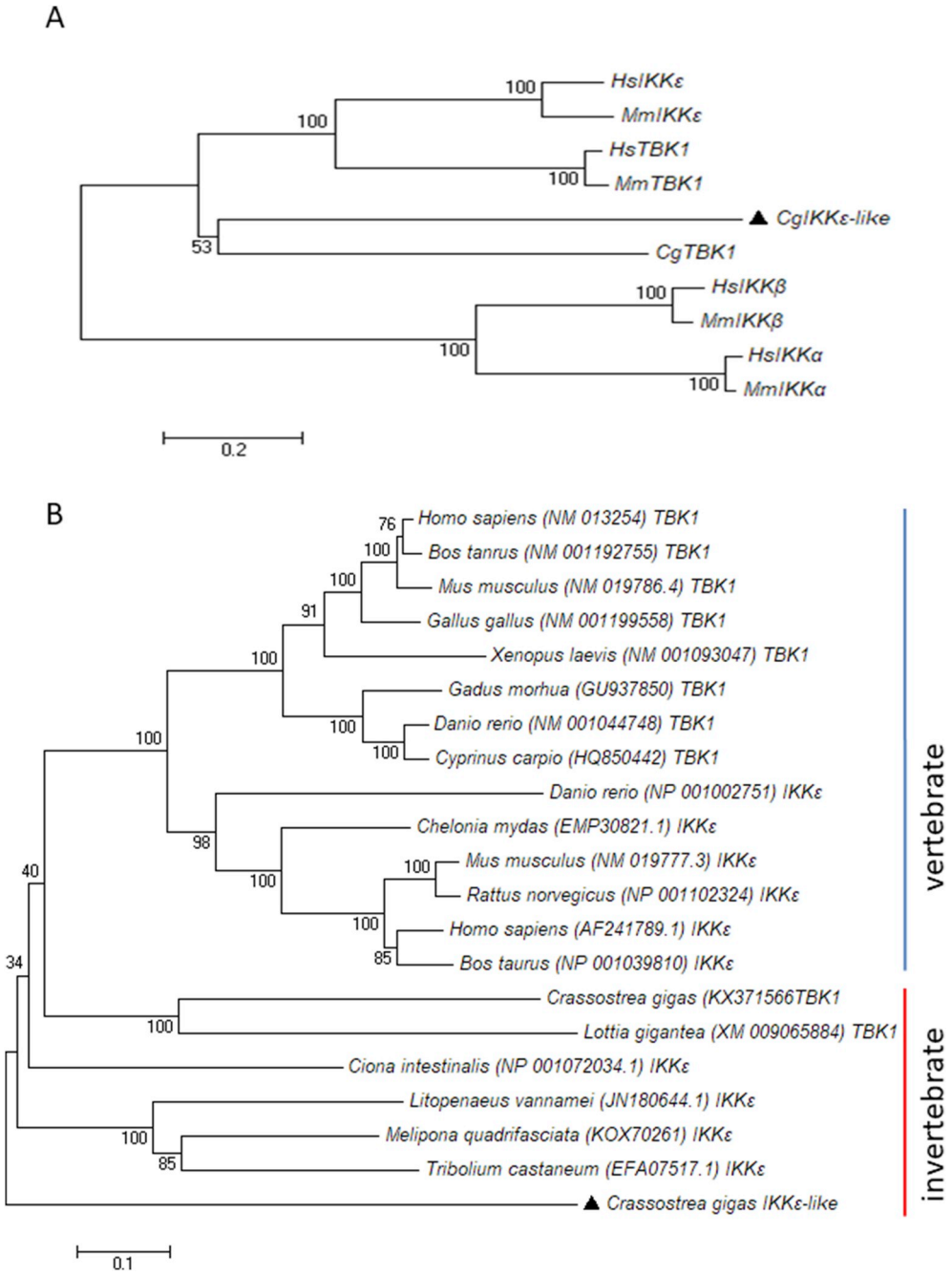

Fig. 2. A phylogenetic tree was constructed using the neighbor-joining method by the program MEGA 6.0 based on an amino acid alignment (Clustal Omega) of the full-length protein. The numbers on the lines indicate the percentage bootstrap values of 1000 replicates. Evolutionary distances were computed using the Poisson correction method. **(A)** The amino acid sequences are based on four members of the IKK family in *Homo sapiens* and *Mus musculus*. HsIKKα: AAC51671.1; HsIKKβ: AAC64675.1; HsIKKε: AAF45307.1; HsTBK1: AAF05989.1; MmIKKα: Q60680.1; MmIKKβ: AAD52095.1; MmIKKε: NP_062751.2; MmTBK1: NP_062760.3. **(B)** The amino acid sequences are based on various species of IKKε and TBK1.

stimulation from three separate experiments, each performed in duplicate. All data were calculated as means ± standard deviations (SD).

2.7. Co-immunoprecipitation assays

HEK293T cells were divided between two Petri dishes (10 cm diameter, Corning, USA) and cultured for 24 h. Fused pCMV-Myc plasmids were co-transfected with vectors expressing flag-tagged fusion proteins or empty flag vector (control). After 24–36 h, cells were harvested in cell lysis buffer (Beyotime, China). Input samples were prepared from the cell lysate and the remaining lysates were mixed with the anti-FLAG M2 magnetic beads (Sigma, USA) under gentle shaking on a roller at 4 °C for 2–4 h. The beads were then washed three times with cell lysis buffer. Input and co-immunoprecipitation (Co-IP) samples were incubated with 2 × protein sodium dodecyl sulphate polyacrylamide gel electrophoresis loading buffer (TaKaRa) at 100 °C for 3–5 min. Proteins were analyzed by western blotting using anti-Myc antibody (Roche, USA) and anti-FLAG antibodies (Sigma).

3. Results

3.1. Sequence analysis of CgIKKε-like

The full-length *CgIKKε-like* cDNA consisted of a 76-bp 5′-untranslated region (UTR), a 2163-bp open reading frame encoding 720 amino acids (with a calculated molecular weight of 82.9 kDa and a theoretical isoelectric point 7.31), and a 657-bp 3′-UTR with a poly A tail (Fig. 1A). SMART program analysis revealed that the typical Serine/threonine protein kinase domain (KD), which is ubiquitously found in all IKK family members, is located at position 12–310 (Fig. 1B). There is a coiled coil domain (507–524) at the C-terminus. Besides, an ATP-binding site (18–41) and kinase-activating site (130–142) is found in

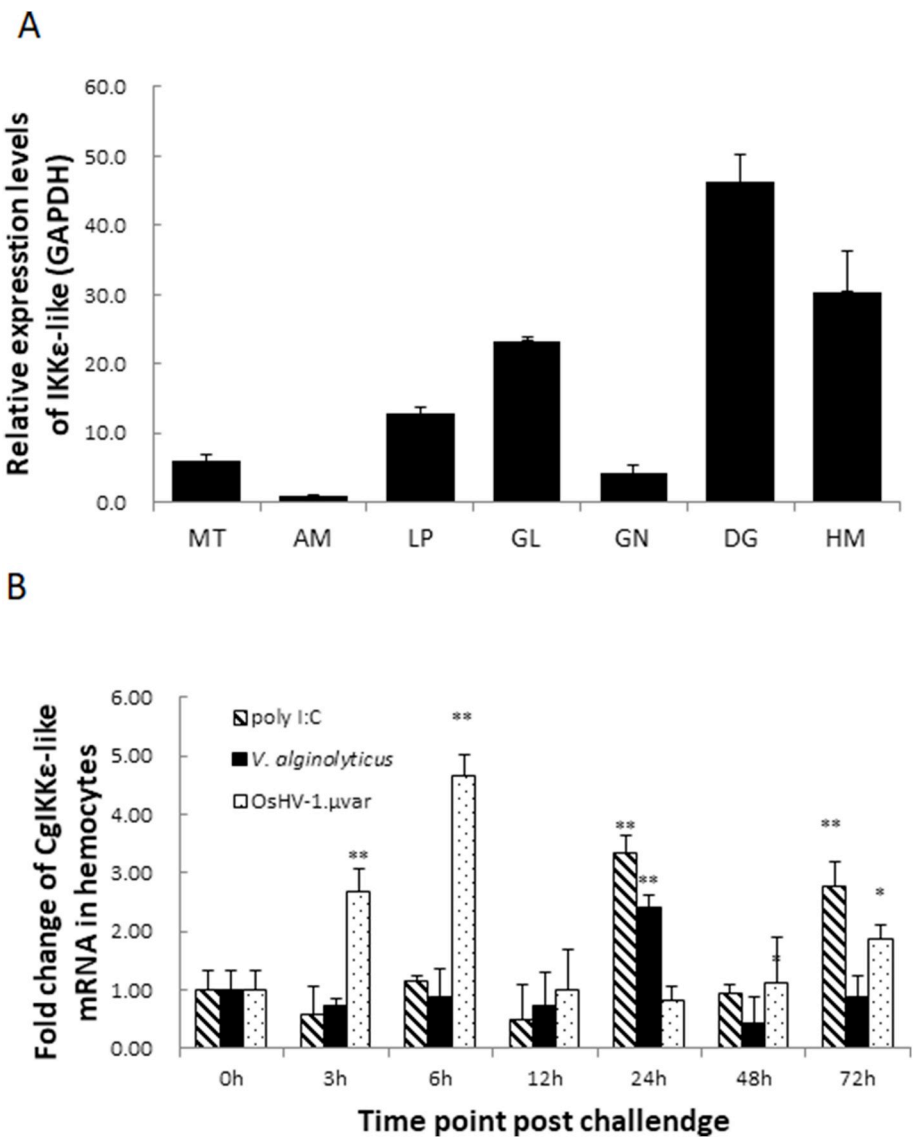

Fig. 3. Expression profile of *CgIKKε-like* transcripts. The data are expressed as means ± standard errors (N = 3). **(A)** Tissue distribution of *CgIKKε-like* in healthy oysters. The mRNA expression levels were normalized to that of *GAPDH*, and the adductor muscle sample was used as the reference sample. The seven tissues examined are MT: mantle; AM: adductor muscle; LP: labial palp; GL: gill; GN: gonad; DG: digestive gland; HM: hemocytes. **(B)** Temporal expression of *CgIKKε-like* relative to that of β-actin was analyzed by real-time PCR in *C. gigas* hemocytes after poly I:C, *V. alginolyticus* and OsHV-1. μVar challenge. Significant difference from the control is indicated with asterisks (*: $p < 0.05$; **: $p < 0.01$). The numbers in the table represent fold relative to the control and p value in the brackets.

CgIKKε-like. Multiple alignments revealed that CgIKKε-like has a high conserved ATP-binding site and kinase activating site (Fig. 1C).

3.2. Bioinformatics analysis of CgIKKε-like

Because the IKK family comprises four members in vertebrates, we constructed phylogenetic trees with the MEGA 6.0 software and Clustal omega to provide evidence that CgIKKε-like is grouped with other non-canonical IKKs (IKKε/TBK1) (Fig. 2A). In addition, another phylogenetic tree was developed based on multiple alignments of IKKε and TBK1 from various species. The TBK1/IKKε was divided into vertebrate and invertebrate branches. Oyster IKKε-like was found to be more closely related to the IKKε branch (Fig. 2B).

3.3. Expression profiles of CgIKKε-like after challenge

The quantitative real-time PCR analysis revealed that the *CgIKKε-like* could be detected in all the collected tissues of oyster, including the mantle, adductor muscle, labial palp, gill, gonad, digestive gland, and hemocytes (Fig. 3A). Moreover, a relatively higher expression level was observed in the digestive gland, followed by the hemocytes.

To support the hypothesis that *CgIKKε-like* is involved in the immune response, we examined the expression of *CgIKKε-like* in the hemocytes after poly I:C, *V. alginolyticus*, or OsHV-1 μVar infection. The poly I:C, OsHV-1 μVar, and *V. alginolyticus* may represent an RNA virus, a DNA virus, and a bacterium, respectively; results are shown in Fig. 3B. Compared to the control group, expression levels of *CgIKKε-like* mRNA were significantly increased at 3 h and peaked at 6 h, followed by a drastic decline at 12 h after OsHV-1 μVar challenge. After *V. alginolyticus* and poly I:C infection, no obvious changes were observed in the expression levels until 24 h, when it reached a maximum ($p < 0.01$), and then rapidly decreased. Finally, the expression levels of *CgIKKε-like* were recovered to the baseline levels at 72 h after *V. alginolyticus* challenge, and were up-regulated at 72 h after poly I:C stimulation.

3.4. CgIKKε-like activates NF-κB and ISRE motif reporter genes

In order to investigate the functional properties of CgIKKε-like, we examined whether transient overexpression of the CgIKKε-like protein might activate the expression of NF-κB or ISRE motif reporter genes. Due to the lack of bivalve mollusk cell lines, transfection experiments were performed in a HEK293T cell line. The function of CgIKKε-like was evaluated by cotransfecting a Myc-tagged CgIKKε-like or CgIKKε-like deletion mutant with the NF-κB-Luc or ISRE-Luc reporter genes construct. Expression of the recombinant CgIKKε-like and its deletion mutant protein in the HEK293T cells was detected using western

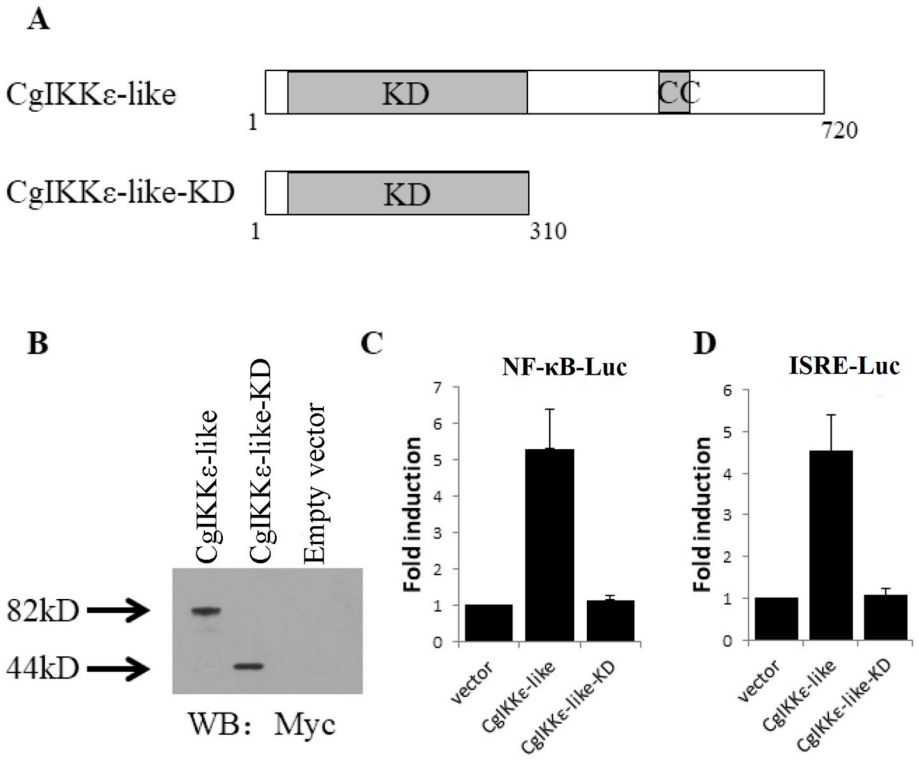

Fig. 4. CgIKKε-like activates NF-κB and ISRE luciferase reporter genes in HEK293T cells. **(A)** Schematic representation of wild-type CgIKKε-like and mutant CgIKKε-like-KD. **(B)** Detection of wild-type CgIKKε-like and mutant CgIKKε-like-KD overexpression in HEK293T cells using western blotting. **(C and D)** HEK293T cells were transiently co-transfected with NF-κB luciferase reporter plasmids (C) or ISRE luciferase reporter plasmids (D), together with vectors control (pCMV-Myc) and wild-type CgIKKε-like or with CgIKKε-like-KD. Firefly and *Renilla* luciferase activities were detected in the cell lysates at 24 h after transfection and the data indicates the fold change relative to the signal in the empty-plasmid-transfected cells. The results are expressed as means ± standard errors (N = 3).

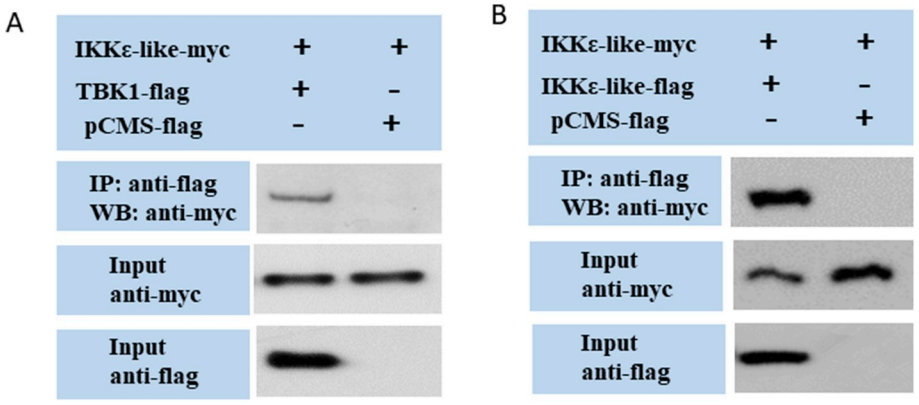

Fig. 5. CgIKKε-like interacts with CgTBK1 and interacts strongly with itself. **(A)** Interaction between CgTBK1 and CgIKKε-like by the Co-IP assay. Co-IP of proteins from CgIKKε-like-myc with CgTBK1-flag and pCMS-flag was performed using anti-FLAG M2 magnetic beads, followed by Western blot analysis against the anti-myc antibody (top). Input samples were detected using the anti-myc antibody (middle) or the anti-FLAG antibody (bottom). CgIKKε-like-myc was detected in anti-FLAG immunoprecipitates (IP: FLAG) of cotransfected cell extracts. **(B)** CgIKKε-like interacts strongly with itself. Co-IP of proteins from CgIKKε-like-myc with CgIKKε-like-flag and pCMS-flag was performed using anti-FLAG M2 magnetic beads, followed by Western blot analysis against the anti-myc antibody (top). Input samples were detected using the anti-myc antibody (middle) or the anti-FLAG antibody (bottom). CgIKKε-like-myc was detected in anti-FLAG immunoprecipitates (IP: FLAG) of cotransfected cell extracts.

blotting with anti-myc antibodies (Fig. 4B). Overexpression of CgIKKε-like was found to activate both NF-κB and ISRE motif reporter genes. However, the CgIKKε-like-KD mutant, which lacked 56% of the amino acids, was unable to activate the NF-κB and ISRE motif reporter genes (Fig. 4C and D).

3.5. *CgIKKε-like interacts with CgTBK1 and forms homodimers*

In mammal cells, IKKε interacts with TBK1 to make a complex and then phosphorylates IRF3 and IRF7. And an oyster TBK1 has been identified in our previous study [18]. Therefore we examined the interaction between oyster TBK1 and IKKε-like using Co-IP assays. The Co-IP results showed that -myc tagged CgIKKε-like protein was co-precipitated by -flag tagged CgTBK1 protein, indicating the interaction between these two proteins (Fig. 5A). Also the Co-IP results confirmed that CgIKKε-like interacts strongly with itself (Fig. 5B).

3.6. *CgIKKε-like may increase CgIκBs phosphorylation and interacts with CgIRF8*

Since CgIKKε-like was able to activate the NF-κB and ISRE motif reporter genes, we wondered the mechanisms that CgIKKε-like could activate NF-κB and “interferon” like pathways. In previous study, three *IκB* genes have been identified in oyster [27,28]. CgIKKε-like expression vector was co-transfected with these three oyster IκB proteins vectors. The results were shown in Fig. 6A. We found that, in contrast to the control, CgIKKε-like expression resulted in obvious shift of CgIκB1 and CgIκB2 western blotting bands but had no effect on CgIκB3 (Fig. 6A). As IRF is the key transcriptional factor of interferon signaling [29], we detect the relationship between CgIKKε-like and CgIRF8, one identified IRF protein in oyster [17], using Co-IP assays. The results showed that CgIKKε-like could bind CgIRF8, although not very strongly (Fig. 6B).

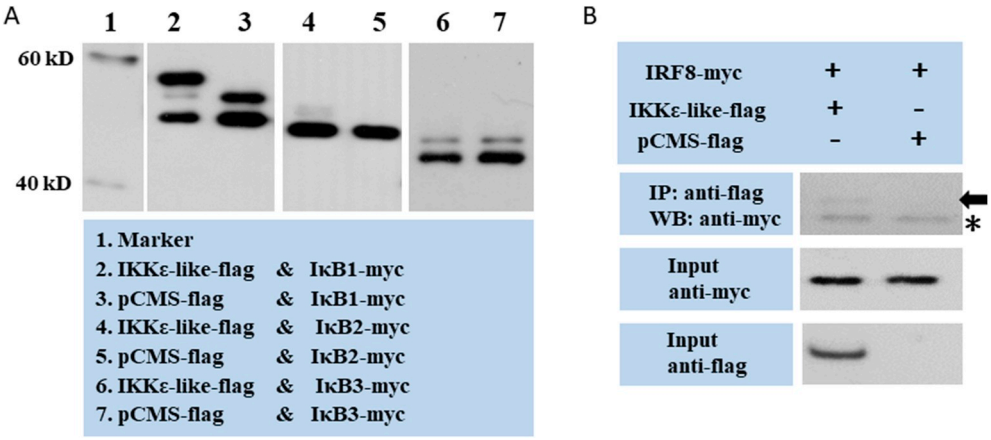

Fig. 6. CgIKKε-like increases CgIκBs phosphorylation and interacts with CgIRF8. **(A)** CgIKKε-like expression vectors were co-transfected with three oyster IκB proteins vectors respectively. CgIKKε-like expression resulted in a shift of CgIκB1 and CgIκB2 western blotting bands but had no effect on CgIκB3. **(B)** Interaction between CgIRF8 and CgIKKε-like by the Co-IP assay. Co-IP of proteins from CgIRF8-myc with CgIKKε-like-flag and pCMS-flag was performed using anti-FLAG M2 magnetic beads, followed by Western blot analysis against the anti-myc antibody (top). Input samples were detected using the anti-myc antibody (middle) or the anti-FLAG antibody (bottom). The asterisk represents the heavy chain of mouse IgG. CgIRF8-myc was detected in anti-FLAG immunoprecipitates (IP: FLAG) of cotransfected cell extracts (marked by black arrow).

4. Discussion

Since their discovery, studies of the transcription factors NF-κB and IRF and their related regulatory proteins have gained increasing attention as their key roles in the innate immune signaling. In recent decades, several mollusk proteins homologous to members of the NF-κB or IRF signal transduction pathway have been characterized [17,18,30–32]. In this study, we reported the isolation of an oyster homologue of *IKKε* and termed it *CgIKKε-like*. Phylogenetic analyses of CgIKKε-like with members of the IKK family from both vertebrates and invertebrates provided evidence that CgIKKε-like is grouped with the IKKε subfamily. In contrast to canonical IκB kinases (IKKs) IKKα and IKKβ, CgIKKε-like lacked a conversed leucine zipper amphipathic α-helix (LZ) and helix-loop-helix (HLH) domain at the C-terminus; instead, it contained a coiled coil domain at the C-terminus. As identified in other IKKε proteins, CgIKKε-like also possessed several characteristic motifs such as protein kinases ATP-binding region signature and serine/threonine protein kinases active-site signature in the KD. It has been revealed that overexpression of wild-type IKKε was shown to phosphorylate serine residues Ser 32 and Ser 36 of IκB-α, and significantly stimulate NF-κB activation [9]. Similar to other IKKε family members, CgIKKε-like also has a highly conserved ATP-binding site and kinase-activating site, as revealed by multiple alignment results (Fig. 1C). This suggests that CgIKKε-like, similar to other IKKε family members, might possess the kinase activity to stimulate NF-κB and IRF activation.

Real time PCR analysis of the *CgIKKε-like* mRNA showed that its expression is significantly higher in digestive gland, hemocytes and gill than in other tissues. The gills and digestive system are presumed to be the first line of defense in mollusks [33]. Hemocytes, particularly in the molluskan immune system, play a key role in innate immune responses [34]. The internal defense mechanisms of oysters can be separated into cell-mediated and humoral types; however, it has become increasingly apparent that both are interrelated and closely associated with hemocytes. In response to conditions of infection, stress, and injury, the diverse expression patterns of NF-κB and IFN as well as related molecules contribute to the stability of the organism. In order to better understand the roles of *CgIKKε-like* in immunity, we analyzed the gene expression pattern under the stimulation of three types of pathogens. All the stimulated groups demonstrated elevated expression of *CgIKKε-like* at some time-points during the sampling period post-injection. Besides, *CgIKKε-like* was up-regulated significantly earlier after OsHV-1 μVar stimulation than after other treatments, which is similar to the expression pattern of TBK1 reported in *C. gigas* [18]. Therefore, it is suggested that CgIKKε-like may have a direct involvement in the immune response against DNA virus infection in organisms.

In mammals, IKKε phosphorylates the N-terminal regulatory region of IκB-α, and sequentially releases NF-κB, which is inhibited by IκB-α. Then, the released NF-κB is transferred to the nucleus through the nuclear pore and activates the transcription of immune-related genes [9]. In addition, IKKε and TBK1 are components of the virus-activated kinase that phosphorylates IRF3 and IRF7, which is critical for expression of type I IFN genes [6,7]. In our study, we demonstrated the ability of CgIKKε-like to induce NF-κB activation by showing that its overexpression stimulates NF-κB-dependent reporter gene expression. IFN-stimulated response element (ISRE) is located in the promoter region of IFN. Our results also demonstrated that overexpression of CgIKKε-like could activate ISRE-dependent IFN reporter gene. In addition, we found that CgIKKε-like-KD mutant, which lacked 56% of the amino acids, was unable to activate the NF-κB and ISRE motif reporter genes. These results indicates that CgIKKε-like could activate both NF-κB and IFN signaling and that the remaining CgIKKε-like sequence besides the KD motif plays an essential role in the regulation of IKK activity.

In vertebrate innate immunity signaling pathways, IKKε and the TBK1 complex phosphorylate IRF3 and IRF7, subsequently activating IRFs that induce IFN gene expression and release. Since the results of the dual reporter gene assays showed that CgIKKε-like could activate the ISRE reporter gene and participate in the IFN signaling pathway in mammal cells, we wondered the specific signal transduction of CgIKKε-like. As an oyster TBK1 has been identified in our previous study [18], we examined the interaction between oyster TBK1 and IKKε-like using Co-IP assays. The Co-IP results showed that CgIKKε-like could interact with CgTBK1 protein (Fig. 5A). The presence of a TBK1-IKKε signal might play important roles in the immune system of oyster. Additionally, the Co-IP results showed that CgIKKε-like interacts strongly with itself (Fig. 5B), as same as the human IKKε [11]. The formation of homodimer or homooligomer might be important for the regulatory function of CgIKKε-like.

Now that oyster IKKε-like was able to activate the NF-κB and ISRE motif reporter genes, the mechanisms of the activation would be of much more interesting. In mammals, IKKε activates NF-κB by phosphorylating IκB [11]. In the previous study, three IκBs had been identified in oyster [27,28]. So in this research, CgIKKε-like and oyster IκBs expression plasmids were co-transfected into HEK293T cells. And as a result, the expression of CgIKKε-like caused significant shift of CgIκB1 and CgIκB2 bands but had no effect on CgIκB3 (Fig. 6A). We speculated that the shift might be due to the modification of CgIκBs by CgIKKε-like, and this modification was probably phosphorylation according to the fact that CgIKKε-like is an IKK family member and possess a typical Serine/threonine protein kinase domain. Phosphorylation abolished the inhibition of NF-κB by IκBs and then activated NF-κB. However,

CgIKKε-like expression had no effect on CgIκB3, which may tell us that the phosphorylation of IκBs are specific to certain IKK proteins and CgIκB3 may be the target of other oyster IKK proteins. Besides, we verified the interaction of CgIKKε-like with CgIRF8 which is considered as a key transcription factor of the oyster "interferon-like" signaling [17]. CgIKKε-like may be involved in the oyster "interferon-like" pathway by binding IRF proteins.

In this study, we successfully cloned and characterized the full-length cDNA of an *IKKε-like* gene from *C. gigas*. *CgIKKε-like* was ubiquitously expressed and the expression were significantly induced after OsHV-1 μVar, poly I:C, and *V. alginolyticus* challenge, suggesting that it could be involved in the innate immunity of oysters against viruses and bacteria. CgIKKε-like could induce NF-κB and ISRE activation, while the CgIKKε-like-KD could not. CgIKKε-like could interact with CgTBK1 and form homodimers strongly, which may be important for signaling. We also found that CgIKKε-like could increase CgIκBs phosphorylation and could interact with CgIRF8, and these results may explain why CgIKKε-like could activate NF-κB and ISRE reporter genes. Together, these results suggest that CgIKKε-like would play an important role in the oyster immune system.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.074.

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