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Short communication

Identification and expression analysis of IκB and NF-κB genes from *Cyclina sinensis*

Shan Gao [a, 1], Yipeng Ren [a, b, 1], Haijing Zhang [a], Baoping Pan [a, *], Hong Gao [a]

[a] Tianjin Key Laboratory of Animal and Plant Resistance, School of Life Sciences, Tianjin Normal University, Tianjin, 300387, PR China
[b] Institute of Entomology, College of Life Sciences, Nankai University, Tianjin, 300071, PR China

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ABSTRACT

With the increasing economic importance of *Cyclina sinensis* aquaculture, interest in its defense mechanisms against pathogenic infection has grown in recent years. Inhibitor of nuclear factor-kappaB (IκB) and nuclear factor-kappaB (NF-κB) are proteins with central roles in many important physiological and pathological processes, such as innate immune responses. In this study, we identified *CsIκB* and *CsNF-κB* genes from a *C. sinensis* transcriptome library. In healthy adult clams, *CsIκB* and *CsNF-κB* genes were widely expressed in various tissues and highly expressed in hemocytes. Further, the expression levels of these genes were significantly increased in hemocytes challenged by *Vibrio anguillarum*, *Micrococcus luteus* and poly I:C. Inhibition of *CsMyD88* expression by RNAi technology significantly altered the mRNA expression patterns of *CsIκB* and *CsNF-κB* as measured using quantitative real-time PCR. These results collectively indicated that the NF-κB signaling pathway, including *CsIκB* and *CsNF-κB* genes, might be involved in early innate immune responses and may be regulated by a MyD88-dependent signaling pathway in *C. sinensis*.

1. Introduction

Cyclina sinensis is one of the most important bivalves in Chinese marine aquaculture and possesses many advantages as a popular type of seafood, including rapid growth, pollution tolerance, temperature and salt resistance, and a high survival ratio [1,2]. This clam species is distributed along Asian coastlines and has a huge consumer market in China, South Korea and Japan. In recent years, germplasm decline, environmental pollution and pathogenic stimulation have resulted in high mortality in this species and resulted in huge economic losses [3,4]. Pathogenic microorganisms such as *Vibrio anguillarum* and *Micrococcus luteus* can cause death in many marine organisms [5].

Nuclear factor-kappaB (NF-κB) is a pleiotropic transcription factor that is sequestered in the cytoplasm by a member of the inhibitor of the nuclear factor-kappaB (IκB) protein family in unstimulated cells. The ANK domain, a defining characteristic of IκB proteins, is the domain responsible for the inhibitory interaction between IκB and NF-κB. Based on previous reports, IκB and NF-κB play major roles in many physiological and pathological processes such as inflammation, immunity, cell proliferation and apoptosis [6,7]. Before, members of the Toll-like receptor (TLR) signaling pathway, such as Toll, MyD88, IRAK4 and TRAF6, were expressed in mollusks. However, the response of IκB and NF-κB genes to bacterial infection in mollusks has not been well studied [8]. So identifying their roles in bacterial defense may shed light on the innate immune pathway and provide valuable information for the prevention of diseases in clam aquaculture.

In this study, we identified IκB (designated *CsIκB*) and NF-κB (designated *Cs NF-κB*) genes from the *C. sinensis* transcriptome and determined their tissue distribution and expression levels in *C. sinensis* after pathogenic stimulation. Further, we evaluated the effect of RNA knockdown of the *MyD88* gene. These data will improve our knowledge of the molecular mechanisms underlying the immune response of clams to bacterial infection and contribute to improved clam breeding and disease control.

2. Materials and methods

2.1. Microorganisms and animals

C. sinensis individuals were collected from the coastal region of

* Corresponding author.
E-mail address: baopingpan@126.com (B. Pan).
[1] Contributed equally to this work.

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Dagang District (117.45°E 38.83°N), Tianjin, P.R. China. They were maintained in tanks containing aerated fresh seawater and 5‰ *Chlorella* sp. for 7 days. Their average features were as follows: shell lengths of 28.12 ± 1.49 mm; shell heights of 28.86 ± 1.57 mm; and shell widths of 18.56 ± 0.47 mm. Clams used in experiments were two-year-old males and females with no damage and no significant differences in morphological index. Seawater was completely replaced when the density reached 1.020–1.040 g/cm^3 with a pH of 7.0. Clams were fed 5% chlorella twice per day.

V. anguillarum and *M. luteus* were cultured in a 2216E medium at 28 °C for 24 h. The bacterial culture was washed three times with sterile saline. Between each wash, the bacteria were centrifuged at 4000*g* for 10 min and resuspended at 1×10^8 cells/ml in each tube. 50 μL (approximately 5×10^6 cells) were injected into each clam.

2.2. Gene identification and sequence analysis

To identify the *CsIκB* and *CsNF-κB* genes, the transcriptome of *C. sinensis* and other sequence databases were searched using all protein sequences available in the databases of NCBI. The ORF (open reading frame) finder was used to predict the amino acid sequences. The predicted amino acid sequences were then confirmed using BLASTP against the NCBI non-redundant protein sequence database. The deduced amino acid sequence was analyzed with the Expert Protein Analysis System. The molecular weight and theoretical isoelectric point were computed using the ProtParam tool. The functional domains in the genes were predicted using the SMART database. Protein sequences were aligned using the ClustalW program. The phylogenetic tree was constructed using the neighbor-joining method in MEGA 5.0 [9].

2.3. Tissue distribution of CsIκB and CsNF-κB mRNA in C. sinensis

Tissue-specific mRNA expression was analyzed in hemocytes, gills, mantle, adductor muscle, gonad and liver from nine randomly selected clams. Hemocytes of three individuals (0.5 mL per individual) were collected from the muscle tissue and centrifuged for 10 min at 8000 rpm at 4 °C. Frozen muscle, gill, gonad, mantle and liver samples from three individuals were homogenated with 9× pre-cooled physiological saline, centrifuged for 15 min at 4500 rpm at 4 °C, and immediately used for RNA extraction with Trizol. The expression level of *CsIκB* and *CsNF-κB* in *C. sinensis* tissues was analyzed using quantitative real-time PCR.

2.4. Effect of V. anguillarum, M. luteus and poly (I:C) on CsIκB and CsNF-κB expression

A total of 150 *C. sinensis* individuals were selected at random, and the adductor muscles of 36 of these individuals were injected with 50 μL of live *V. anguillarum* or *M. luteus* suspended in sterilized seawater ($OD_{600} = 0.4, 1 \times 10^8$ cell/mL) or with 1 μg/mL (optimized final concentration) poly I:C (Sigma-Aldrich, China; Catalog No. P1530). Additionally, the same 36 individuals were treated with sterilized seawater (control group), and six were left untreated (blank group) with only hemolymph samples were taken. Hemolymph samples were centrifuged (3000 rpm, 4 °C, and 10 min) to collect hemocytes. Samples were taken from 6 clams from each group at 3, 6, 12, 24, 48 and 96 h post-injection (hpi) to analyze the expression of genes of interest by quantitative real-time PCR.

2.5. The expression profiles of CsIκB and CsNF-κB by CsMyD88 interference

T7 promoter-linked primers *CsMyD88*-dsRNA-T7-F/R and EGFP-dsRNA-T7-F/R (Table 1) were used to amplify a *CsMyD88* cDNA fragment (521 bp) from cDNA of *C. sinensis* and an EGFP DNA fragment (657 bp) from pEGFP vector, and the PCR products were used as templates to synthesize dsRNA. The dsRNAs were generated via in vitro transcription according to the method described by previous reports, with slight modifications [10]. RNA integrity was examined using electrophoresis, and the concentration was quantified by the absorbance at 260 nm and was adjusted to a final concentration of 1 mg/mL. The control group received an injection of EGFP dsRNA, and untreated clams were used as the blank group. Hemocyte samples from 3 clams per treatment were collected at 0, 24, 48, 72 and 96 h post-dsRNA injection for analysis of *CsIκB and CsNF-κB* gene expression using quantitative real-time PCR.

2.6. RNA extraction and cDNA synthesis

Total RNA was extracted from *C. sinensis* samples using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. First-strand synthesis was carried out based on Promega M-MLV RT Usage Information using the DNase I (Promega, USA)-treated total RNA as a template and oligo (dT)-adaptor as a primer. The synthesis reaction was performed at 42 °C for 1 h, then terminated by heating at 95 °C for 5 min. RNA integrity was examined using electrophoresis and visualized on a GelDoc system. Finally, synthesized cDNA was diluted in ultrapure water before storing at −20 °C for subsequent SYBR Green fluorescent (TaKaRa, Japan) quantitative real-time PCR.

2.7. Quantitative real-time PCR and statistical analysis

Quantitative real-time PCR was carried out in a 20 μL reaction volume containing 1 μL of original cDNA, 10 μL of Top Green, 0.4 μL of Reference solution, 0.4 μL of each primer and 7.8 μL of DEPC water. The real-time PCR running program was as follows: one cycle of 95 °C for 30 s, 40 cycles of amplification (95 °C, 5 s; Tm, 30 s), one cycle of 95 °C for 15 s, and one cycle of 60 °C for 60 s. All primers for *CsIκB* and *CsNF-κB* used in real-time PCR were designed by Primer Premier 5.0 (Table 1). All data were given as the means ± S.D (n = 3). The data were subjected to one-way analysis of variance (one-way ANOVA) followed by a multiple comparison using the SPSS 17.0 software. The comparative Ct method ($2^{-\triangle\triangle CT}$ method) was used to analyze gene expression level [11,12]. Differences were considered statistically significant at $p < 0.05$ and extremely significant at $p < 0.01$.

3. Results

3.1. Sequence characterization of CsIκB and CsNF-κB

The cDNA sequences of *CsIκB* and *CsNF-κB* were identified from the *C. sinensis* transcriptome library. The ORF of the *CsIκB* cDNA was determined to be 1134 bp and to encode a polypeptide of 377 amino acids. This polypeptide has six ANK domains, a theoretical molecular mass of 41.79 kDa and an isoelectric point of 4.98. *CsIκB* was deposited in GenBank under accession No. KP067202.

The ORF of the *CsNF-κB* cDNA was determined to be 2019 bp and to encode a polypeptide of 672 amino acids. This polypeptide has a theoretical molecular mass of 74.46 kDa and an isoelectric point of 5.96. Sequence analysis revealed that *CsNF-κB* is a cytosolic protein because there was no signal peptide or transmembrane domain structure in its amino acid sequence. *CsNF-κB* gene was deposited in GenBank under accession No. KR732937.

3.2. Phylogenetic analysis of CsNF-κB and CsIκB

Based on the deduced amino acid sequences of the *CsIκB* and