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Two homologues of inhibitor of NF-kappa B (IκB) are involved in the immune defense of the Pacific oyster, *Crassostrea gigas*

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ABSTRACT

A novel homologue of IκB was cloned from a hemocyte cDNA of *Crassostrea gigas* (designed as *CgIκB2*). The complete cDNA of *CgIκB2* includes an open reading frame (ORF) of 1032 bp, and 3′ and 5′untranslated regions (UTR's) of 141 bp and 279 bp, respectively. The ORF encodes a putative protein of 343 amino acids with a calculated molecular weight of approximately 37.8 kDa. Alignment analysis reveals that CgIκB2 contains a conserved degradation motif and six ankyrin repeats. A phylogenetic analysis suggests that a gene duplication event prior to the gastropod-bivalve divergence resulted in the emergence of two IκB homologues in *C. gigas*. Distinct maximal expression patterns of *CgIκB1* in hemocytes and *CgIκB2* in the gonad were observed. *CgIκB1* and *CgIκB2* expression in response to bacterial challenge is similar and inducible. Moreover, both CgIκB1 and CgIκB2 are able to inhibit NF-κb/Rel activating transcription in S2 or HEK293 cells. Our findings demonstrate that both *CgIκB1* and *CgIκB2* are involved in immune defense in *C. gigas* through regulation of NF-κB/Rel activity.

1. Introduction

The nuclear factor (NF)-κb family of transcription factors are evolutionary conserved proteins that play a central role in diverse biological processes, including immunity, inflammation, proliferation, apoptosis, the cellular-stress response and tissue remodeling [1,2]. All of these family members contain a conserved domain, the Rel-homology domain (RHD) of approximately 300 amino acids that mediates DNA binding, dimerization, and nuclear translocation. In quiescent cells, NF-κb proteins are predominantly retained in the cytoplasm in an inactive complex with inhibitors of NF-κb (IκB), that interact with the RHD domain and mask the nuclear localization signal (NLS) of NF-κb [3]. However, exposure of cells to various stimuli leads to a rapid cascade of IκB phosphorylation, polyubiquitination, and proteasomal degradation, by which NF-κb can be released from its cognate inhibitor and translocated into the nucleus to drive transcription of target genes [4].

In mammals, the IκB family is composed of IκBα, IκBβ, IκBγ, IκBϵ, IκBζ, BCL3 and the precursor protein p100 and p105. All family members contain five to seven ankyrin repeats (ARs) that are required for interaction with NF-κb proteins. Despite obvious structural similarity, each of the IκB proteins appear to have a distinct and non-overlapping function [4]. For example, IκBα, IκBβ, and IκBϵ inhibit NF-κb, but other members such as IκBζ and BCL3, can function as NF-κb activators [2]. IκBα is the primary regulator of the classic NF-κb heterodimer p65/p50, whereas IκBϵ participates in the regulation of p65 homodimers and c-Rel/p65 heterodimers [5,6]. Moreover, each family member is expressed in a tissue- specific manner and responds to different external stimuli [7]. Knocking out multiple isoforms of IκB demonstrates that the distinct characteristics of IκBα, IκBβ and IκBϵ are attributable to differential transcriptional regulation and specificity in gene expression [8].

In invertebrates, the functions of IκB homologues have been identified in arthropods, such as fruit fly and horseshoe crab [9,10]. Although the NF-κb signaling pathway is absent in *C. elegans* [11], this pathway plays a pivotal role in triggering innate immune response and regulating the expression of critical immune defense molecules in humans, *Drosophila*, and horseshoe crab indicating evolutionary conservation from arthropods to mammals. Recently, an IκB gene has been characterized in mollusks, including the sea sleeve, the Pacific oyster, pearl oyster, and bay scallop [12–15], and its expression pattern in response to infection indicates that IκB is likely to be involved in immune defense in these organisms. However, all available evidence shows that only one IκB gene exists in arthropods and mollusks. It is not yet known whether additional

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IκB genes are present in other invertebrate species. Moreover, our understanding of how IκB functions in innate immunity in marine bivalves is also limited.

In the current study, we identified and cloned a novel IκB gene in the Pacific oyster *Crassostrea gigas* that shares high amino acid similarity with other mollusk IκB genes. To better understand the function of the two IκB genes in marine bivalves, we compared their tissue-specific expression patterns and expression profiles in hemocytes during infection, and investigated the ability of CgIκB1 and CgIκB2 to inhibit NF-κB/Rel transcriptional activity.

2. Materials and methods

2.1. Cloning the full-length cDNA of CgIκB

The homologues of IκB were identified using BLAST (http://www.ncbi.nlm.nih.gov/blast) to search the Pacific oyster hemocyte EST library. Based on the identified EST sequence, gene specific primers CgIκB2F1, CgIκB2F2, CgIκB2R1 and CgIκB2R2 were designed to amplify the full-length cDNA of *CgIκB2* by rapid amplification of cDNA ends (RACE). We isolated the 5′- and 3′- ends using the GeneRacer™ kit (Invitrogen, CA, USA). Briefly, 5 μg of total RNA isolated from oyster hemocytes was treated with calf intestinal phosphatase to remove the 5′-phosphate from truncated RNAs and non-mRNAs, leaving a 5′ -OH end. Total RNA was then treated with tobacco acid pyrophosphatase to remove the 5′-cap from full-length mRNAs, leaving a 5′-phosphate to which a GeneRacer™ RNA oligo was ligated with T4 RNA ligase. Ligated mRNAs were then reverse-transcribed with GeneRacer™ Oligo(dT) Primer to obtain the RACE-ready cDNA. The first PCR used primers pairs GR5P/CgIκB2R1 and GR3P/Cg IκB2F1 for 5′RACE and 3′RACE, respectively, and GR5NP/CgIκB2R2 and GR3NP/CgIκB2F2 for nested PCR (see Table 1). PCR was performed in a 25 μL volume containing 2.5 μL 10× Ex-Taq Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 μM of each primer, and 1.25 U TaKaRa Ex-Taq DNA Polymerase (TaKaRa, DaLian, China) and 1 μL of RACE-ready cDNA. The target PCR products were gel-purified using a Gel Extraction System (Omega, USA) and cloned into pGEM-T Easy Vector (Promega, WI, USA). The plasmid DNA was sequenced with forward and reverse universal primers using the Bigdye-Terminator kit and an ABI Prism 3730 DNA sequencer (Perkin–Elmer, Wellesley, MA, USA). Full-length cDNA sequences were obtained by combining the 3′- and 5′-end sequences.

Table 1
Sequences of oligonucleotide primers used.

Primer	Sequence (5′ → 3′)	Comment
CgIκb2F1	ATCTCGCAGTGCTGATGAAACA	5′RACE of CgIκb2
CgIκb2F2	TCTGGTGCCGATGTGAATGTTG	
CgIκb2R1	CCAACATTCACATCGGCACCAG	3′RACE of CgIκb2
CgIκb2R2	GGGTCTGCCTCAGTTTGTCGTTGTA	
GR5P	CGACTGGAGCACGAGGACACTGA	Adaptor of 5′RACE
GR5NP	GGACACTGACATGGACTGAAGGAGTA	
GR3P	GCTGTCAACGATACGCTACGTAACG	Adaptor of 3′RACE
GR3NP	CGCTACGTAACGGCATGACAGTG	
CgIκb1F1	CCCTTCACATTGCCAGTAG	Realtime-PCR of
CgIκb1R1	ATTGGGAGATGGGTGTTCT	CgIκb1
CgIκb2F3	GCTCGGAAGTAAATGAAGTG	Realtime-PCR of
CgIκb2R3	CTGGAGTTCTTGAAGTCTGC	CgIκb2
β-actinF1	AAGATATTGCAGCTTTAGTCGT	Realtime-PCR of
β-actinR1	TTCTGTCCCATACCAACCAT	β-actin
CgIκb1F2	AGCC GGTACC AAAATGTCGAACAGAGACTTT	Vector of pAc-Iκb1
CgIκb1R2	AGCG CTCGAG ACTCATATCTTCCTCACTATC	
CgIκb2F4	AGAC GGTACCGTCATGCTTGCAAAAGTGTTC	Vector of pAc-Iκb2
CgIκb2R4	TCAC GAATTC ATCTTCTTCCTCCTCCGATTC	
CgRelF1	AGAC GGTACC GACATGGCTGAGCTCAACTTT	Vector of pAc-Rel
CgRelR1	AGTT CTCGAG GGACTGGCCCAGCGACTGGAC	
CgIκb1F3	GTAAGGATCCACCATGTCGAACAGAGACTTTG	Vector of
CgIκb1R3	CACTCTCGAGTGAGTTTCCTGCCAGAGTTAG	pcDNA-Iκb1
CgIκb2F5	GTAAAAGCTTACCATGGATTTGAACGACCTGGAA	Vector of
CgIκb2R5	CACTGAATTCCAATCAGCCAATCACAGCTCG	pcDNA-Iκb2
CgRelF2	GTGTAAGCTTGTCTATACTGACATGGCTGAGCT	Vector of
CgRelR2	CTCTCTCGAGTAACAATGCCTCTGTCAGAGG	pcDNA-Rel

"F" indicates forward primer and "R" indicates reverse primer.

2.2. Sequence analysis of two CgIκBs

Deduced amino acid sequences of two CgIκBs were compared with previously published sequences of representative invertebrates and vertebrates. The percent identity of amino acid sequences was calculated using Megalign of the DNAstar software package (DNASTAR Inc., Madison, WI, USA). The alignment of amino acids sequences was performed using ClutalX 1.81. The protein domains were predicted with the Simple Modular Architecture Research Tool (SMART) version 4.0 (http://smart.embl-heidelberg.de/). The casein kinase II phosohorylation site was predicted through web-based KinasePhos 2.0 (http://kinasephos2.mbc.nctu.edu.tw/index.html). Finally, the phylogenetic tree was generated by the neighbor-joining method of MEGA 4.0 [17], with bootstrap values estimated by 1000 replications.

2.3. Animals, tissue collection and bacterial challenge

Pacific oysters, *C. gigas*, (two-years old with averaging 90 mm in shell height) were obtained from Qingdao, Shangdong Provice, China, and maintained at 22–25 °C in tanks with circulating seawater for one week before use. The oysters were fed twice daily with *Tetraselmis suecica* and *Isochrysis galbana*. All procedures and investigations were performed in accordance with the Guide for the care and use of laboratory animals [37]. The experiment for *in vivo* infection was performed according to previous work with slight modification [16]. Briefly, 120 oysters were randomly divided into bacteria challenge group and control group. The oysters were challenged by injecting 100 μl bacteria *Vibrio alginolyticus* (1×10^9 bacteria per liter) suspended in sea water, and the control group was injected with an equal volume of sea water. Hemolymph was collected at different times (2, 4, 6, 8, 12, 24, 48 h after challenge) from the pericardial cavity and immediately centrifuged (700×*g* for 10 min at 4 °C) to separate blood cells from plasma. Five individuals were randomly sampled in each group at every time point after injection. For the tissue distribution analysis, total RNA was extracted from gill, mantle, adductor muscle, digestive gland, gonads, and hemocytes of three healthy animals.

2.4. Isolation of total RNA and real-time quantitative RT-PCR analysis of two CgIκBs

Total RNA was isolated from frozen tissues using TRIzol reagent (Invitrogen, CA, USA), and quantified based on the absorbance at 260 nm. The integrity of RNA was verified with agarose gel electrophoresis. Real-time PCR analysis was used to quantify mRNA expression levels in tissues and during bacterial challenge of two oyster *IκBs*. Total RNA (2 μg) isolated from different tissues was treated with DNase I (Invitrogen, Amplification Grade, CA, USA) to remove any genomic DNA contamination. The RNA was then reverse-transcribed using an oligo (dT) primer and ThermoSript™ RT-PCR System (Invitrogen, CA, USA) according to the manufacturer's instructions. The primers for real-time PCR analysis were CgIκB1F1 and CgIκB1R1 for CgIκB1, CgIκB2F3 and CgIκB2R3 for CgIκB2, β-actinF1 and β-actinR1 for the internal control gene, β-actin (EW779066). Real-time PCR was performed on a Light-Cycler 480II System (Roche, USA) in a volume of 20 μL containing 0.2 μM of each primer, 1 μL bovine serum albumin (1 mg/ml), 10 μL