Fish and Shellfish Immunology 80 (2018) 46–55

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Identification and functional characterization of NEMO in *Crassostrea gigas* reveals its crucial role in the NF-κB activation

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ARTICLE INFO

Keywords:
CgNEMO
Crassostrea gigas
NF-κB activation
Immune response
IκK complex

ABSTRACT

NEMO (NF-κB essential modulator) is one of the important regulatory subunits of the IκB kinase (IκK) complex that controls the activation of the NF-κB signaling pathway. Here, we have identified the homolog of NEMO from the pacific oyster *Crassostrea gigas*. *Cg*NEMO harbors the conserved the IκK binding region, NEMO ubiquitin binding domain and Zinc finger domain. In terms of tissue distribution, *Cg*NEMO is expressed in various tissues with an observed highest expression in the hemocytes. Furthermore, infection by two related *Vibrio* strains significantly increased *Cg*NEMO expression in the hemocytes. Cell culture based luciferase reporter assays showed that *Cg*NEMO activates the NF-κB reporter in a dose-pendent manner. Moreover, *Cg*NEMO was also found to counter the IkB-dependent inhibitory effect on NF-κB activation, providing a plausible mechanism of NF-κB activation by *Cg*NEMO. Meanwhile, site-directed mutagenesis demonstrated that the putative ubiquitination site K535 is required for the activation of NF-κB, implying that ubiquitination of NEMO may be involved in regulating its activity. Finally, RNAi mediated knockdown of *Cg*NEMO *in vivo* significantly compromised the bacterial induction of key cytokines TNF-α and IL-17, strongly suggesting a role for *Cg*NEMO in acute immune defense in oyster. In conclusion, this study provides new insights into our understanding about the evolution of NEMO mediated NF-κB activation and the induction of cytokine. Our findings may provide valuable information about diseases control and management in oyster aquaculture.

1. Introduction

NF-κB signaling pathway plays crucial role in diverse physiological processes, including immunity, inflammation, apoptosis and stress response [1–3]. In resting cells, NF-κB proteins are predominantly located in the cytoplasm, bound by its cognate inhibitor (inhibitors of NF-κB, IκB) [4]. However, the response to various stimuli, such as microbial pathogens, inflammatory cytokines, signaling from pattern recognition receptor, converge at the multi-protein IκB kinase (IκK) complex, resulting in the degradation of IκB protein via a cascade of phosphorylation, polyubiquitination, and proteasomal degradation [4]. The degradation of IκB protein leads to release of NF-κB and it's translocated into the nucleus and subsequent activation of target genes [5]. Generally, the IκK complex is composed of four subunits-two structurally related catalytic subunit (IκKα and IκKβ), one scaffold protein rich in glutamate, leucine, lysine and serine (ELKS) and one non-catalytic regulatory subunit, NF-κB essential modulator (NEMO) [6]. Among them, activation of IκK complex mainly depends on NEMO protein that is responsible for the initiation of the cascade that degrades IκB [7]. Therefore, in most situations NEMO, by being the core regulatory subunit of IκK complex, controls the activation of NF-κB signaling pathway. NEMO was originally identified to be an essential component of the IκK complex in the rodent cell line through genetic complementation experiments [8]. In human, the homolog of NEMO is located on the X-chromosome, and mutations within this locus results in many diseases and a defective activation of the NF-κB pathway [9].

Usually, the NEMO protein is composed of several function domains, including the IκK binding region, the NEMO ubiquitin binding domain and the Zinc finger domain. Several studies have shown that NEMO, through its different binding domains, interacts with various partner proteins resulting in successful activation of NF-κB signaling pathway. The IκK binding region of the NEMO protein is responsible for recruiting the catalytic subunit of IκK [10], while the proline-rich region near the zinc finger domain binds CYLD (cylindromatosis) protein

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https://doi.org/10.1016/j.fsi.2018.05.054
Received 6 April 2018; Received in revised form 17 May 2018; Accepted 29 May 2018
Available online 31 May 2018
1050-4648/

[11]. The recruitment of CYLD negatively regulates NF-κB signaling pathway [10]. Moreover, lysine site-specific ubiquitination of NEMO is required for tumor necrosis factor α (TNF-α) mediated IκK activation [12]. Therefore, NEMO could serve as a platform to recruit both activators and inhibitors of IκK, acting as a convergence point for serial signaling or multi-stumuli regulating NF-κB signaling pathway [12]. Thus, NEMO acts as a central modulator for and consequently converge serial signaling or multi-stimuli to regulate situation of NF-κB, emphasizing its central role in modulating NF-κB activation [13].

However, functional characterization of NEMO homolog in invertebrates revealed that its role as NF-κB activator is not evolutionary conserved. In arthropods, particularly in *Drosophila*, NEMO promotes Wnt/β-catenin and Notch signaling pathways to affect lateral inhibition during neurogenesis and cell movement during eye development, respectively [14–16]. Thus, it is still unclear whether NEMO homologs in other invertebrates are involved in the activation of NF-κB signaling pathway. Recently, genomic analysis of NF-κB signaling pathway in *Crassostrea gigas* revealed presence of all the components of NF-κB signaling pathway [16]. The existence of one homolog of oyster NEMO (*Cg*NEMO) was also identified in this genomic study [17]. Based on these evidences, it would be interesting to investigate the possible role of NEMO in the activation of NF-κB signaling pathway in this ecologically and economically important oyster species.

Here, our study demonstrates that *Cg*NEMO is highly expressed in oyster hemocytes and is rapidly induced in response to bacterial infection. Overexpression of *Cg*NEMO activates NF-κB reporter and relieves IκB-dependent inhibitory effect on NF-κB activity, indicating its role in activation of NF-κB. Mutagenesis of potential ubiquitination site implies CgNEMO-dependent NF-κB activation may rely on specific lysine modification. Moreover, knockdown of CgNEMO effectively suppresses bacterial infection caused cytokines induction. Therefore, this study shed light on the role of CgNEMO as a key regulator of NF-κB activation in the pacific oysters.

2. Methods

2.1. Cloning, sequencing and bioinformatic analysis of CgNEMO

The ORF sequence of *Cg*NEMO were identified from genome database of *C. gigas* [18]. On the basis of this sequence, the GeneRacer™ kit (Invitrogen, CA, USA) was used to obtain its 3′ and 5′ ends of the gene according to manufacturer's protocol. All primers used were listed in Table 1. The PCR products was cloned into the pGEM-T Easy Vector (Promega, WI, USA) and sequenced by ABI Prism 3730 DNA sequencer (PerkineElmer, Wellesley, MA, USA). The sequence of *Cg*NEMO was analyzed using the BLAST algorithm at NCBI (http://www.ncbi.nlm.nih.gov/blast) and the Expert Protein Analysis System (http://www.expasy.org/). 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/). Finally, the phylogenetic tree was generated by the neighbor-joining method of MEGA 7.0 [19], with bootstrap values estimated by 1000 replications.

2.2. Oysters, tissue collection and bacterial challenge

The Pacific oysters were purchased from Qingdao fishery market (Shangdong province, China) and acclimatized at 22–25 °C in tanks with circulating seawater for two weeks, prior to experiments. For the tissue distribution analysis, portions of different tissues such as the gills, mantle, adductor muscle, digestive gland, gonads as well the hemocytes were extracted from there different oysters. For the bacterial challenge analysis, each oyster was injected with 100 μl of either *Vibrio alginolyticus* strain GS03024 or *Vibrio parahaemolyticus* strain GS03002 (10^7 CFU) into adductor muscles using the G20 syringe needle with the outer diameter of 0.91 mm. The control groups were injected with equal volume of PBS. Hemocytes were collected at 3, 6, 12, 24, 48, 72 h post bacterial challenge from the pericardial cavity and immediately centrifuged at 1000 × g for 5 min at 4 °C. Five individuals were randomly sampled in each group at every time point.

2.3. Isolation of total RNA and real-time quantitative RT-PCR

Total RNAs were isolated from different tissue with TRIzol Reagent (Invitrogen, USA) and the integrity was checked by agarose gel electrophoresis. To synthesize cDNA, 1 μg of total RNA was utilized to perform reverse transcription reaction using PrimeScript™ RT Reagent Kit (TaKaRa, Japan). The expression of the target gene was measured using Quantitative real-time PCR. The reference gene GAPDH was employed here to normalize the expression level of the target gene. All the primers used for the real-time PCR analysis are listed in Table 1., and negative controls were also operated to avoid any genomic contamination. The Real time real-time PCR was performed on the Light-Cycler 480 (Roche) platform with a 20 μL reaction system, containing 10 μL of 2 × Master Mix (Roche, USA), 0.4 μL of the desired primer pair (10 mM), 1 μL of 1:10 diluted cDNA, and 8.2 μL of water. The dissociation curve was analyzed to confirm specificity of the amplicons. All experiments were performed in triplicates.

2.4. Plasmid construction and site-directed mutagenesis

To construct the expression vector for *Cg*NEMO, the sequence containing *Cg*NEMO ORF was amplified using primers NEMO-F4 and NEMO-R4 with the restriction sites *Hin*d III and *Bam*H I, respectively. The target amplicon was digested and inserted into pcDNA4.0. The four different *Cg*NEMO mutants were constructed with Q5® Site-Directed Mutagenesis Kit (NEB) utilizing the original pcDNA4.0-CgNEMO construct. All the primers used for the construction of mutants are listed in Table 1. Finally, all the resulting clones were confirmed by DNA sequencing and clones with the correct sequences were used for downstream experiments. The endotoxin free plasmids were obtained using Ezgene EndoFree Plasmid Kit (BioMIGA, USA).

2.5. Cell culture and transfection

The HEK293T cell were cultured in high-glucose DMEM (Gibco) containing 10% FBS and 10^5 U/L penicillin and 100 mg/L streptomycin at 37 °C in a humidified incubator under 5% CO2 as described previously. The transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, HEK293 cells were plated on 24-well culture plates (10^5 cells/well) for 24 h, followed by transient transfection with NF-κB reporter vectors, the desired expression vector and the control pRL-TK vector. The expression vector pcDNA-CgNEMO was synthesized in our lab while pcDNA-CgIκB and pcDNA-CgRel were gifts from Dr. Yu's Lab at South China Sea Institute of Oceanology [20]. Finally, Luciferase activities were measured 48 h later after transfection.

2.6. Dual-luciferase reporter assay

The Firefly and Renilla luciferase activities were measured for each sample using Dual-Luciferase Reporter Assay System (Promega life science, Madison, WI). After transfection, HEK293 cells in the culture plates were washed with 100 ml PBS twice, then lysed with 100 ml passive lysis buffer at room temperature for 10 min. Cell lysates were transferred to a culture plate and 100 ml of luciferase assay reagent II were added followed by the measurement of firefly luciferase activity. Then 100 ml Stop&Glo reagent were added to the plate prior to Renilla luciferase activity measurements. The Firefly luciferase data were corrected for transfection efficiency by using the Renilla luciferase activity. All experiments were performed in triplicates and the data was represented as means of the three biological replicates. All data are