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Molecular profiles and pathogen-induced transcriptional responses of prawn B cell lymphoma-2 related ovarian killer protein (BOK)

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ABSTRACT

In this study, we have reported a molecular characterization of the first B cell lymphoma-2 (BCL-2) related ovarian killer protein (BOK) from freshwater prawn *Macrobrachium rosenbergii* (*Mr*). BOK is a novel pro-apoptotic protein of the BCL-2 family that entails in mediating apoptosis to remove cancer cells. A cDNA sequence of *Mr*BOK was identified from the prawn cDNA library and its full length was obtained by internal sequencing. The coding region of *Mr*BOK yields a polypeptide of 291 amino acids. The analysis revealed that *Mr*BOK contains a transmembrane helix at V^{261}–L^{283} and a putative BCL-2 family domain at V^{144}–W^{245}. *Mr*BOK also possessed four putative BCL-2 homology domains including BH1, BH2, BH3 and weak BH4. The BH3 contains 21 binding sites and among them five residues are highly conserved with the aligned BOK proteins. The homology analysis showed that *Mr*BOK shared maximum similarity with the *Caligus rogercresseyi* BOK A. The topology of the phylogenetic tree was classified into nine sister groups which includes BOK, BAK, BAX, BAD, BCL-2, BCL-XL, NR13 and MCL members. The BOK protein group further sub-grouped into vertebrate and invertebrate BOK, wherein *Mr*BOK located within insect monophyletic clad of invertebrate BOK. The secondary structural analysis showed that *Mr*BOK contains 11 α-helices (52.2%) which are connected over random coils (47.7%). The 3D structure of *Mr*BOK showed three central helices (α6, α7 and α8) which formed the core of the protein and are flanked on one side by α1, α2 and α3, and on the other side by α4, α5 and α11. *Mr*BOK mRNA is expressed most abundantly ($P < 0.05$) in ovary compared to other tissues taken for analysis. Hence ovary was selected to study the possible roles of *Mr*BOK mRNA regulation upon bacterial (*Aeromonas hydrophila* and *Vibrio harveyi*) and viral [white spot syndrome virus (WSSV) and *M. rosenbergii* nodovirus] infection. During bacterial and viral infection, the highest *Mr*BOK mRNA transcription was varied at different time points. In bacterial infected ovary tissue, the highest mRNA expression was at 24 h post-infection, whereas in viral infection, the expression was highest at 48 h post-infection. Thus we can conclude that *Mr*BOK functions as an apoptotic protein in intracellular programmed cell-death pathway to counteract the anti-apoptotic proteins released by bacterial and viral pathogens at the time of infection. This is the first study that emphasizes the importance of BOK during bacterial and viral infection in crustacean.

1. Introduction

Apoptosis or programmed cell death (PCD) is a strongly conserved cellular process [especially, mitochondrial-endoplasmic reticulum (ER) dependent apoptosis] throughout evolution. Failure of this normal apoptotic process results in several pathological changes, most notably accumulation of damaged cell such as cancer

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or inappropriate cell loss, where the imbalance between cell growth and apoptotic rate leads to tumor development and metastasis [1,2]. To maintain the balance between cell division and cell death, cells possessing the B cell lymphoma 2 (BCL-2) family protein play a central role in the regulation of apoptosis (especially, in mitochondria) in response to various intrinsic and extrinsic stimuli [3,4]. The regulation of apoptosis by the BCL-2 family protein is vital for tissue homeostasis, embryo development, development of the immune and nervous systems and for the maturation of blood cells [5]. Cancer is mediated when there is an up-regulation of anti-apoptotic transcripts (e.g. BCL-2) and down-regulation of pro-apoptotic transcripts [e.g. B cell associated X protein (BAX)].

The recent investigation showed that BCL-2 family proteins are also involved in controlling neuronal activity, autophagy, immune responses, mitochondrial dynamics, calcium handling and ER-mitochondria cross talk [6–12]. BCL-2 family is a large and diverse group of globular protein that is composed of approximately 25 members [13,14]. The BCL-2 family protein are identified by the presence of specific sequence homology in their protein sequence called BCL-2 homology (BH1, BH2, BH3, and BH4) domains which are important for the functions including cell survival, dimerization, apoptotic regulators and mediating interaction among BCL-2 family members [15]. Based on the number of BH domains, BCL-2 family proteins are categorized into 1-4 BH motifs, BH-3 only and multi-domain with BH1-3 [3,4].

Based on the function, BCL-2 family is divided into two groups *i)* anti-apoptotic (BCL-2, BCL-XL, BCL-W, MCL-1, BCL-B) which protect cells from apoptosis and *ii)* pro-apoptotic proteins which are further subdivided into two sub-groups namely effector proteins (BAX, BAK, BOK and BOX) and BH3 only proteins (BID, BIM, PUMA, BAD, NOXA, BMF, HRK, and BIK). Both anti-apoptotic and pro-apoptotic BCL-2 family proteins are critical for tissue homeostasis [8,16,17]. All the anti-apoptotic proteins possess BH domains 1–4 and contain a hydrophobic transmembrane (TM) region to localize the cytoplasmic faces of intracellular membranes and apoptotic activity. In pro-apoptotic group, the effector proteins (BAX and BOK) contain BH domains 1–3 and a TM domain whereas, BH3-only proteins (BAD and BID) harbor only the BH3 domain and lacked in TM domain [16–18].

BCL-2 family genes have been reported in vertebrates including fish [19], amphibian [20] and mammal [21] and in invertebrates including mollusk [22], sea urchin [23], planarian [24], sea anemone [25] and sponge [26]. Recently we have reported bioinformatics characterization and gene expression studies on anti-apoptotic gene BCL-2from striped murrel [27]. BOK is an important novel pro-apoptotic protein of BCL-2 family that entails in mediating apoptosis to remove cancer cells. BOK was initially isolated and cloned from a rat ovarian fusion cDNA library established by two-hybrid screen method [28]. The same gene from mouse EST database was also identified and named as Metador (Mtd) [29].

The giant freshwater prawn *Macrobrachium rosenbergii* is a commercially important aquatic crustacean which has high local and international market value. In recent years, the commercial prawn cultivation industry is suffering from severe viral and bacterial infections which results in extreme economic loss. Thus it is necessary to characterize the immune molecules which are involved in immunological process against bacterial and viral pathogens. In this study, we report the first molecular characterization of BCL-2 related ovarian killer protein (BOK) from *M. rosenbergii* (*Mr*). The study includes bioinformatics analysis, tissue distribution and mRNA transcription upon viral [white spot syndrome virus (WSSV) and *M. rosenbergii* nodovirus (*Mr*NV)] and bacterial (*Aeromonas hydrophila* and *Vibrio harveyi*) infection of the pro-apoptotic protein *Mr*BOK. According to the best of our knowledge, this is the first study which correlates the activity of BOK against bacterial and viral pathogens in prawn.

2. Materials and methods

2.1. cDNA library, sequence identification and cloning of MrBOK

*M. rosenbergii*c cDNA library was established using total RNA isolated from *M. rosenbergii* tissue pool including muscle, gills, hemocyte, hepatopancreas and brain. The methodology on *M. rosenbergii* cDNA library development was given in our previous reports [30,31]. The developed library was annotated using Blast2GO program (http://www.blast2go.com/b2ghome), while screening a partial *Mr*BOK sequence was observed. To obtain its full length, we used ABI Prism-Bigdye Terminator Cycle Sequencing Ready Reaction kit and applied internal sequencing method using the forward (*Mr*BOK F1: CCTCTCCCATTCCTGCTTATC) and reverse (*Mr*BOK R2: GTCCTTTGAGGGCAAGGATTAG) primers and the sequence was obtained using an ABI 3730 sequencer.

2.2. Bioinformatics characterization of MrBOK

DNAssist (ver. 2.2) program was applied to obtain physico-chemical properties, coding, non-coding and protein sequence of *Mr*BOK. Homology analysis was carried out using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast). N-terminal transmembrane region was predicted using DAS transmembrane prediction program (http://www.sbc.su.se/~miklos/DAS). Domain and motif were analyzed using the following PROSITE program: http://prosite.expasy.org/scanprosite/. Multiple sequence alignment was constructed on ClustalW 2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Evolutionary relationship of *Mr*BOK was analyzed by constructing a Neighbor-Joining phylogenetic tree at MEGA (ver. 5.05). *Mr*BOK secondary structure was established in SOPMA program and it was viewed through Polyview program (http://polyview.cchmc.org) for further analysis. Three dimensional structure of *Mr*BOK was established using the following I-Tasser server: http://zhanglab.ccmb.med.umich.edu/I-TASSER. Further, the obtained 3D structure was validated using Ramachandran plot analysis (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php).

2.3. M. rosenbergii

Prawns (15 ± 2 g) were collected from a commercial prawn farm (Nellore, Andhra Pradesh, India). They were transported to the aquarium at Division of Fisheries Biotechnology & Molecular Biology, SRM University in oxygenated polyethylene bags. They were maintained in 15 rectangular plastic containers (50 L) with aerated and de-chlorinated freshwater (water quality: dissolved oxygen, 5.8 ± 0.2 mg/L; water temperature, 28 ± 1 °C and pH, 7.2 ± 0.1). A maximum of 10 prawns per container was maintained during the experiment. The prawns were acclimatized for a week before being challenged to microbial pathogens. During acclimatization, the prawns were fed *ad libitum* two times daily at 09.00 and 16.00 h with a commercial pellet diet (Cargill Animal Nutrition, Andhra Pradesh, India).

2.4. Construction of disease challenge model and collection of tissue

In our earlier studies [32,33] we have reported the construction of disease challenge model between prawn and pathogenic microbes. Briefly, WSBV and *Mr*NV infected prawn tail tissue was homogenized in 2% NaCl solution and injected to the prawn. Tissue homogenate prepared from healthy tissue served as control. For bacterial infection, the individuals were injected (50 μL per 15 g