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*Ch*Bax/Bak as key regulators of the mitochondrial apoptotic pathway: Cloned and characterized in *Crassostrea hongkongensis*

Zhiming Xiang [a, 1], Fufa Qu [a, b, 1], Fuxuan Wang [a, b], Shu Xiao [a], Li Jun [a], Yang Zhang [a], Ziniu Yu [a, *]

[a] *CAS Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, Guangdong, China*
[b] *University of Chinese Academy of Sciences, 19A Yuquan Road, Beijing 100049, China*

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ABSTRACT

Apoptosis has been primarily investigated in mammals, and little is known about apoptosis in mollusks. The proteins Bax and Bak play critical roles in the mitochondrial apoptosis pathway and in determining cell fate. In this study, *Ch*Bax and *Ch*Bak, homologs of the well-known Bax and Bak proteins, were identified from the oyster *Crassostrea hongkongensis*. The *Ch*Bax/Bak proteins consist of 207/232 amino acids with the typical domains found in BCL-2 family members. *Ch*Bax and *Ch*Bak mRNA expression were detected in all 8 of the selected oyster tissues and at the different stages of development. Fluorescence microscopy revealed that the full-length proteins of *Ch*Bax/Bak were located in the cytoplasm and mitochondrial outer membrane, of HEK293T cells, respectively. Furthermore, both of the genes' expression levels were found to increase in the hemocytes of oysters challenged with pathogens. The over-expression of *Ch*Bax or *Ch*Bak activates the p53-Luc reporter gene in HEK293T cells in a dose-dependent manner. These results indicate that *Ch*Bax and *Ch*Bak may play important roles in the mitochondrial apoptotic pathway in oysters.

1. Introduction

Apoptosis is an important mechanism in adaptive responses to environmental stress, which may be responsible for the ecological success of an organism and reflect their ability to modify different physiological functions when environmental conditions become unfavorable [1]. Mitochondria play a pivotal role in the process of apoptosis and are considered to be essential for the completion of this cell death program involving many signals from different cellular compartments and functional pathways [2].

By changing the MOM (mitochondrial outer membrane) permeability, mitochondria initiate apoptosis and lead the so-called executive phase, which is under the control of the Bcl-2 family of proteins. In mammals, the mitochondria apoptosis pathway is controlled by Bcl-2 family members, which have one or more Bcl-2 homology domains (BH1, -2, -3, and -4); these domains are important for the heterodimeric interactions that regulate apoptosis, and the Bcl-2 family is divided into pro- and anti-apoptotic members [3]. Anti-apoptotic Bcl-2 family members, including Bcl-2, Bcl-xL (Bcl-extra long), A1, Bcl-wand Boo (Bcl-2 homolog of ovary) protein, have a transmembrane domain and a BH domain (BH1-4) [4]. The myeloid cell leukemia factor-1 (Mcl-1) is the only anti-apoptotic Bcl-2 protein with three BH domains (BH1, BH2, and BH3) and a transmembrane domain, and it does not contain a BH4 domain [5]. The pro-apoptotic proteins of the Bcl-2 family consist of two subgroups, one includes BH1-3 domains (e.g., Bax, Bak, and Box), and the other only includestheBH3 domain (e.g., Bid, Bim, Bad, Bid, Noxa and Puma). Notably, the pro-apoptotic protein Bcl-xS only has BH3 and BH4 domains.

Abbreviations: HIV-1, human immunodeficiency virus-1; MOMP, mitochondrial outer membrane permeabilization; IMS, inter-membrane space; TM, transmembrane; ORF, open reading frame; UTR, untranslated region; Bcl2, B-cell CLL/Lymphoma 2; Bax, Bcl2-associated X protein; Bak, Bcl2-antagonist/killer; VDAC, voltage-dependent anion channel; IAPs, inhibitor of apoptosis proteins; Smac, second mitochondria derived activator of caspase; Bid, BH3 interacting domain death agonist; Mcl-1, myeloid cell leukemia factor-1; ANT, adenine nucleotide translocator; Mw, molecular weight; pI, isoelectric point; qPCR, real-time quantitative PCR; RACE, rapid-amplification of cDNA ends; EGFP, enhanced green fluorescent protein; hpi, hour post infection.

* Corresponding author. Tel./fax: +86 20 8910 2507.
E-mail address: carlzyu@scsio.ac.cn (Z. Yu).

[1] These authors contributed equally to this work.

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Bax and Bak are pro-apoptotic proteins that contain BH1, BH2, and BH3 domains and are activated by the truncated BID (tBID) [6]. The activated Bax and Bak hetero-oligomerize and participate in the formation of pores in the outer mitochondrial membrane that lead to MOM permeabilization [7]. The latter results in the release of pro-apoptotic factors, such as cytochrome c and second mitochondria derived activator of caspase (Smac), from the intermembrane space (IMS). Then, the released cytochrome c leads to the activation of caspases, which are proteases that cleave key cellular proteins. This leads to many physical processes of apoptosis, including condensed nuclei, DNA laddering and exposure of phosphatidylserine to the outer leaflet of the plasma membrane [8]. Anti-apoptotic proteins sequester BH3-only proteins to prevent the activation and homo-oligomerization of both Bax and Bak. Both of these proteins play key roles in apoptosis caused by MOM permeabilization [1,9,10]. Many hypotheses have been proposed to explain the apoptosis mechanisms involving Bak and Bax. In general, both of these proteins have been suggested to oligomerize and form pores in the mitochondrial outer membrane [11] or to interact with mitochondrial proteins, such as megapore (VDAC and ANT), to release apoptosis inducing factors from the mitochondria [12]. In response to stress activation, Bax/Bak form hetero- or homodimers to each other, both of these proteins may be able to partially substitute for each other. Bax and Bak double-knockout cells fail to undergo MOMP in response to many different apoptosis inducers, while the single knockout of either gene in cells has far less of an effect on sensitivity [9].

It is known that bacterial infection can trigger apoptosis by modulating the expression of apoptosis-related genes in response to a variety of pro-apoptotic or inflammatory stimuli [13,14]. In oysters, transcriptomic analysis indicated that many mitochondrial genes respond in immunity defense [15]. However, the oyster has a complicated apoptosis regulation system, including 48 genes that code for inhibitor of apoptosis proteins (IAPs), while only several have been identified in humans [15]. To further characterize oyster apoptosis, particularly the mitochondrial apoptosis genes, Bax and Bak were identified and cloned from *Crassostrea hongkongensis*. These identified genes (*Ch*Bax/Bak) were found to be key genes associated with the mitochondrial apoptotic pathway and characterized in the present study.

Table 1
Sequences of designed primers used in this study.

Primer	Sequence (5′ to 3′)	Comment
5′RACE-OU Bax R	CTTCAAAAGGTAGAGGCCAGCACAA	5′RACE
5′RACE-IN Bax R	ACTGTTAATCCTAGGCCGACGACTC	
3′RACE-OU Bax F	TCATAGATAGAGGCGGATGGGAT	3′RACE
3′RACE-IN Bax F	ACCAGTTTTTTGGAGTCGTCGGCCT	
Bax ORF F	CCAGAAAGGTTGAATGAAAGAGGTT	ORF amplification
Bax ORF R	AATACCAGATGTGTCCCATTCGAAC	
pCDNA3.1Bax F	CTTGGTACCATGACATCGTCTGACA	Bax-his fusion
pCDNA3.1Bax R	CCCTCTAGATTTGAGCGCCTTCAAA	protein
pEGFPN1Bak F	TTTAAGCTTATGACATCGTCTGACA	Bax-EGFP fusion
pEGFPN1Bak R	AAAGTCGACTTGAGCGCCTTCAAAA	protein
qPCR Bax F	TTTACAAGACCCTGGCACAC	qPCR
qPCR Bax R	GTAGGCAAAGTAGAATAGACATCC	
5′RACE-OU Bak F	CCTATTTATGTCATCTCCAATCCTA	5′RACE
5′RACE-OU Bak R	CACTTCATAGGCTGTATCTTCATTC	
3′RACE-OU Bak F	CAAGTCAAGGGGGATGGAGTGG	3′RACE
3′RACE-OU Bak R	CAAGTCAAGGGGGATGGAGTGG	
Bak ORF F	GGATGACACAACCCAAACAAAGACG	ORF amplification
Bak ORF R	AAGTTTTTCATTTCAGTTTTTGTCAGT	
pCDNA3.1Bak F	GGATCCATGGCTTACTGGGACGGTG	Bak-his fusion
pCDNA3.1Bak R	CTCGAGACTCTTCTTGCTGAAGTAG	protein
pEGFPN1Bak F	TTTAAGCTTATGGCTTACTGGGACG	Bak-EGFP fusion
pEGFPN1Bak R	AAAGTCGACCTCTTCTTGCTGAAGT	protein
qPCR Bak F	CGCCCGACACTGAGGAGAAT	qPCR
qPCR Bak R	TTTATGTCATCTCCAATCCTAGCCA	
GAPDH-qPCR F	AACTCGAGATGTCAGAGTCACGCC	qPCR
GAPDH-qPCR R	GTATGATGCCCCTTTGTTGAGTC	

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

2. Materials and methods

2.1. Characterization of a full-length cDNA of ChBax and ChBak

Through a homolog search of the *C. hongkongensis* hemocyte EST library using a blast program (http://www.ncbi.nlm.nih.gov/blast), two EST were found to be homologous to the known Bax/Bak genes and were designated accordingly as *Ch*Bax/Bak (GenBank # KM262836/KM262837). Next, RACE-PCR was performed using cDNA from *C. hongkongensis* and the BD SMART RACE cDNA Amplification kit (Clontech, USA). Based on the identified EST sequences, *Ch*Bax/Bak gene-specific primers for RACE were designed, which included 5′RACE-OU/IN and 3′RACE-OU/IN (Table 1) for 5'- and 3' -RACE, respectively. For construction of a full-length *Ch*Bax/Bak cDNA, the sequences obtained by over lapping ESTs and fragments were amplified via RACE. Following the full-length cDNA sequence, the open reading frames of *Ch*Bax/Bak were amplified using the up-and downstream primers Bax/Bak ORF-F and-R (Table 1). PCR products were then cloned into the pGEM-T easy vector (Promega, USA) for sequencing by an ABI 3730DNA sequencer (Applied Biosystems, USA).

Amino acid sequences were deduced using DNAstar. Protein domains and mitochondrial targeting sequences were predicted using an online program (http://smart.embl-heidelberg.de andhttp://www.cbs.dtu.dk/services/TargetP). The amino acid sequences of *Ch*Bax/Bak were aligned with sequences of representative invertebrate and vertebrate Bax/Bak proteins by MegAlign in DNAstar. Phylogenetic analyses of *Ch*Bax/Bak were performed using ClustalW, which was chosen to correct the distances for multiple substitutions at a single site.

2.2. Animals, tissue collection, and microbe challenge

Healthy *C. hongkongensis* (two years old, shell height 10.00 cm ± 0.05 cm) were obtained from our experimental station in Zhanjiang, Guangdong province, China. Oysters were maintained at 24 ± 1 °C in tanks with circulating seawater for one week before the experiments. Oysters were fed twice daily with marine algae, *Tetraselmis suecica* and *Isochrysis galbana*.

For expression profiles in different tissues, equal amounts of tissue from five healthy oysters were pooled for each tissue-specific expression analysis. The tissues collected included the gill, mantle, adductor muscle, heart, digestive gland, gonads, labial palps and hemocytes. For the expression analysis at different developmental stages, samples were collected at the following stages: fertilized egg, 2-cell, 4-cell, blastula, gastrula, trochophore and D-larvae.

For the pathogen challenge, oysters were randomly divided into challenge and control groups. For the preparation of microbial samples, *Vibrio alginolyticus* (gram-negative bacteria), *Staphylococcus haemolyticus* (gram-positive bacteria), and *Saccharomyces cerevisiae* (fungus) were cultured separately. Bacteria and fungi were collected by centrifugation and then resuspended in 0.1 M phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH7.4) with 1.0×10^9 cells/L. Equal volumes of the three suspensions were mixed to generate a stock suspension for injection. An equal volume of PBS was used as a control. A microbial suspension or PBS was injected into the adductor muscle of *C. hongkongensis*. After injection, the oysters were returned to seawater tanks, and 5 individuals's hemocytes were randomly sampled at 0, 3, 6, 12, 24, 48, 72 and 96 h post-injection. Samples were stored in liquid nitrogen until analyzed.