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Cloning, characterization and expression analysis of a caspase-8 like gene from the Hong Kong oyster, *Crassostrea hongkongensis*

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ABSTRACT

Apoptosis plays a key role in many biological processes, including homeostasis within the immune system. A family of cysteine proteases, the caspases, constitutes the core of the apoptotic machinery. We have characterized the first bivalve caspase-8 ortholog from the Hong Kong oyster *Crassostrea hongkongensis* (designated *Ch*Caspase-8). The full-length cDNA is 1945 bp in length encoding a putative protein of 557 amino acids that contains two N-terminal DED domains, and a CASc domain at the C-terminus. *ChCaspase-8* is ubiquitously expressed in oysters, with highest expression levels in the gonad and labial palps. Following microbial infection, the expression of *ChCaspase-8* increased in hemocytes from 12 to 72 h post-challenge. When expressed in HeLa cells, *Ch*Caspase-8 is located in the cytoplasm, while over-expression of *Ch*Caspase-8 in HEK293T cells activates the transcriptional activities of NF-κB. These results indicate that *Ch*Caspase-8 might play an important role in the immune and apoptotic responses of oysters.

1. Introduction

Apoptosis, or programmed cell death, plays an important role in many biological processes, including embryogenesis, maintenance of normal tissue, and immune system homeostasis [1–3]. The caspase family of proteins constitutes the core of the apoptotic machinery and the proteins are categorized as either initiators or effectors of apoptosis [4,5]. As a member of the caspase family, caspase-8 is well characterized as a proximal enzyme involved in death receptor mediated or "extrinsic" apoptosis [6–8]. The known caspase-8 proteins consist of two death effector domain (DED) motifs at the N-terminus that cause homotypic interactions and can trigger cell death following death effector domain-mediated recruitment to the "death-inducing signaling complex (DISC)" [9]. In a word, caspase-8 plays a critical role in the regulation of programmed cell death (apoptosis).

Caspase activity has been characterized in detail in mammals as well as in invertebrate phyla in organisms such as the fruit fly and *Crassostrea elegans* [10]. Only a few reports describe initiator and executioner caspases in mollusks, even though they are the second most diverse group of animals (next only to arthropods) with about 93,000 extant species [11]. Although an *ab*Caspase-8 like protein was predicted in abalone (*Haliotis diversicolor*), the protein contains only 336aa with no typical DED domains [12]. On the other hand, *Cg*Caspase-1 and *Cg*Caspase-2 were identified in *Crassostrea gigas* [13]. Here we report the identification and characterization of a full-length cDNA of a *ChCaspase-8* gene in the Hong Kong oyster *Crassostrea hongkongensis*. We investigated the expression of this gene in multiple tissues, including hemocytes following bacterial challenge, as well as the subcellular location of the resulting protein and its role in activating cellular signaling pathways, in the hopes of better understanding its association with immune and apoptotic responses of oysters.

2. Materials and methods

2.1. Characterization of a full-length cDNA of ChCaspase-8

The cDNA ready for RACE-PCR was prepared using the BD SMART RACE cDNA Amplification kit (Clontech). *Ch*Caspase-8 (GenBank# KC822928) gene specific primers for PCR amplification were designed according to orthologs of *Ch*Caspase-8 identified by BLAST (www.ncbi.nlm.nih.gov/blast) when searching a *C. hongkongensis* hemocyte EST library which was constructed and sequenced by our

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lab (data not yet published). The protein motifs were analyzed with PROSITE (http://www.expasy.ch/prosite/) and SMART (http://smart.embl-heidelberg.de/smart). The *Ch*Caspase-8 gene-specific primers for 5′RACE were 5′-GACACTAAGTTTCTATTTTTGTATTGG-3′ (outer primer) and 5′- CTGTTCGAGTGTCTTCACGTCA-3′ (inner primer), and gene specific primers for 3′RACE primers were 5′-ACATCAACATCCTGGCT-3′ (outer primer) and 5′-GATCTGAGGTAAATCCCAGTGAC-3′ (inner primer). There was an overlay between target sequences of RACE PCR for later reconstruction of *Ch*Caspase-8 full-length cDNA sequences. The open reading frame of *Ch*Caspase-8 was amplified with up- and down-stream primers 5′-TCAGTTTATTATTAGACAGCAGGAA-3′ and 5′-GTTTGCTGACACCAGTCTTATG-3′, and then the PCR products were cloned into pGEM-T easy vector (Promega) for sequencing with an ABI3730 DNA sequencer (Applied Biosystems). Analyses of *Ch*Caspase-8 sequences from representative species and phylogenetic tree construction were performed with DNAstar software.

Comparison of *Ch*Caspase-8 and their homologues were performed using MegAlign in DNAstar. Phylogenetic analyses of amino acid sequences deduced from *Ch*Caspase-8 sequences were also conducted using the same program. The Clustal method was used to correct the distances for multiple substitutions at a single site. GenBank accession numbers of *Ch*Caspase-8 sequences from available model species used for the analysis were: NP_001073594 [*Homo sapiens*], CAA07677 [*Mus musculus*], NP_989923 [*Gallus gallus*], AAS91705 [*Danio rerio*], ACN10768 [*Salmo salar*], ADB80148 [*Branchiostoma floridae*], ACP41139 [*Tubifex tubifex*], KC822928 [*C. hongkongensis*] and HM582204 [*Haliotis discus*].

2.2. Animals, tissue collection, and bacterial challenge

C. hongkongensis (two-years old with averaging 90 mm in shell height) were obtained from Zhanjiang, Guangdong province, China, and maintained at 22–25 °C in tanks with circulating seawater for one week before use. The oysters were fed twice daily with *Tetrasel missuecica* and *Isochrysis galbana*. Eight tissues including gill, mantle, labial palps, adductor muscle, heart, digestive gland, gonads, and hemocytes were collected from five healthy oysters for analysis of tissue-specific expression.

For bacterial challenge, 150 oysters were randomly divided into a bacterial challenge groups and a control group. Live *V. alginolyticus* and *Salmo cerevisiae* were resuspended in 0.1 M phosphate buffered saline (PBS) at 1.0×10^9 cells/L. A 100ul bacterial suspension, or PBS, was injected into the adductor muscle of *C. hongkongensis*. Hemocytes were collected from five individuals at 0, 3, 6, 12, 24, 48, 72 and 96 h post injection.

2.3. RNA isolation and quantitative PCR analysis of ChCaspase-8

RNA was isolated from *C. hongkongensis* samples using TRIzol (Invitrogen, USA) and DNase I treatment (Promega, USA) according to the manufacturers' protocols. Gene-specific primers (up-and down-stream primers, 5′-TCTCCGACAGATGAAATGAATG-3′, 5′-ATGGAGGTCAATGAGGGATAGA-3′) for qPCR were designed to amplify an approximately 150 bp fragment. *C. hongkongensis GAPDH* primers (up-and down-stream primers: 5′-GGATTGGCGTGGTGGTAGAG-3′ and 5′- GTATGATGCCCCTTTGTTGAGTC-3′) were used as an internal control.

Quantitative PCR was performed on a Light-Cycler 480II System (Roche, USA) as a three-step RT-PCR using SYBR Premix Ex TaqTM (TOYOBO, Japan) according to the manufacturer's instructions. Reactions were performed in a volume of 20 μl containing 10 μl of 2 × SYBR Green PCR Master Mix, 1 μl of 10 μM primers, 8 μl of nuclease-free water, and 1 μl of cDNA template. Cycling parameters included 95 °C for 5 min, followed by 40 cycles of 95 °C for 10s, 56 °C for 15s and 72 °C for 15s. Data were quantified using the $2^{-\Delta\Delta Ct}$ method based on Ct values of *ChCaspase-8* and *ChGAPDH*. The PCR efficiencies of target and reference genes were verified as being approximately equal. Values were considered significant at $P < 0.05$. Statistical analysis was performed using SPSS15.0 for Windows.

Table 1
Primers used in this study.

Primers name	5′–3′
*Ch*Casp-8 GFPXhoI F:	5′-AAACTCGAGATGAAGATGACACACC-3′
*Ch*Casp-8 GFPHindIII R:	5′-TTCAAGCTTACACTTATCGAACTGC-3′
*Ch*Casp-8 HisHindIII F:	5′-TAAAGCTTCTATGAAGATGACACAC-3′
*Ch*Casp-8 HisXhoI R:	5′-TTTCTCGAGACACTTATCGAACTGC-3′
*Hu*Casp-3 HisBamH I F:	5′-CTCGGATCCCGCTATGGAGAACACT-3′
*Hu*Casp-3 HisXho I R:	5′-GACCTCGAGAAAAATAGAGTTCTTT-3′

2.4. Plasmid construction

The following plasmids were constructed and used for mammalian cell transfection. The pMD18-T vector containing the full length *Ch*Caspase-8 sequence was used as a template. Primers for construction of various expression vectors are listed in Table 1: 1) Construction of pEGFP-N1-*Ch*Caspase-8: a DNA fragment containing the full-length coding region of *ChCaspase-8* was amplified to generate a fusion protein of *Ch*Caspase-8 and the enhanced green fluorescent protein; 2) Construction of pcDNA-His-*Ch*Caspase-8 and *Hu*Caspase-3(Genbank#:NM_032991): the DNA fragment containing the full-length coding region of *Ch*Caspase-8 and *Hu*Caspase-3 were amplified to generate a fusion protein of *Ch*Caspase-8 or *Hu*caspase-3 with a His tag. Inserted fragments of each recombinant plasmid sequenced.

2.5. Cell culture, transient transfection and subcellular localization

HeLa and HEK293T cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal calf serum (FCS, Gibco) and antibiotics (streptomycin and penicillin, Gibco) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Transient transfection was conducted with lipofectine 2000 (Invitrogen) according to the manufacturer's instructions. HeLa cells were seeded on sterile microscope cover glasses placed in a 6-well plate, and HEK293T cells were directly seeded in 24-well plates prior to transfection. Cells were transfected in a serum-free culture medium. After 4–6 h, the medium was replaced by complete medium with 10% fetal calf serum (FCS) and antibiotics. The transfected HEK293T cells were lysed for the luciferase assay and the HeLa cells were fixed for laser confocal imaging at 48 h post transfection.

Forty-eight hours after transfection, HeLa cells were washed with PBS buffer for 5 min, then fixed with 4% paraformaldehyde for 10 min and stained for the nuclear fractions with 4′, 6-diamidino-2-phenylindole hydrochloride (DAPI). The cells transfected with fluorescent vectors were directly observed under fluorescent microscopy.

2.6. Luciferase reporter assay

The analyses of NF-κB and p53/p21 response promoter (human) activation were conducted using luciferase reporter genes. The NF-κb-luc (Cat No: 219077) and p53-luc (Cat No: 219083) contain the binding sites for NF-κB and p53, respectively, both of them were purchased from Stratagene. The p21-luc (Cat No: 16451) was purchased from Addgene, which contains a promoter of P21. During transfection, HEK293T cells Cells were plated on a 24-well plate, grown to 50% density and transfected as above. Cells were co-transfected with 100 ng/well of each pRL-TK and luciferase