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Cloning and characterization of an apoptosis-related DNA fragmentation factor (DFF) from oyster, *Crassostrea hongkongensis*

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ABSTRACT

Apoptosis plays an important pathophysiological role in the homeostasis of immune systems. DNA fragmentation factors (DFFs) have been shown to be essential for DNA fragmentation, and the resultant DNA fragments follow a laddering pattern during apoptosis in vertebrates. In invertebrates, the functions of the DFF orthologs are not well characterized; therefore, we cloned and characterized a bivalve DFFA ortholog from the Hong Kong oyster *Crassostrea hongkongensis* (designated *Ch*DFFA). The full-length cDNA of *Ch*DFFA is 1186 bp in length and encodes a putative protein of 200 amino acids that contains an N-terminal CAD domain and a DFF-C domain at its C-terminus. Real-time RT-PCR results showed that *Ch*DFFA is ubiquitously expressed in several tissues, and its highest expression is in gill. Following a 3- to 48-h challenge by microbial infection, the expression of *Ch*DFFA increased in hemocytes. Using fluorescence microscopy, *Ch*DFFA was localized in nuclei when exogenously expressed in HeLa cells. In addition, overexpression of *Ch*DFFA inhibited the transcriptional activities of p53/p21-Luc reporter genes in HEK293T cells. These results suggest that *Ch*DFFA may be involved in immune response reactions in the Hong Kong oyster *C. hongkongensis*.

1. Introduction

Apoptosis plays important roles in the development, cellular homeostasis and, especially, oncogenesis of mammals; and it is characterized by a series of remarkable changes in cell morphology, including membrane blebbing, chromatin condensation and DNA fragmentation [1]. Among the changes in cell morphology, DNA fragmentation caused by the activation of endonucleases is one of the hallmarks of apoptosis in a variety of animal models [2]. Several nucleases, such as the DNA fragmentation factors (DFFs), endonuclease G, AIF, DNase I and DNase II, were found to be involved in the degradation of genomic DNA during apoptosis [3–5]. Internucleosomal DNA fragmentation is a major sign of the degradation of genomic DNA, and the DNA fragmentation factors (DFFs) that are responsible for this type of DNA degradation belong to the CAD family [6]. The DFF families, including DFFA and DFFB, have been shown to be essential for apoptosis. DFFA, which is a 45-kD protein, is the substrate for caspase-3 and triggers DNA fragmentation during apoptosis [7], while DFFB, which is a 40-kD protein, has been shown to trigger DNA fragmentation and chromatin condensation during apoptosis. The former inhibits DFFB by serving as a specific chaperone that forms a heterodimer with DFFB in normal cells. In humans, the DFFs become activated when DFFA is cleaved by caspase-3 at amino acids 117 and 224 and dissociates from DFFB, resulting in the induction of the nuclease activity of the DFFs [3,7].

DNA fragmentation is a necessary step for the disposal of large DNA fragments from dying cells, which is critical for maintaining normal tissue homeostasis [8]. Many genes, such as p53, which is a DNA-damage checkpoint gene that monitors cell cycle progression, are involved in this biological process [9]. Although no strong evidence exists for the tight association between p53 and DNA fragmentation factors, recent studies suggested that DNA fragmentation factors maintain chromosome stability in a p53-independent manner [10]. However, when cells receive apoptotic bodies, p53 or p21-deficient cells are able to resist apoptosis when DFFA is overexpressed, but apoptosis in normal cells is induced [11–13]. The relationships between p21/p53 and the DFFs are relatively complicated; therefore, more detailed studies are required.

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Although DFF activity has been well characterized in mammals, no DFF homolog has been described in invertebrates, even in *Crassostrea elegans* [14]. In mollusks, the second most diverse group of animals with approximately 93,000 extant species [15], an ortholog/homolog of DFFA (named *Mg*DFFA) was recently reported in *Mytilus galloprovincialis*. The gene expression of *Mg*DFFA was up-regulated following UV irradiation and was suggested to be involved in UV-induced apoptosis [16]. To investigate the DFF ortholog in ***Crassostrea hongkongensis***, which is an aquaculture species with high commercial value along the coastal waters of the South China Sea, we cloned and characterized a homolog of DFFA named *Ch*DFFA. We found that *Ch*DFFA was expressed in multiple tissues and localized in the nucleus. Importantly, *Ch*DFFA expression was up-regulated in hemocytes when challenged by bacterial infection. In line with its function in cell cycle arrest and apoptosis pathways, exogenously expressed *Ch*DFFA resulted in inhibition of the activation of p53/p21-Luc reporters in 293T cells. Our results suggest that *Ch*DFFA may provide an opportunity for better understanding the function of DFF in immune and apoptotic responses in mollusks.

2. Materials and methods

2.1. cDNA cloning and recombinant plasmid construction of ChDFFA

We constructed an EST library from *C. hongkongensis* hemocytes, and 39,792 sequences were obtained following 454 sequencing and data analysis through a homologue search using the BLAST program (http://www.ncbi.nlm.nih.gov/blast). An EST was found to be homologous to the DNA fragmentation factor subunit alpha of *Crassostrea gigas* (EKC41810.1) and was designated *Ch*DFFA. To obtain the full-length *Ch*DFFA sequence, RACE-PCR was performed using cDNAs from *C. hongkongensis* and the BD SMART RACE cDNA Amplification kit (Clontech, USA). Based on the identified EST sequence, the *Ch*DFFA gene-specific primers for RACE amplification were designed. The *Ch*DFFA gene-specific primer pairs included 5′RACE-OU/IN and 3′RACE-OU/IN for 5′- and 3′ -RACE (Table 1), respectively. Sequences were obtained by overlapping the ESTs and amplifying the fragments via RACE to reconstruct the full-length *Ch*DFFA cDNA. Using the full-length cDNA sequence, the open reading frame of *Ch*DFFA was amplified using the up-stream primer ORF-up and down-stream primer ORF-down (Table 1). The PCR products were then cloned into the pGEM-T easy vector (Promega, USA) and sequenced using an ABI 3730 DNA sequencer (Applied Biosystems, USA).

Amino acid sequences were deduced using DNAstar. Protein domains and a nuclear localization signal (NLS) were predicted using the networks program cNLS Mapper (http://smart.embl-heidelberg.de and http://nls-mapper.iab.keio.ac.jp). Multiple sequence alignment of the deduced amino acids was performed using ClustalX 1.81, and a neighbor-joining (NJ) phylogenetic tree was constructed using the MEGA5.0 package. Reliability of branching was tested using bootstrap re-sampling (with 1000 pseudo-replicates). To examine whether the gene was under positive or purifying selection, selection pressures were examined using site-specific and branch-site models, as implemented in the PAML 4.4 package. The codons of the DFFs were aligned using ClustalW implemented into the MEGA5.0 program. Gap characters were removed, and the likelihood value was calculated using the Codeml program in PAML, a non-constrained model (allowing each branch to have distinct dN and dS values) and a same evolutionary rate model [17]. Asymmetric divergence was assessed using the Chi-square test of the twice log-likelihood difference between the two models. Values of $\omega > 1$ indicated positive selection, while $\omega = 1$ and $\omega < 1$ indicated neutral evolution and purifying selection, respectively.

For subcellular localization of *Ch*DFFA, the pEGFP-N1-*Ch*DFFA plasmid was constructed, and the recombinant protein was expressed in mammalian cells. The primers for the various expression vectors (pEGFP-N1 and pCDNA3.1) are listed in Table 1. The full-length cDNA of *Ch*DFFA was cloned in-frame into the pEGFP-N1 (CAT#6085-1, Clontech) vector to produce a GFP-*Ch*DFFA fusion protein for subcellular localization. In parallel, the ORF of *Ch*DFFA was cloned into the pCDNA3.1 (CAT#V810-20, Invitrogen) vector to express the *Ch*DFFA-His tagged recombinant protein.

2.2. Oysters, tissue collection and immune challenge

Healthy *C. hongkongensis* (two-year-old, shell height 10.00 cm–0.05 cm) were obtained from an oyster culture farm in Zhanjiang, Guangdong province, China. The oysters were maintained at 24 ± 1 °C in tanks with circulating seawater for one week before the experiments and fed twice daily with the marine algae *Tetraselmis suecica* and *Isochrysis galbana*.

For the gene expression profiles in different tissues, equal amounts of tissues from five healthy oysters were pooled for each tissue-specific expression analysis. Tissues were collected from the gill, mantle, adductor muscle, heart, digestive gland, gonads and hemocytes. For bacterial challenge, oysters were randomly divided into challenge and control groups. *Vibrio alginolyticus* (Gram-negative bacteria), *Staphylococcus haemolyticus* (Gram-positive bacteria) and *Saccharomyces cerevisiae* (fungus) were cultured separately. Bacteria or fungi were collected using centrifugation followed by re-suspension in 0.1 M phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) with 1.0×10^9 cells/L. Equal volumes of the three suspensions were mixed to provide the stock suspension for injection, and the same volume of PBS was used as a control. The microbial suspension or PBS was injected into the adductor muscle of *C. hongkongensis*. After injection, the oysters were returned to the seawater tanks, and 5 individuals were randomly sampled at 0, 3, 6, 12, 24 and 48 h post-injection. The samples were stored in liquid nitrogen until use. Total RNA samples were extracted with TRIzol (Invitrogen) according to the manufacturer's instructions.

2.3. The mRNA expression analysis of ChDFFA by quantitative RT-PCR

The total RNAs of each sample were treated with DNase I (Promega) and verified by agarose gel electrophoresis. The RNA concentration was determined by measuring the absorbance at 260 and 280 nm; and purified RNA was diluted to a concentration of 1 μg/μL, and 2.5 μg of RNA was reverse-transcribed using random primers and a SuperScript III first-strand cDNA synthesis kit

Table 1
Primers used in this study.

Primer name	Sequence (5′–3′)
5′RACE-OU	5′-AGTCTTCATCTGAGATTTCTGTTCC-3′
5′RACE-IN	5′-TCTAATGTAGCTGCCACTATTCCCT-3′
3′RACE-OU	5′-TTGTAGAAATGGATGTTGCCTTGTT-3′
3′RACE-IN	5′-TTGGATGAAAGAACTCAAACAGCAG-3′
ORF-up	5′-GAAGTTGAAAGCGAAACACATCCCA-3′
ORF-down	5′-CAAAGTATAATTTATTATATTGATCATTTGCA-3′
DFFA-qPCR F	5′-AGAGTCACGCCCATTTAAGATA-3′
DFFA-qPCR R	5′-GATTTCTGTTCCATCCTCCTC-3′
GAPDH-qPCR F	5′-AAACTCGAGATGTCAGAGTCACGCC-3′
GAPDH-qPCR R	5′-GTATGATGCCCCTTTGTTGAGTC-3′
*Ch*DFF His-HindIII F	5′-AAAAAGCTTCCATGTCAGAGTCACG-3′
*Ch*DFF His-XhoI R	5′-TTTCTCGAGTGTATGAGCATGTTTCTGT-3′
*Ch*DFF GFP-XhoI F	5′-AAACTCGAGATGTCAGAGTCACGCC-3′
*Ch*DFF GFP-HindIII R	5′-TTTAAGCTTTGTATGAGCATGTTTCTGTC-3′