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# Molecular cloning, expression and functional characterization of a teleostan cytokine-induced apoptosis inhibitor from rock bream (*Oplegnathus fasciatus*)

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A B S T R A C T

Apoptosis plays a key role in the physiology of multicellular organisms and is regulated by different promoting and inhibitory mechanisms. Cytokine-induced apoptotic inhibitor (CIAPI) was recently identified as a key factor involved in apoptosis inhibition in higher vertebrate lineages. However, most of the CIAPIs of lower vertebrate species are yet to be characterized. Herein, we molecularly characterized a teleostan counterpart of CIAPI from rock bream (*Oplegnathus fasciatus*), designating as RbCIAPI. The complete coding region of *RbCIAPI* was consisted of 942 nucleotides encoding a protein of 313 amino acids with a predicted molecular mass of ~33 kDa. RbCIAPI gene exhibited a multi-exonic architecture, consisting 9 exons interrupted by 8 introns. Protein sequence analysis revealed that RbCIAPI shares significant homology with known CIAPI counterparts, and phylogenetic reconstruction confirmed its closer evolutionary relationship with its fish counterparts. Ubiquitous spatial distribution of RbCIAPI was detected in our quantitative real time polymerase chain reaction (qPCR) analysis, where more prominent expression levels were observed in the blood and liver tissues. Moreover, the *RbCIAPI* basal transcription level was found to be modulated by different bacterial and viral stimuli, which could be plausibly supported by our previous observations on the transcriptional modulation of the caspase 3 counterpart of rock bream (Rbcasp3) in response to the same stimuli. In addition, our *in vitro* functional assay demonstrated that recombinant RbCIAPI could detectably inhibit the proteolysis activity of recombinant Rbcasp3. Collectively, our preliminary results suggest that RbCIAPI may play an anti-apoptotic role in rock bream physiology, likely by inhibiting the caspase-dependent apoptosis pathway. Therefore, RbCIAPI potentially plays an important role in host immunity by regulating the apoptosis process under pathogenic stress.

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## 1. Introduction

Development of a multicellular organism highly depends on the equilibrium between cell proliferation and cell death; processes that are tightly regulated by different mechanisms including programmed cell death (PCD) (Danial and Korsmeyer, 2004). Apoptosis is a type of PCD, and is considered as a key component of the development and aging processes, as well as a homeostatic mechanism to maintain cell populations in tissues (Elmore, 2007). Moreover, apoptosis is known to be induced as a host defense mechanism through mediating immune responses, especially immune responses mounted against viral infections (Everett and McFadden, 1999; Sun and Shi, 2001), and counterbalance the consequences of pathological conditions. However, proper regulation of apoptosis, in terms of its activation and inhibition, is also required to maintain a proper life cycle. In this regard, BCl-2 family proteins are known to be prominent players in pro-apoptotic and anti-apoptotic processes (Burlacu, 2003), whereas inhibitor of apoptosis proteins (IAPs) are widely known to obstruct the apoptotic process (Deveraux and Reed, 1999).

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Besides the prominent modulators of apoptosis mentioned earlier, another potent inhibitor of apoptosis, designated as cytokine-induced apoptosis inhibitor (CIAPI) or anamorsin has been identified from mice as an essential component of definitive hematopoiesis (Shibayama et al., 2004). CIAPI deficiency was found to induce significant apoptosis in hematopoietic cells in fetal livers of mice, which was accompanied by downregulated expression levels of Bcl-xL and Jak2, suggesting CIAPI as a potential candidate for inducing Bcl-xL and Jak2 expression.

CIAPI exhibits an extensive spatial distribution in both fetal and adult tissues of animals. For instance, more pronounced expression levels of CIAPI were observed in various regions of the rat central nervous system including the cerebral cortex, hippocampus, midbrain, cerebellum medulla, and spinal cord (Park et al., 2011). Moreover, cytosolic CIAPI in rats could be translocated into the nucleus upon reactive oxygen species (ROS) production, and is potentially involved in the regulation of transcription of vital proteins that are important in dopaminergic neurodegeneration (Park et al., 2011). A recent report showed that CIAPI of the well-known human parasite *Schistosoma japonicum* (schistosoma) could inhibit the caspase activity induced by cytokines such as interleukin-β and tumor necrosis factor-α in either human cell lines or schistosome lysates, and it could therefore be considered as a potential drug target against schistosomiasis (Luo et al., 2012).

Abundant expression of CIAPI was observed under some neoplastic conditions in different types of cancer cells including hepatocellular carcinoma, gastric cancer, leukemia and B-cell lymphoma cells, and was associated with clinicopathological characteristics of tumor aggressiveness. This observation further supports the candidature of CIAPI as a prognostic marker of cancer in humans (Gastric and Cells, 2006; Li et al., 2007, 2008; Shizusawa et al., 2008). Moreover, another study showed that the multidrug resistance (MDR) of human gastric cancer cells could be triggered by CIAPI through up-regulating MDR1 at the transcriptional and translational levels (Gastric and Cells, 2006), demonstrating the mediatory properties of CIAPI on gastric cancer MDR. However, the exact physiological function of CIAPI is yet to be elucidated in animals.

Although information on CIAPI of higher vertebrates, such as mice and humans, is currently available, characterization studies on CIAPI of lower vertebrates, especially from fish, are lacking.

Edible marine fish are considered as a protein-rich resource in human diets; thus, mariculture farming of fish has been widely adopted to compensate for the increasing demand. However, as a consequence of intensive, large-scale culturing of fish in restricted areas, different stress factors, particularly pathogenic stress, have adversely affected the yield of fish mariculture farming worldwide, resulting in considerable economic loss. Considering this background, investigations on the molecular mechanism underlying the pathophysiology of mariculture fish species can be considered as a preliminary step toward developing disease management strategies to combat the growing threat of pathogenic infections on farmed fish populations.

Herein, we attempted to characterize a teleostan counterpart of CIAPI, identified from rock bream (*Oplegnathus fasciatus*), as the first such characterization report from fish, evaluated its transcriptional modulation under pathogenic stress, and further demonstrated its potent inhibitory properties against the previously identified ortholog of caspase 3 from the same species.

## 2. Materials and methods

### *2.1. Identification and sequence characterization of rock bream CIAPI (*RbCIAPI*)*

Analysis of our previously established cDNA sequence database using the Basic Local Alignment Search Tool (BLAST) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) led to the identification of the complete cDNA sequence of *RbCIAPI*, which was analyzed and compared with its orthologs using bioinformatics. The complete coding region of *RbCIAPI* and its corresponding amino acid sequence were derived using DNAsist 2.2 software, and the domains of the protein were predicted using the SMART online server (http://smart.embl-heidelberg.de/). Some of the physicochemical properties of RbCIAPI were determined using the ExPASy ProtParam tool (http://web.expasy.org/protparam). The derived protein sequence of RbCIAPI was compared with its orthologs through pairwise sequence alignments and multiple sequence alignment using the EMBOSS Needle (http://www.Ebi.ac.uk/Tools/emboss/align) and ClustalW2 (http://www.Ebi.ac.uk/Tools/clustalw2) programs, respectively. The evolutionary relationship of RbCIAPI with other vertebrate as well as invertebrate counterparts at the molecular level was determined using phylogenetic analysis using Molecular Evolutionary Genetics Analysis (MEGA) software version 4 (Tamura et al., 2007), following the neighbor-joining method, supported by 1000 bootstrapped replications.

In addition, we identified the complete genomic sequence of *RbCIAPI* using our custom-constructed random sheared rock bream BAC genomic DNA (gDNA) library (Lucigen®; USA). The BAC clone containing the genomic *RbCIAPI* gene was analyzed using a two-step polymerase chain reaction (PCR)-based screening approach of our gDNA library with a gene-specific primer pair (RbCIAPI_qF and RbCIAPI_qR; Table 1) according to the manufacturer's instructions. After localizing the putative clone bearing gDNA of *RbCIAPI*, it was sequenced by GS-FLX™ system (Macrogen, Korea), and the complete genomic sequence of *RbCIAPI* was obtained. Thereafter, the obtained gDNA sequence was compared with the previously identified complete cDNA sequence using the National Center for Biotechnology Information (NCBI) 'Spidey' online server (http://www.ncbi.nlm.nih.gov/spidey) to obtain the annotation of exon–intron arrangement.

### *2.2. Cloning, over expression, and purification of recombinant* RbCIAPI *(rRbCIAPI)*

Recombinant RbCIAPI was expressed as a fusion protein with maltose binding protein (MBP), and purified as described in the pMAL protein fusion and purification protocol (New England BioLabs, USA). Briefly, the complete coding sequence of *RbCIAPI* was cloned into a pMAL-c2X expression vector after successful PCR amplification using the respective cloning oligomers (Table 1) designed with

**Table 1**
Oligomers used in this study.

| Name | Purpose | Sequence (5′ →3′) |
|---|---|---|
| RbCIAPI_qF | BAC genomic library screening and qPCR amplification of *RbCIAPI* | GACTGGGTGCTCTCTTGCCT |
| RbCIAPI_qR | BAC genomic library screening and qPCR amplification of *RbCIAPI* | ACAACTTCAGAGCTGACATCAGCTTCT |
| RbCIAPI-F | ORF amplification (*Eco*RI) | GAGAGAgaattcATGGCAGACCTCGGCATCAA |
| RbCIAPI-R | ORF amplification (*Hin*dIII) | GAGAGAaagcttTCAAGCGTCCGTCAGCGT |
| Rb-βF | qPCR amplification of rock bream β-actin gene | TCATCACCATCGGCAATGAGAGGT |
| Rb-βR | qPCR amplification of rock bream β-actin gene | TGATGCTGTTGTAGGTGGTCTCGT |

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