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Molecular cloning, characterization and expression patterns of HSP60 in the grass carp (*Ctenopharyngodon idella*)

Xiao-Yan Xu [a], Yu-Bang Shen [a], Jian-Jun Fu [a], Feng Liu [a], Shi-Zhao Guo [a], Xiao-Meng Yang [a], Jia-Le Li [a,b,*]

[a] Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Shanghai Ocean University, Ministry of Education, Shanghai 201306, China
[b] E-Institute of Shanghai Universities, Shanghai Ocean University, Shanghai 201306, China

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ABSTRACT

HSP60 is a highly immunogenic molecule, which is able to activate a large number of T cell types and is implicated in a variety of autoimmune diseases. The grass carp (*Ctenopharyngodon idella*), a freshwater fish species of the family Cyprinidae, accounts for the third biggest value (USD 4.8 billion) at single species level of major cultured fish species in the world. Here, we isolated and characterized the HSP60 cDNA from grass carp (designated as CiHSP60). Its cDNA was 2434 bp in length and encoded a putative protein of 575 amino acids. BLAST analysis revealed that the CiHSP60 gene shared a high similarity with other known HSP60 sequences. CiHSP60 contained all three classical HSP60 family signatures. The mRNA of CiHSP60 was constitutively expressed in all tested tissues of untreated grass carp, including brain, muscle, trunk kidney, liver, head kidney, skin, spleen, heart, gill, intestine, and fin, with the highest expression level in the blood. CiHSP60 transcript was present in unfertilized eggs, which suggests that CiHSP60 transcription is maternally inherited. Fluorescent real-time quantitative RT-PCR was used to examine the expression of the CiHSP60 gene in grass carp after the challenge with the bacterium *Aeromonas hydrophila*. A clear time-dependent expression pattern of CiHSP60 was found after the bacterial challenge, and the mRNA expression reached a maximum level at three days post challenge, and returned to control levels after seven days. The upregulated mRNA expression of CiHSP60 in grass carp after bacterial challenge indicates that the HSP60 gene is inducible and involved in immune responses. These results suggest that CiHSP60 plays an important role in *A. hydrophila*-related diseases and in early embryonic development stages in grass carp.

1. Introduction

Heat-shock proteins (HSPs) are a group of molecular chaperones that are highly conserved from prokaryotes to higher eukaryotes [1]. The HSP genes have been divided into families such as HSP110, HSP100, HSP90, HSP70, HSP60, HSP40, and HSP20, based on molecular weight and sequence homology of proteins [2].The initial observation that led to the discovery of the HSPs was made by Ritossa [3]. Recent years have seen the emergence of compelling evidence that HSPs possess unique properties that permit their use in generating specific immune responses against infectious agents and in innate immune responses [4,5]. Bacterial HSPs, in particular HSP60, attracted attention initially as highly immunogenic molecules able to activate a large number of T cells and were implicated in a variety of autoimmune and inflammatory conditions [6]. HSP60 is a group of proteins with distinct ring-shaped, or toroid (double donut) quaternary structures [7]. It is a well-characterized chaperone, mainly localized in the mitochondria of eukaryotic cells [8,9]. Inside all cells – both prokaryotic and eukaryotic – HSP60 functions as a highly connected chaperone with links to most cellular proteins [10]. Although HSP60 is a major node in the intracellular chaperone network, evidence is lacking for the immune-specific function of HSP60 inside the cell [5].

Recent reports suggest that HSP60 plays an important role in health, in particular in the development of inflammation and the specific and non-specific immune responses to bacterial and viral infections in shrimp [11]. However, Deane & Woo [12] found that the expression of HSP60 remained unchanged throughout the disease process. A study on Chinook salmon (*Oncorhynchus tshawytscha*) [13] demonstrated that the intensity of the HSP60 response is not only dependent on the induction, but also organ specific. A study on zebrafish (*Danio rerio*) indicated that HSP60 is required for the formation and maintenance of regenerating tissues.

* Corresponding author. Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Shanghai Ocean University, Ministry of Education, Shanghai 201306, China. Tel.: +86 021 61900401; fax: +86 021 61900405.
E-mail address: jlli2009@126.com (J.-L. Li).

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Grass carp is one of the most important farmed fish species in China, with a cultural history dating back to the 7th century (Tang Dynasty) [14]. According to the FAO, the value of grass carp reached more than 4.8 billion USD at single species level in 2008, accounts for the third biggest value at single species level of major cultured fish species in the world [15]. Despite its favorable growth traits, farmed grass carp are rather susceptible to various diseases. Outbreaks of disease associated with bacteria such as *Aeromonas hydrophila* have caused high fish mortality, resulting in reduced production and considerable economic losses [16]. Immune responses in fish can be experimentally stimulated by injection of bacteria [17]. Therefore, an immunological challenge with *A. hydrophila* has great potential to understand immune regulatory effects.

In the present study, the full-length cDNA of grass carp HSP60 was cloned and characterized, and the tissue-specific and embryogenetic expression patterns were analyzed by qRT-PCR. Transcriptional analysis was carried out to test the immune responses of HSP60 after an experimental challenge of grass carp using *A. hydrophila.* The qRT-PCR data indicate that HSP60 is functional during the embryogenesis of grass carp. In addition, the expression patterns of HSP60 in grass carp exposed to a bacterial challenge suggested that HSP60 has potential roles in innate immune responses.

2. Materials and methods

2.1. Animal treatment and RNA extraction

Grass carp with an average weight of 200 g were cultured individually in Wujiang National Farm of Chinese Four Family Carps, Jiangsu Province, China. Animals were raised at 28 °C in 400 l aerated tanks for one week before the experiment and fed twice daily (in the morning and late in the afternoon) at a ratio of 5% of the total biomass. Embryos and fry were obtained from the Wujiang National Farm of Chinese Four Family Carps, and reared in a hatching trough with constant pool water flow at 21 ± 1 °C. On day 5 post hatching, the fry could swim steadily and were fed with freshwater rotifers captured from the pool. For full-length cDNA cloning and expression pattern analysis, grass carp brain, muscle, trunk kidney, liver, head kidney, skin, spleen, heart, gill, intestine and fin were dissected from three unchallenged fish. Blood samples (approximately 1–2 ml/fish) were taken from the caudal vein using a 2 ml syringe. Samples were immediately centrifuged at 3000 × rpm at 4 °C for 10 min to separate blood cells. Total RNA was isolated by using the RNAiso Plus kit (TaKaRa, Japan) and stored at −80 °C after incubation with RNase-free gDNA Eraser (TaKaRa, Japan). The RNA concentration and purity were determined by measuring the absorbance at 260 nm and 280 nm in the Nanodrop 2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

2.2. Full-length cDNA cloning of HSP60

Full-length cDNA of HSP60 was obtained through reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The first cDNA was synthesized from the isolated RNA using the High Fidelity PrimeScript RT-PCR Kit (TaKaRa, Japan). Primers (H1-F and H1-R, Table 1) were based on conserved regions of this gene from other fishes. The PCR program was: 1 cycle of 94 °C/3 min; 31 cycles of 94 °C/30 s, 55 °C/30 s, 72 °C/1 min; 1 cycle of 72 °C/10 min. The PCR product was ligated into a pGEM-T easy vector (Promega), transformed into competent *E*scherichia *coli* DH5α cells, plated on a LB-agar Petri dish and incubated overnight at 37 °C. Positive clones containing the insert with the expected size were identified by colony PCR.

Table 1
Primer sequences used in this study.

Name	Sequence (5′–3′)	PCR objective
H1-F	ATGCTGCGTTTACCTAGTG	cDNA cloning
H1-R	GGTCCTTCTTCTCATTCAC	cDNA cloning
H6-5	CTTCCACCGCCATCATTACTCCTCTACG	5′-RACE
H6-3	GGACAACCGAAAGAACCA	3′-RACE
RH-F	GAAGTTTGACCGTGGCTACA	Real-time RT-PCR
RH-R	CTGCCACAACCTGAAGTCCA	Real-time RT-PCR
b-F	CCTTCTTGGGTATGGAATCTTG	Real-time RT-PCR
b-R	AGAGTATTTACGCTCAGGTGGG	Real-time RT-PCR

Three of the positive clones were picked and sequenced on an ABI PRISM 3730 Automated Sequencer, using BigDye terminator v3.1 (Applied Biosystems, USA).

Based on the sequences of the conserved region we obtained in grass carp, 5′- and 3′-RACE-PCR were performed to define the putative 5′ and 3′ ends of HSP60, respectively. The gene-specific primers (H6-5 and H6-3, Table 1) were designed according to the conserved region sequences. RACE and RACE-PCR were conducted with the SMART RACE cDNA Amplification Kit and Advantage 2 PCR Kit (Clontech). The conditions for the PCR were five cycles of (94 °C/30 s; 70 °C/1 min; 72 °C/3 min), followed by 31 cycles (94 °C/1 min; 68 °C/1 min; 72 °C/3 min) and 72 °C for 10 min after the last cycle. PCR products were cloned and sequenced as described above.

2.3. Sequence analysis

The open reading frame (ORF) of CiHSP60 cDNA was determined using the ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Nucleotide and amino acid sequence identity and the prediction of conserved domains in the peptide were performed using the BLAST program (GenBank, NCBI). The putative amino acid sequence of the CiHSP60 protein was analyzed for the presence of signal peptides using SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) [18]. Multiple sequence alignments were performed using the CLUSTALW 1.8 program [19]. Needle program (http://www.ebi.ac.uk/Tools/emboss/align/) was used to calculate identities among different HSP60 genes of different species. A phylogenetic tree was constructed, based on the deduced full-length amino acid sequences alignment by the neighbor joining (NJ) algorithm and Maximum Likelihood method embedded in MEGA 5.0 [20]. The reliability of the estimated tree was evaluated by the bootstrap method with 1000 pseudo-replications. The accession numbers of the 25 sequences used in this analysis are listed in Fig. 2.

2.4. Tissue and embryogenetic expression analysis

HSP60 mRNA contents in tissues were detected on the samples from three unchallenged fish to illustrate the spatial expression patterns of HSP60 in grass carp. Various tissues were tested, including blood, brain, muscle, trunk kidney, liver, head kidney, skin, spleen, heart, gill, intestine and fin. Total RNA was extracted as described above.

To study the embryogenetic expression profiles, embryos and early larvae from different development stages (10 specimen from each stage) were collected and stored in liquid nitrogen. These stages included unfertilized eggs, 0 h post fertilized, embryos at 16-cell stage, morula stage, gastrula stage, eye sac-appearance stage, caudal fin-appearance stage, muscular effect stage, heart beating stage, mental stages and 1, 2, 3, 4, 5, 6, 7, 10 and 15 days post hatching [21]. Total RNA was extracted as described above.

Quantitative Real-time PCR was performed. The specific primers of HSP60 (RH-F and RH-R, Table 1) were designed according to the