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Overexpression of Hsp90 from grass carp (*Ctenopharyngodon idella*) increases thermal protection against heat stress

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ABSTRACT

With homologous DNA probes, we had screened a grass carp heat shock protein 90 gene (*CiHsp90*). The full sequence of *CiHsp90* cDNA was 2793 bp, which could code a 798 amino acids peptide. The phylogenetic analysis demonstrated that *CiHsp90* shared the high homology with Zebrafish *Grp94*. Quantitative RT-PCR analysis showed that *CiHsp90* was ubiquitously expressed at lower levels in all detected tissues and up-regulated after heat shock at 34 °C or cold stress at 4 °C. To understand the function of *Ci*Hsp90 involving in thermal protection, an expression vector containing coding region cDNA was expressed in *E. coli* BL21 (DE3) plysS. Upon transfer from 37 °C to 42 °C, these cells that accumulated *Ci*Hsp90 peptides displayed greater thermoresistance than the control cells. While incubated at 4 °C for different periods, it could also improve the cell viability. After transient transfected recombinant plasmid pcDNA3.1/*Ci*Hsp90 into mouse myeloma cell line SP2/0, we found that *Ci*Hsp90 could contribute to protecting cells against both thermal and cold extremes. On the contrary, the mutant construct △N-*Ci*Hsp90 (256–798 aa) could abolish the protection activity both in prokaryotic cells and eukaryotic cells. Additionally, both *Ci*Hsp90 and △N-*Ci*Hsp90 peptides could reduce the level of citrate synthase aggregation at the high temperature.

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1. Introduction

Heat shock proteins (Hsps) presented in all cells, both prokaryotic and eukaryotic. Many studies demonstrated that various stressors transiently would increase the production of Hsps as protection against harmful insults in cells. The exposure of organisms to elevated temperatures and other various stimuli could induce and increase the expression of Hsps [1]. According to their apparent molecular mass, these HSPs had been classified into several families: the small heat shock proteins (sHsps), the 40-kDa Hsps, the 70-kDa Hsps, the 60-kDa Hsps, the 90-kDa Hsps, and the 104-kDa Hsps [2,3]. All of these classes had important functions in fundamental cellular processes.

Members of the Hsp90 family were the most abundant heat shock proteins under physiological conditions that account for 1–2% of cellular proteins in most cells [4]. Hsp90 was a major chaperon in eukaryotes for it could form complexes with over 400 different proteins [5–7]. Hsp90 was up-regulated by a range of stressors such as heat or cold shock [8,9], food-deprivation [10], heavy metals [11] and diseases [12]. It had been revealed that Hsp90 would play crucial roles in protein folding, protein degradation and signal transduction [13–15]. These primary functions could help Hsp90 to exert its profoundly broad biological activities, such as cellular differentiation [16], cell proliferation and apoptosis [17], and cytoprotection [18]. In additional, studies in zebrafish demonstrated that Hsp90 was required for embryogenesis and organism development [19–21]. The importance of Hsp90 made it serve as an evolutionary capacitor of morphological changes during embryogenesis [22,23].

As aquatic vertebrates, fish were apt to suffer from a wide variety of stressors including heat shock, osmotic stress, and environmental contaminants. Hsps were key elements of the stress response system at the cellular level in fish. Although some fish Hsp90s had been cloned [24–26], limited information was available regarding Hsp90 in grass carp (*Ctenopharyngodon idella*) at present and its molecular mechanisms were still far from clear.

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Here we screened and identified a heat shock protein 90 gene (*CiHsp90*, GU258544) in the grass carp. Then, the coding region of *CiHsp90* and the amino-terminal deletion mutant (△N-*CiHsp90*) (868–2496) were cloned into both the vector of pET-32a(+) and pcDNA 3.1, which were transformed into *E. coli* and transfected into mouse myeloma cells SP2/0 for temperature treatment experiment, respectively. Moreover, the *Ci*Hsp90 and △N-*Ci*Hsp90 (256–798 aa) peptides were expressed and purified for the citrate synthase aggregation assays. The results showed that *Ci*Hsp90 could contribute to protecting cells against both heat stress (42 °C) and chilling temperature (4 °C), while the mutant construct △N-*Ci*Hsp90 abolished the protection activity. In addition, both *Ci*Hsp90 and △N-*Ci*Hsp90 could reduce the level of citrate synthase aggregation at the heat stress (42 °C).

2. Materials and methods

2.1. Animals, temperature stress and sample collection

The grass carp liver cDNA library was kept by our laboratory. Grass carps (about 100 g body weight) were obtained from Jiangxi Provincial Fisheries Research Institute and acclimatized to the laboratory conditions for 2 weeks in a quarantine area. For temperature stress experiments, 10 fish were put into 25 L aerated aquaria at 34 ± 1 °C or at 4 °C for 2 h and then transferred back to room conditions for 20 min. Then, tissues including liver, brain, head kidney, spleen, and intestine were sampled and frozen in liquid nitrogen (we focused on the functions of Hsp90 in immune-related tissues, so other tissues such as muscle tissue were not included in this study). Non-heat shock animals were used as a control group. Total RNA was extracted according to the manufacturer's instruction of SV Total RNA Isolation System (Promega). Intact RNA was incubated with RNase-free DNase I to remove contaminated genomic DNA before being reverse transcribed into cDNA.

2.2. Cloning of CiHsp90

To screen *CiHsp90* in the grass carp liver cDNA library, probe primers were designed based on a *Danio rerio* heat shock protein 90 (Hsp90b) cDNA sequences (NM_198210). The positive cDNA clone numbered YG001_F07 (pBluescript II SK/*CiHsp90*) was found by hybridization. Then, plasmid universal primers: T7 (5′-TAA-TACGACTCACTATA-3′) and T3 (5′-ATTAACCCTCACTAAAGGGAA-3′) were used to identify the clone. The PCR program was: 1 cycle of 94 °C/5 min; 30 cycles of 94 °C/30 s, 59 °C/30 s, 72 °C/3 min; 1 cycle of 72 °C/10 min. The full length cDNA sequence was confirmed by sequencing (Beijing Genomics Institute).

2.3. Sequence analysis and phylogeny of Hsp90

The cDNA sequence was analyzed by ORF finder and the deduced amino acid sequence was analyzed with SMART and PROSITE. All homology sequences were obtained using BLAST program. Phylogenetic and molecular evolutionary analyses were conducted using PHYLIP 3.69 and optimized manually.

2.4. Tissue expression analysis of CiHsp90

First strand cDNA was synthesized using oligo(dT)$_{18}$ primers with M-MLV Reverse Transcriptase (TaKaRa). Expression of *CiHsp90* mRNA was assessed in different tissues using quantitative real-time RT-PCR (qRT-PCR) in a Mastercycler ep realplex (Eppendorf). *CiHsp90* gene expression was detected using primers: RT-F (5′-CGTTGACGTTGACGGCACAGT-3′) and RT-R (5′-CCAAGGCATCGGAAGCATTAG-3′). β-actin (primers were 5′-CACTGTGCCCATCTACGA-3′ and 5′-CCATCTCCTGCTCGAAGT-3′) was utilized as an internal control for cDNA normalization. Amplifications were carried out at a final volume of 20 μl, containing 1 μl DNA sample, 10 μl SYBR Premix Ex Taq™ (TaKaRa) and 0.5 μl of each forward and reverse primer. The PCR cycling conditions were: 1 cycle of 94 °C/5 min, 40 cycles of 94 °C/30 s, 59 °C/30 s, 72 °C/30 s, followed by dissociation curve analysis to verify the amplification of a single product. Each sample was run in triplicate. The data were subjected to one-way ANOVA followed by an unpaired, two tailed *t*-test. $P < 0.05$ was considered statistically significant.

2.5. Prokaryotic expression and purification of recombinant peptides in bacteria

With pBluescript II SK/*CiHsp90* as the template, the full-length coding region of the putative *Ci*Hsp90 molecule and the amino-terminal deletion mutant (256–798 aa) (△N-*Ci*Hsp90) were amplified by PCR and inserted into the *Bam* H I/*Xho* I-cleaved expression vector pET-32a(+) (Novagen). The primers for amplification of *Ci*Hsp90 were Hsp90-F (5′-CGGGATCCATGAGGC-GACTGTGGATTATC-3′) and Hsp90-R (5′- CGCTCGAGCTACAGCTCA TCTTTGCCTGTG-3′). And the primers for amplification of △N-*Ci*Hsp90 were F (5′-CGGGATCCTCTGACTACCTTGAGCTGGAG-3′) and R (5′-CGCTCGAGCTACAGCTCATCTTTGCCTGTG-3′). The recombinant expression vectors were pET-32a(+)/*Ci*Hsp90 and pET-32a(+)/△N-*Ci*Hsp90 that yielded the N-terminal 6×-histidine-tagged protein, which was confirmed by PCR and sequencing.

These recombinant expression vectors were transformed into *E. coli* BL21 (DE3) plysS (Novagen) and the recombinant proteins were prepared. The proteins were purified and then used in the thermal aggregation assays.

2.6. Bacterial cells survival assays

To estimate the growth curves, cells of *E. coli* BL21 (DE3) plysS and those were transformed with pET-32a(+)/*Ci*Hsp90, pET-32a(+)/△N-*Ci*Hsp90 or pET-32a(+) (control) were grown at 37 °C in LB media to an A_{600} of 1.5. Then, cultures were diluted 1:100 into fresh LB media supplemented with appropriate ampicillin. Continuously, optical densities (A_{600}) of these cultures were measured every 30 min, and the means of three experiments were determined (with SD being less than 5%).

A single colony of bacteria transformed with pET-32a(+)/*Ci*Hsp90, pET-32a(+)/△N-*Ci*Hsp90 or control plasmids was transferred to 20 ml of LB media containing 50 μg/ml of ampicillin. Cells were grown at 37 °C with vigorous shaking until they reached an OD of 0.6–0.8 at A_{600} nm, which were induced by the addition of IPTG to a final concentration of 1.0 mM. After 3 h of growth, the cultures were divided into three aliquots. For heat shock experiments, the first aliquot of cultures was placed at 42 °C. Then 1 ml of the cultures was taken at 60, 90, 120, and 150 min, and serial dilution of 1:10^6 was plated onto LB agar plates containing 50 μg/ml of ampicillin. After incubation overnight at 37 °C, cell viability was estimated by counting the number of colony-forming units. For cold treatments, the second aliquot of cultures was diluted 1:10^6 and plated onto LB agar supplemented with ampicillin. Plates were incubated at 4 °C for different periods (2, 4, 6, 8 and 10 d) and then cultured overnight at 37 °C. Cell viability was estimated as described above. The third aliquot of cultures was to be a control which were diluted and plated and then cultured overnight at 37 °C. For both heat and cold treatments, the means of three experiments were determined (with SD being less than 5% in all cases).

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