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Identification, tissue distribution and characterization of two heat shock factors (HSFs) in goldfish (*Carassius auratus*)

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ABSTRACT

Heat shock proteins (HSPs) are synthesized rapidly in response to a variety of physiological or environmental stressors, whereas the transcriptional activation of HSPs is regulated by a family of heat shock factors (HSFs). In vertebrates, multiple HSFs (HSF1-4) have been reported to have different roles in response to a range of stresses. This paper reports the cDNA cloning of two goldfish (*Carassius auratus*) HSF gene families, HSF1 and three isoforms of HSF2. Both HSF1 and HSF2s showed high homology to the known HSFs from other organisms, particularly the zebrafish. Different patterns of HSF1 and HSF2 mRNA expression were detected in several goldfish tissues, highlighting their distinct roles. In cadmium (Cd)-treated tissues, the responses of HSP70 showed less difference. However, the increase in HSF1 and HSF2 in these tissues differs considerable. In particular, HSF2 was induced strongly in the heart and liver. On the other hand, in heart tissue, HSF1 showed the smallest increment. These results suggest the potential role of HSF2 in assisting HSF1 in these tissues. In another in vitro experiment of hepatocyte cultures, Cd exposure caused similar patterns of goldfish HSF1 and HSF2 mRNA expression and induction of the HSP70 protein. On the other hand, an examination of the characterization of recombinant proteins showed that HSF1 undergoes a conformation change induced by heat shock above 30 °C and approaches each other in the trimer, whereas HSF2 could not sense thermal stress directly.

Furthermore, immune-blot analysis of HSFs showed that both monomers and trimmers of HSF1 were observed in cadmium-induced tissues, whereas HSF2 were all in monomeric. These results show that HSF1 and HSF2 play different roles in the transcription of heat shock proteins.

1. Introduction

Environmental contamination is a severe problem worldwide that has prompted many studies on the effects of various pollutants on the biological functions of aquatic organisms, particularly the defense mechanisms in fish [1]. The first response of cells to a stress is a general increase in the level of stress proteins. When exposed to heat shock and other insults, such as oxidative stress or toxic substances, organisms synthesize a common set of proteins, which are known as heat shock proteins (HSPs) [2].

HSPs are synthesized in response to various environmental stressors, and pathophysiological or growth-related signals [3–5]. HSPs are normally expressed to assist misfolded proteins in stressed cells by acting as molecular chaperones. Transcription activation of the HSP genes is regulated by various heat shock factors (HSFs), in which the binding of HSFs to the heat shock elements (HSEs) mediates the transcription of the heat shock genes. Four HSFs have been characterized in vertebrates and are named HSF1, HSF2, HSF3 and HSF4 in the order of their discovery. Both HSF1 and HSF2 cooperate either during stress or in physiological conditions [6–8]. Heat inducible HSF3 was thought to be expressed exclusively in birds, however recently it has also been found in mammals [9]. HSF4 is expressed in a tissue-specific manner, predominantly in lens and brain. Different isoforms of mammalian HSF1, HSF2 and HSF4 have been identified and are generated by alternative splicing of transcripts [10].

HSF members are characterized by their conserved domain structure consisting of a winged helix-turn-helix motif and an extended hydrophobic heptad repeat (HR-A/B) [11]. The helix-turn-helix DNA binding domain (DBD) can bind to the HSEs, as a head-to-head (nGAAnnTTCn) and tail-to-tail (nTTCnnGAAn) repeat [12]. The HR-A/B domain facilitates trimerization through the formation

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of a coil depending on intermolecular hydrophobic non-covalent interactions [13,14]. HSFs have been suggested to play different roles in response to a variety of physiological and environmental stimuli [10,15]. In mammals, HSF1 acts as a classical stress-responsive factor that regulates the expression of HSPs, and HSF2 is rather active under developmentally related conditions [10–18]. Another difference between the two factors is that while HSF1 is expressed evenly, the levels of HSF2 fluctuate [19]. Hetero-trimerization of HSF1 and HSF2 provides a transcriptional switch in response to distinct stimuli [19]. In contrast to mammalian HSFs, HSFs in aquatic organisms are important because of the wide variety of inducers available, such as elevated temperatures, oxidants, heavy metals and chemical pollutants. Although the induction of HSF1 activity by external stimuli is well known, the mechanism of HSF2 activation is less well understood.

Goldfish (*Carassius auratus*) are a freshwater species that are distributed widely. This species has been used as a model to assess environmental contamination [20]. At least two forms of HSFs (HSF1 and HSF2) have been identified in fish, each of which has several isoforms [21–25]. In zebrafish, three isoforms of HSF1 have been reported [26,27]. This study examined the HSFs from goldfish and cloning of the putative HSF genes. In addition, the tissue expression profiles and characterization of goldfish HSFs have been studied.

2. Materials and methods

2.1. Animals and materials

Goldfish (*C.auratus*), approximately 10–15 cm in length and 20 g in weight, were purchased from a local market and kept in dechlorinated freshwater tanks. The fish were killed by a sharp blow to the head followed by severance of the spinal cord, and were dissected on ice. The animal protocol used in this study was reviewed and approved by the Pusan National University-Institutional Animal Care and use Committee (PNU-IACUC) according to their ethical procedures and scientific care. The fish were killed by an anesthetic overdose with MS-222 (Sigma), and the organs and tissues, namely the brain, kidney, spleen, heart, intestine, gill, stomach, bladder, and liver, were collected, frozen immediately in liquid nitrogen, and stored at −80 °C until RNA extraction. $CdCl_2$, 17β-estradiol (E2) and PCB77, were purchased from Sigma–Aldrich.

2.2. Isolation of HSF cDNA

The total RNA was isolated from the liver of *C. auratus* in liquid nitrogen using an easy-spin™ total RNA extraction kit (Invitrogen). The first-strand cDNA was synthesized from 5 μg of the total RNA with an Oilgo (dT) primer by AMV reverse transcriptase (Promega). A polymerase chain reaction (PCR) was carried out using cDNA as the template, containing 20 μM forward and reverses primers, Taq DNA polymerase and 2.5 μM of each dNTP (Bioneer, Korea). The primers were designed based on the conserved regions of zebrafish HSF1 (accession number NM_131600), zebrafish HSF2 (accession number NM_131867), rainbow trout HSF1 (accession number M21310), and rainbow trout HSF2 (accession number AB062548). Twenty-five cycles were carried out with each cycle of the following: 1 min at 94 °C for 30 s, 30 s at 54 °C, and 1 min at 72 °C. The PCR products were confirmed by electrophoresis, and the nucleotide sequence was determined using a 3730xl DNA analyzer (Macrogen).

2.3. Rapid amplification of cDNA 5′ and 3′ ends (RACE)

The full-length HSF1 and HSF2 sequences were obtained by rapid amplification of the cDNA ends using a SMART cDNA amplification kit (Clontech). The gene specific primers (GSPs) were designed according to the determined partial *C. auratus* HSFs sequences. The 5′-RACE-PCR product was amplified using the GSP (3′RACE-1and 2) and the adaptor primer included in the kit. 5′-RACE-PCR was carried out using the primers, 5′-RACE-1/2, and the adaptor primer. The final PCR products were cloned into the TOPO PCR 2.1 vector (Invitrogen) and the sequence was determined to establish the full-length gfHSF1 and HSF2 cDNA.

2.4. Phylogenetic analysis

The HSFs amino acid sequences from different species were aligned and used to construct the phylogenetic tress. The alignment was generated from these sequences using a T-COFFEE Multiple Sequence Alignment Server. The alignment output was analyzed with MEGA 5.0 using the maximum likelihood statistical method with a nearest-neighbor-interchange strategy, while allowing for the detection of gaps in <50% of the sequences [28].

2.5. Isolation and primary culture of hepatocytes

The isolation and primary culture of the hepatocytes were performed using the methods reported elsewhere [29]. Briefly, the fish were euthanized with an overdose of MS222 (Sigma) and their liver tissue was isolated carefully, and perfused with a hepatocyte buffer (HB, 136.9 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4, 5.0 mM $NaHCO_3$, pH 7.6) for 10 min at room temperature. The liver tissue was digested with HB buffer containing collagenase (0.3 mg/ml) (Sigma, St. Louis, MO). The softened tissue was minced and sieved through a nylon mesh (50 μm). The resulting cell suspension was washed three times with HB containing 1.5 mM $CaCl_2$. After isolation, the cells were seeded in a Microtest™ tissue culture plate and cultured in Leibovitz-15 medium (L-15, Sigma, St. Louis, MO).

2.6. Semi-quantitative reverse transcriptase-PCR amplification and measurement of the HSP70 levels

Semi-quantitative RT-PCR was performed to examine the changes in the goldfish HSF1 and HSF2 mRNA levels from the tissue samples and hepatocyte cultures. One microgram of the total RNA extracted from the brain, kidney, spleen, heart, intestine, gill, stomach, bladder, and liver of *C. auratus* were reverse transcribed with the oligo(dT) primer and M-MLV reverse transcriptase (Gibco/BRL) according to the manufacturer's instructions. The reaction conditions were as follows: 94 °C/4 min; 25 cycles of 94 °C/30 s, 56 °C/30 s, and 72 °C/30 s; and 72 °C/5 min. The β-actin gene was used as a reference to normalize the relative expression of the samples. The PCR products were subjected to agarose gel electrophoresis and ethidium bromide staining. The amplified products were detected using an UVitec Cambridge Scan system, and the analysis was performed using ImageQuant TL (GE Healthcare) software.

After pre-culturing for 48 h, the hepatocytes were exposed to the ethanol control, 10 nM, 50 nM, 100 nM and 200 nM of $CdCl_2$, E2 or polychlorinated biphenyls (PCB77). The control received the ethanol solvent only. After 48 h and 96 h treatment with the pollutants, the cells were harvested and the HSP70 levels were measured. The HSP70 levels were measured quantitatively using a sandwich enzyme-linked immunosorbent assay (ELISA) method developed by Chang et al. [30].

2.7. Expression and purification of gf HSF1, HSF2 and HSP70 in Escherichia coli

The full-length gfHSF1 and 2 cDNA were constructed into the *E. coli* expression vector, pET21b vector. The overexpression of HSF1