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Cloning of an orange-spotted grouper *Epinephelus coioides* heat shock protein 90AB (HSP90AB) and characterization of its expression in response to nodavirus

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ABSTRACT

The heat shock proteins (HSPs) family which consists of HSP90, HSP70, and low molecular mass HSPs are involved in chaperone activity. Here, we report the cloning and characterization of *HSP90AB* gene from orange-spotted grouper, *Epinephelus coioides*. The full-length of grouper HSP90AB was 727 amino acids and possessed an ATPase domain as well as an evolutionarily conserved molecular chaperone. The HSP90AB-green fluorescent protein fusion protein was evenly distributed in the cytoplasm. Immunohistochemistry (IHC) and real-time polymerase chain reaction (PCR) analyses indicated that the expression of grouper *HSP90AB* was marginally increased following nodavirus infection. Grouper *E. coioides* that received HSP90 inhibitor geldanamycin (GA) showed an increase in HSP90AB expression and growth of nodavirus supporting nodavirus replication.

1. Introduction

Betanodavirus belongs to the family of nodaviridae that usually infects a wide variety of larval and juvenile marine fish, while *Alphanodavirus* infects insect species [1,2]. The molecular chaperone heat shock protein 90 (HSP90) has been identified as important factors involved in the regulation of the RNA polymerase synthesis of viral replication of *Alphanodavirus* [3], the genome of which contains two positive-sense RNA strands known as RNA1 and RNA2. The former encodes RNA-dependent RNA polymerase (RdRp), an enzyme responsible for viral replication that is located in mitochondria, whereas the latter encodes coat protein, which assembles to form the viral particles.

Recent studies demonstrated the possibility that *betanodavirus* requires cellular stress protein [4], grouper HSP90AB, a stimulatory host factor for its coat protein synthesis. The abundance of this protein may be advantageous to viral infections among animals, such as hepatitis C virus (HCV) [5] and influenza virus [6]. Temperature, an important parameters influence virus growth, regulate heat shock proteins (HSPs), and interrelate HSPs and virus growth.

References have pointed out that there are four main types of HSP90 isoforms of the vertebrates found in the expressing areas. One of the two types of the cytosolic isoforms is called HSP90AA (HSP90 alpha or inducible form) and the other is called HSP90AB (HSP90 beta or constitutive form). Another type is found in the endoplasmic reticulum region of all the eukaryotes, except the fungi, and is referred to as HSP90B (94 kDa glucose regulated protein, Grp94). Lastly, a HSP90 homologue is found in the mitochondrial region, the TRAP (tumor necrosis factor receptor-associated protein) [7]. Although cytosolic HSP90 isoform is involved in cell proliferation and differentiation, HSP90AA has also similarities with growth promotion, cell cycle regulation, and stress-induced cytoprotection, on the other hand, HSP90AB is also associated with early embryonic development, germ cell maturation, cytoskeletal stabilization, cellular transformation, signal transduction, and long-term cell adaptation [8].

Viruses induce a heat shock response indicative of a stressed cell state [9–13]. HSP90 is involved in the immune response especially in lipopolysaccharide (LPS) recognition [14–16], and is regulated by a range of stressors such as heat or cold shock [17–20], hyperosmotic stress [21], food-deprivation [22], reduced oxygen level

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[23], and the presence of polychlorinated biphenyl (PCB) [24], arsenates [25], and heavy metals [26,27]. However, little is known on the function of HSPs in nodavirus infection.

HSP90 is an atypical member of the family of heat shock-induced chaperones that promote folding, cellular translocation and assembly of newly synthesized polypeptides of viruses [28]. HSP90 participates in the growth of vaccinia virus [29], as well as in the function of translocation and the assembly of polypeptides of polyomavirus and SV40 [30,31]. In the case of hepatitis B virus, HSP90 interacts with viral reverse transcriptase to facilitate the formation of a ribonucleoprotein (RNP) complex [32]. These activities of HSP90 centers on a specific set of client proteins that facilitate their folding into stable or active conformations, and are assisted by the proteins ATP-dependent chaperone capability.

The purpose of the present study was 1) to present cloning and molecular characterization of *HSP90AB* cDNA from orange-spotted grouper *Epinephelus coioides*, 2) to compare its sequences and conduct phylogenetic analyses with other HSP90, 3) to examine the expression of HSP90AB and protein level from different tissues, and 4) to evaluate the changes of HSP90AB expression whether or not the groupers were infected by nodavirus, and 5) to evaluate the changes of HSP90AB expression after geldanamycin (GA) treatment of the grouper cells (GF-1), whether viral coat protein expression will be affected or not.

2. Materials and methods

2.1. Cell culture and reagents

The grouper cell line GF-1 [33], derived from the fin tissue of orange-spotted grouper *E. coioides*, was grown at 28 °C in Leibovitz's L-15 medium (GibcoBRL, Gaithersburg, MD, USA) supplemented with 5% fetal bovine serum (FBS). Grouper cells (GF-1), which are susceptible to nodavirus infection and nodavirus replication, obtained from the Bioresources Collection and Research Center in Taiwan BCRC960094 was used. Transient transfection was performed by introducing 1–2 μg of plasmid encoding grouper HSP90-fused green fluorescent protein (GFP) into cells using Lipofectamine (Invitrogen). After transfection, cells were grown for 24–30 h. Intracellular localization of GFP-fused proteins was examined using an Olympus IX70 microscope (Olympus, Tokyo, Japan). GA was dissolved in 20% dimethylsulfoxide (DMSO; Sigma–Aldrich) in sterile 0.9% saline. An alkaline phosphatase-conjugated substrate Western blotting detection system kit was purchased from Bio-Rad (Hercules, CA, USA). Alkaline phosphatase-conjugated anti-mouse, anti-rabbit, and anti-goat IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted 1:5000 prior to use.

2.2. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from grouper at 40–45 days post-hatching using a previously described single-step acid guanidinium thiocyanate–phenol–chlorofrom extraction method [34]. Extracted cellular total RNA (5 μg) as template was incubated at 42 °C for 60 min in 20 μL of 1X reaction buffer containing 2 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA), 0.25 mM dNTP and 4 μM oligo$(dT)_{15}$ primer, and lastly 0.4 U RNase (Boehringer Mannheim Biochemicals, Mannheim, Germany) was added. Putative *HSP90AB* sequences were examined for homogeneity by comparison with published *HSP90AB* sequences using Blast (http://www.ncbi.nlm.nih.gov/BLAST). In the first experiment, the grouper, 40–45 days post-hatching nodavirus naturally-infected, were obtained from hatchery farms. We excised the eye from healthy and nodavirus naturally-infected grouper, and total RNA was extracted for *HSP90AB expression*. First-strand cDNA was synthesized using total RNA (5 μg) and cDNA (250 ng) as a template for the PCR. The grouper *HSP90AB* nucleotide sequences of forward and reverse primers were as follows: HSP90-RT-S, 5′-ATGCCTGAAGAAATGCGCCAAGAGGAG-3′ (forward primer) and HSP90-RT-A, 5′-CCAATGGGCTCACCGTTGTC-GACTCTG-3′ (reverse primer), were designed to specifically amplify a 523-bp PCR fragment. Grouper β-*actin* gene expression was analyzed as an internal marker using the following primers: β-ACTIN-RT-S436 5′-AGCCAACAGGGAGAAGATGACCC-3′ (forward primer) and β-ACTIN-RT-A1170 5′-TGATCCACATCTGCTGGAAG-3′ (reverse primer). PCR was conducted under the following conditions: 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, with 25 cycles; the last cycle was followed by extension for 5 min at 72 °C. The total amount of cDNA was calibrated based on the amplification of *β-ACTIN* cDNA from the same template, and PCR products were analyzed by agarose gel electrophoresis. In the second experiment, real-time RT-PCR was used to quantify the expression of mRNA for *HSP90AB* with *β-ACTIN* as control. First-strand cDNA was synthesized using 2 μg total RNA and the SuperScript First Strand cDNA synthesis kit (Invitrogen). The amplification was performed using the qPCR core kit for SYBR Green (Qiagen, Valencia, CA, USA) and Step-One™ Real Time PCR system (Applied Biosystems, Foster City, CA, USA). Typical profile times used were initial step, 95 °C for 15 min, followed by a second step at 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s for 40 cycles with melting curve analysis. After the PCR program, fluorescent real-time PCR data from three replicate samples were analyzed.

2.3. Suppression subtractive hybridization

cDNA subtraction was performed to generate subtracted cDNA library between 40 and 45 days post-hatching nodavirus naturally-infected grouper (tester) and healthy grouper (driver) using PCR-Select cDNA subtraction Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. Briefly, poly(A^+) RNA was extracted using the FastTrack® mRNA isolation kit (Clontech). cDNA was synthesized using SMART™ PCR cDNA Synthesis Kit according to the manufacturer's instruction. Tester and driver cDNAs were purified by ethanol precipitation, and then digested with *Rsa1* at 37 °C overnight to obtain shorter blunt-ended molecules. Two different adaptors, adaptor 1 and adaptor 2R, were ligated to the 5′-end of each strand of tester cDNA, both of which were separately hybridized at 68 °C for 8 h with an excess of driver cDNA after denaturation at 98 °C for 90 s. After the first hybridization, the two samples were mixed together without denaturation and hybridized again with freshly heat-denatured driver cDNAs for 18 h at 68 °C. The resulting mixture was diluted 1:50, and then amplified by two rounds of PCR to enrich desired cDNAs containing both adaptors by exponential amplification of these products [35]. Nested PCR amplicons were subcloned into a pGEM-T easy Cloning Kit (Promega). Finally, the efficiency was evaluated by PCR with β-actin forward and reverse primers performed on tester (unsubtracted) and subtracted cDNAs for 25 cycles.

2.4. Rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR)

RACE-PCR was conducted following the previously described method [36]. Based on the verified sequence of the 2437 bps fragment of *HSP90AB* cDNA from the subtracted plasmid library, HSP90-5RACE and HSP90-3RACE primers (Table 1) were applied in PCR amplification and cloning of the cDNA 5′ end and 3′ end, respectively. Two adapter primers for both ends were provided in the Marathon cDNA Amplication Kit (Clontech). The RACE-PCR thermal cycle profile was as follows: 94 °C for 1 min; 94 °C for 30 s, 72 °C for 4 min, 72 °C for 10 min with thirty cycles; extension at 72 °C for 10 min. The amplified fragment was verified with subcloning into