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Hypothermal stress induced differential expression profiles of the immune response gene, warm-temperature-acclimation associated 65-kDa protein (Wap65), in the liver of fresh water and seawater milkfish, *Chanos chanos*

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ABSTRACT

The milkfish (*Chanos chanos*), an important aquaculture species, is intolerant to cold environments. Temperature fluctuations in the environment affect the physiological response, behavior, and survival rate of the fish. The warm-temperature-acclimation associated 65-kDa protein (Wap65) of teleosts was identified after heat shock treatment and has two isoforms. Both the isoforms were involved in the induction of immune responses in fish. They showed high degree of sequence conservation with the mammalian hemopexin and had high affinity for heme, which helped in the neutralization of free-heme and its transport to the liver. In this study, we isolated and characterized the two isoforms of *wap65* genes (*Ccwap65-1* and *Ccwap65-2*) from the liver of milkfish. The *Ccwap65-1* and *Ccwap65-2* are mainly expressed in livers of milkfish. In hypothermal treatment, the expression levels of *Ccwap65-2* in the livers of SW and FW milkfish were up-regulated after exposure to low temperature (18 °C) for 12 h and 96 h compared to those in the normal temperature (28 °C) group, respectively. After intraperitoneal injection of lipopolysaccharide (LPS), the expression of *Ccwap65-2* was elevated in both SW and FW milkfish, whereas that of *Ccwap65-1* was not affected in both the groups. Thus, *Ccwap65-2* expressed in the milkfish liver under hypothermal stress was identified as a novel immune biomarker. In addition, according to the transcriptome database, up-regulation of the other immune-response genes indicated increased pathogen infection status under hypothermal stress. Acute increase in the expression of hepatic *Ccwap65-2* in response to pathogen infection might lead to better cold tolerance of SW milkfish compared to that of the FW individuals upon cold challenge.

1. Introduction

The warm-temperature-acclimation associated 65-kDa protein (Wap65) is an ortholog of mammalian hemopexin (Hpx), which is a plasma glycoprotein involved in iron homeostasis and antioxidant function because of its high affinity to bind to and neutralize free-heme, present in blood and the hepatocytes. Because free radicals can be generated from heme, it can cause cellular damage when attached to cellular protein, lipid, and DNA. Hpx is an acute-phase protein expressed during inflammation and infection in mammalian tissues [1–3]. Wap65, first identified using two-dimensional electrophoresis and northern blot analysis in the hepatopancreatic tissue of goldfish, plays an important role in warm temperature acclimation [4]. Two isoforms of Wap65 protein (Wap65-1 and Wap65-2) have been identified in teleosts, which contain several highly conserved heme-binding pockets, like the hemopexin functional domain [5,6]. These two isoforms of Wap65 may play different roles in mRNA and protein expression in response to immune stimulation [5–15], increasing temperatures [5–7,9,10,12,13,16], and challenge with heavy metals [5–7,10,12]. The expression of the isoform Wap65-1 was observed to alter

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slightly with fluctuations in temperature, whereas Wap65-2 responded strongly to increase in temperature and pathogen infection in hepatocytes of the turbot (*Scophthalmus maximus*), Kumgang fat minnow (*Rhynchocypris kumgangensis*), and rock-bream (*Oplegnathus fasciatus*) [5,6,9].

Because fishes are ectothermic, changes in water temperature directly affect their physiological responses [17]. In winter, sudden drop in water temperatures sometimes cause the "winter syndrome" of the gilthead sea bream (*Sparus aurata*), affecting their health and increasing their mortality [18]. The "winter syndrome" is caused by several factors, including fasting, metabolic depression, ionic imbalance, and immune suppression. With lower immune capacity, the fish have higher risks of infection [18]. In the mouse model, blood degradation products (heme or hemoglobin) induce synergistic inflammation upon infection. The levels of pro-inflammatory factors, like tumor necrosis factor (TNF), interleukin 6 (IL-6), and toll-like receptors (TLR) are also induced. In addition, Hpx, which also functions as a TLR-agonist, was found to be capable of reducing the inflammatory response [2].

Milkfish (*Chanos chanos*) is one of the crucial economic teleosts in the Southeast Asia and Taiwan. Milkfish have high euryhalinity and can be cultured in fresh water (FW), brackish water (BW), or seawater (SW) environments [19]. In winter, high mortality of milkfish during cold snap usually causes huge economic loss in Taiwan. SW-acclimated milkfish, however, exhibit better cold tolerance than the FW individuals [20]. Based on the results of next-generation sequencing (NGS) of their transcriptome, SW milkfish were found to increase their energy budget by up-regulating the aerobic metabolism-related genes whereas FW-acclimated milkfish down-regulated the basal metabolism-related genes to reduce their energy loss [21]. Moreover, profiles of the hepatic proteomes in hypothermal SW milkfish revealed that the individuals experienced oxidative stress, which caused apoptosis. The analysis of the hepatic proteome profiles in milkfish also helped in identifying the hemopexin-like protein (Wap65) under cold stress, for the first time [22].

Earlier studies have demonstrated the expression of Wap65 in several teleosts under heat shock or pathogen infection. The present study, based on previous proteomic identification of Wap65 in SW milkfish upon cold challenge [22] and physiological findings of salinity-dependent cold tolerance in milkfish [20], aimed to identify the two isoforms of Wap65 (*Ccwap65-1* and *Ccwap65-2*) in milkfish livers and to compare their responses to hypothermal stress in SW and FW. Moreover, we evaluated the potential roles of *Ccwap65* in reducing the inflammatory responses in SW and FW milkfish by administering lipopolysaccharide injection. The effects of salinity and cold on the expression of some immune-response genes were also determined for comparison.

2. Materials and methods

2.1. Experimental fish

The juvenile milkfish were purchased from a local fish farm (Changhua, Taiwan). The experimental fish were maintained in tanks filled with 400 L fresh water (FW) or seawater (SW; 35‰) that was continuously circulated through fabric-floss filters. SW was prepared from the local tap water by adding appropriate amounts of RealOcean™ Synthetic Sea Salt (Camrillo, CA, USA). The milkfish were raised at 28 ± 1 °C with a 12/12 h light/dark photoperiod for at least one month. All experimental fish were fed with commercial pellets daily. The experimental groups including the SW- and FW-acclimated milkfish were subjected to increased cooling by reducing the temperature at a rate of 2 °C/h (from 28 °C to 18 °C) using a cooling system (PF-225M, PRINCE, Tainan, Taiwan). The body weight and total length of experimental fish were measured before sampling. The average body weight was 10.4 ± 0.5 g and average total length was 9.3 ± 0.1 cm. The mRNA expression levels of *wap65* in various tissues, including brain, gill, intestine, kidney, liver, muscle, and spleen of SW milkfish at normal temperature, were analyzed by the quantitative real-time PCR. Subsequently, in the one-week hypothermal experiments, the livers of milkfish of 18 °C and 28 °C groups were sampled at 7 days, respectively, for the following analyses and comparisons. On the other hand, milkfish of the 18 °C group were sampled at 1, 3, 6, 12, 24, 48, 96, and 168 h post-cooling, for comparisons of the acute-phase changes in livers in response to hypothermal challenges between FW and SW. Six individuals from each time point were used in the one-week and acute-phase experiments. Before sampling, the fish were anesthetized with 0.5% 2-phenoxyethanol, then sacrificed by cutting their spinal cord. The protocol describing the experiments of fish was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the National Chung Hsing University (IACUC Approval No. 104–126R to THL).

2.2. Obtaining the cDNA sequence

Total RNA samples were extracted from the liver of *C. chanos* using Tripure isolation reagent (Roche, Mannheim, Germany). The genomic DNA contamination in the RNA preparation was eliminated using the RNA cleanup protocol provided with the RNAspin Mini RNA isolation kit (GE Health Care, Piscataway, NJ, USA). The quality of the extracted total RNA was determined using NanoDrop 2000 (Thermo, Wilminton, CA, USA) and was visually assessed using electrophoresis, following the methods described in Ref. [23]. The first-strand cDNA was synthesized from 1 μg of total RNA using iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturer's instructions.

2.3. Cloning of full-length Ccwap65-1 and Ccwap65-2 cDNAs from milkfish liver

The partial DNA sequences with homology to *wap65-1* and *wap65-2* were identified from the milkfish NGS database (Hu et al., 2015). The cDNA template for rapid amplification of cDNA ends (RACE) was made from the total RNA extracted from the liver of milkfish using SMART RACE amplification kit (Clontech, Mountain View, CA, USA). For PCR amplification, 2 μL of 5′- or 3′- RACE-cDNA was used as a template in a 50 μL reaction containing 0.25 mM dNTPs, 2.5 U EX-Taq polymerase (Takara, Shiga, Japan), and 0.2 μM of each primer. The specific primers for 5′- and 3′- RACE were designed according to the sequence of the conserved regions listed in Table 1. The RACE products were subcloned into pGM-T vector (Genemark, Taipei, Taiwan) and the amplicons were confirmed by sequencing. BLAST (http://www.ncbi.nlm.nih.gov/) was used to identify the sequences of *Ccwap65-1* and *Ccwap65-2*.

2.4. Sequence analysis

The cleavage sites for the signal peptide were predicted using SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The open reading frame (ORF) of *Ccwap65-1* and *Ccwap65-2* were predicated with ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The molecular mass and theoretical pI were predicted using pI/Mw program (http://web.expasy.org/compute_pi/). The multiple alignment of amino acid sequences was also performed (accession numbers of the amino acid sequences of other fish and vertebrates are listed in Supplementary Table 1). The phylogenetic tree was constructed using Mega 6 by neighbor-joining method with 1000 bootstraps.