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Molecular cloning, characterization and expression analysis of a novel *wap65-1* gene from *Plecoglossus altivelis*

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ABSTRACT

Warm temperature acclimation associated 65-kDa protein 1 (WAP65-1) is a specific fish plasma glycoprotein that is possibly involved in various physiological or pathological processes. In this study, we obtained the cDNA and genomic DNA sequences of the *Plecoglossus altivelis wap65-1* (*Pawap65-1*) gene. Multiple sequence alignment showed that *Pawap65-1* is similar in structure to *wap65-1* in fish. Phylogenetic analysis revealed that *Pawap65-1* is most closely related to that of a rainbow trout. *Pawap65-1* transcripts are present in various tissues and are most abundant in the liver. We expressed recombinant PaWAP65-1 in *Escherichia coli* and raised antiserum against it in mouse. Western blot analysis revealed that the higher molecular mass of PaWAP65-1 in blood plasma was caused by post-translational N-glycosylation. Quantitative real-time quantitative PCR (qPCR) and Western blot analysis data showed that the hepatic mRNA and blood plasma levels of PaWAP65-1 were both influenced by warm temperature acclimation and cadmium exposure, but not by *Listonella anguillarum* infection, hypo-osmotic, or cold temperature acclimation. In conclusion, our data reveals that PaWAP65-1 is a stress-related protein, and may play a role in fish acclimation to warm temperature and cadmium exposure.

1. Introduction

The warm temperature acclimation associated 65-kDa protein (WAP65), which is homologous to mammalian hemopexins, is a fish-specific glycoprotein that was first identified in the plasma of goldfish (*Carassius auratus*) (Kikuchi et al., 1993), and is believed to play an important role in the acclimation of fish to warm temperatures (Kikuchi et al., 1995). Until now, two types of WAP65 proteins, WAP65-1 and WAP65-2, were identified in fish based on their sequence diversity, mRNA expression profile, and function (Hirayama et al., 2003, 2004; Nakaniwa et al., 2005; Sha et al., 2008; Pierre et al., 2010; Cho et al., 2012). The evolution rate of *wap65-2* is much lower than that of *wap65-1*. In addition, *wap65-2* has a restricted tissue distribution (mainly in the liver), whereas *wap65-1* is commonly found in various tissues (Hirayama et al., 2003, 2004; Sha et al., 2008; Cho et al., 2012).

In teleosts, WAP65-1 and WAP65-2 were both found to function as multifunctional agents in several biological processes. The transcription of *wap65-2* in Japanese pufferfish (*Takifugu rubripes*) and channel catfish (*Ictalurus punctatus*) was activated by warm temperature (Hirayama et al., 2003; Sha et al., 2008). In blue catfish (*Ictalurus furcatus*) and channel catfish, *wap65-2* mRNA expression was induced up to 12.3- and 23.4-fold in response to *Edwardsiella ictaluri* infection, respectively (Peatman et al., 2007, 2008). Warm temperature and bacterial infection appear to synergistically induce the expression of *wap65-2* in channel catfish (Sha et al., 2008). Heavy metals such as copper (Cu), cadmium (Cd), or nickel (Ni) can remarkably increase the expression of the *wap65-2* gene in green swordtail (*Xiphophorus helleri*) (Aliza et al., 2008) and pond loach (*Misgurnus mizolepis*) (Cho et al., 2012). In contrast, *wap65-1* mRNA expression in response to stimuli varies in different fish species. For example, *wap65-1* is constitutively expressed in channel catfish (Sha et al., 2008), but in blackhead seabream (*Acanthopagrus schlegelii*) (Choi et al., 2008), European seabass (*Dicentrarchus labrax*) (Pierre et al., 2010, 2011), and Japanese flounder (*Paralichthys olivaceus*) (Choi, 2010), it is regulated by both warm temperature and bacterial infection. In European seabass, *wap65-1* mRNA expression can be induced by Cd exposure, but its expression is slightly inhibited by Cu exposure (Pierre et al., 2011). Thus, it is clear that the two paralogues, WAP65-1 and WAP65-2, have different functions in fish. The WAP65-1 protein, which is not well studied like WAP65-2, may also play an important role in the molecular mechanisms underlying the environmental acclimation of fish.

Plecoglossus altivelis, the sole member of the Osmeriformes family Plecoglossidae, is an important cultured freshwater fish in Japan, China, and Korea. It is one of the most common types of coldwater fish, and prefers higher temperatures (15–25 °C) and clear water. *P. altivelis* are severely affected by environmental factors of water, such as temperature, salinity, heavy metals, and pathogens (Kishino and Shinomiya, 2004; Kishino et al., 2008; Chen et al., 2009; Li et al., 2011; Lu et al., 2012). In our previous report, we identified

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PaWAP65-2 as a positive acute-phase protein in fish upon *Listonella anguillarum* infection (Shi et al., 2010; Li et al., 2011). In this study, we cloned the cDNA and genomic DNA sequences of the *Pawap65-1* gene, and determined the association between environmental acclimation and changes in its expression.

2. Materials and methods

2.1. Experimental fish and conditions

Juvenile male *P. altivelis* (Osmeriformes: Plecoglossidae), weighing 20–25 g, were obtained from a commercial farm in Ningbo City, China. Fish were kept in freshwater tanks at 20 ± 0.5 °C in a recirculating system with filtered water, fed with commercial pellets once a day, and acclimatized to laboratory conditions for two weeks before experiments. All fish used in this study were healthy without any pathological symptoms. All animal work in this paper was conducted according to relevant national and international guidelines. All animal care and experimental procedures were approved by the Committee on Animal Care and Use and the Committee on the Ethics of Animal Experiments of Ningbo University.

2.2. Obtaining the cDNA sequence encoding Pawap65-1

Total RNA was extracted from the liver of healthy *P. altivelis* using RNAiso regents (TaKaRa, Kyoto, Japan), and mRNA was obtained using Oligotex-dT30<super>mRNA Purification Kit (TaKaRa). The TaKaRa cDNA Library Construction Kit (TaKaRa) was used according to the manufacturer's protocols for cDNA library construction (Shi et al., 2010). A random set of 287 clones were partially auto-sequenced by an ABI 3730 automated sequencer (Invitrogen, Shanghai, China). BLASTX search (http://www.ncbi.nlm.nih.gov/) was used to find *wap65-1*-like sequences (Gish and States, 1993).

2.3. Obtaining the genomic sequence of the Pawap65-1 gene

The genomic DNA of *P. altivelis* was isolated from the liver tissue with a DNA Extraction Kit (TaKaRa), dissolved in TE buffer, and kept at −70 °C before use. To amplify the genomic DNA sequence of *Pawap65-1*, long-distance PCR amplification was carried out using LA Taq DNA polymerase (TaKaRa). Based on the cDNA sequence of *Pawap65-1*, one pair of primers was designed, and the sequence of each primer is as follows: PaWAP65-1F: 5′-ATGCTGCTCCTCCACAC TCT-3′ (nt 128–147) and PaWAP65-1R: 5′-CTAGTGGTCACAGCC AAATA-3′ (nt 1437–1456). The conditions of PCR were denaturing at 94 °C for 10 min, 30 cycles of denaturing at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 6 min, and a final extension step at 72 °C for 10 min. The PCR product was cloned into the pMD19-T Simple vector and sequenced (Invitrogen).

2.4. Sequence analysis

The cleavage site of signal peptides was predicted by the SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/). The potential N-linked glycosylation site was predicted by the NetNGlyc program (http://www.cbs.dtu.dk/services/NetNGlyc/). Molecular mass and theoretical pI were predicted by the pI/Mw program (http://web.expasy.org/compute_pi). Multiple alignment was done using ClustalW (http://clustalw.ddbj.nig.ac.jp/), and multiple alignment of genomic sequences was performed using Multi-PipMaker (Schwartz et al., 2000). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0 (Tamura et al., 2007).

2.5. Temperature treatment

Juvenile fish were subjected to heat or cold water. In the heat-treated group, water temperature increased by 1 °C per hour from 20 °C to 22 °C or 24 °C, while in the cold-treated group, water temperature decreased down to 16 °C or 18 °C with a decrease rate of 1 °C per h. On the other hand, the remaining tanks were kept constant at 20 °C. 40 fish per temperature group (a total of 120 fish) were used. Throughout the experiment, no feed was supplied, and the water temperature was controlled to range within ±0.5 °C using electric thermostat-assisted heaters and coolers. Whole blood and liver samples were collected from each group 0 and 24 h after temperature treatment. After been kept at 4 °C overnight, blood plasma was collected by centrifugation of whole blood at 1500 *g* for 15 min.

2.6. Bacterial infection

L. anguillarum infection was performed as previously reported (Li et al., 2011). In brief, overnight cultures of *L. anguillarum* were diluted 1:50 in nutrient broth, grown at 28 °C with shaking, and harvested in the logarithmic phase of growth. The final concentration of bacteria was confirmed by plating serial dilutions on solid media. Each juvenile fish was given an intraperitoneal injection of *L. anguillarum* at a dose level of 3.8×10^5 CFU. 16 biological replicates were used per treatment group (a total of 48 fish), and sampled at 4, 8, 12, and 24 h respectively. Another 16 fish per control group (a total of 48 fish) were injected with an equal volume of PBS and sampled at 4, 8, 12, and 24 h respectively. During the experimental periods, all fish were kept at 20 ± 0.5 °C, and no feed was supplied. Blood plasma and liver samples were collected and treated as above.

2.7. Hypo-osmotic challenge

Juvenile fish maintained in freshwater were challenged with hypo-osmotic treatments as previously reported (Chen et al., 2009). In brief, 16 fish per treatment group (a total of 48 fish) were transferred and sequentially subjected to brackish water at a concentration of 10 mg/L NaCl for 24 h. After accumulation, fish were sampled at 4, 8, 12, and 24 h respectively. Another 16 fish per control group (a total of 48 fish) were maintained at freshwater for 24 h, and sampled at 4, 8, 12 and 24 h respectively. Blood plasma and liver samples were collected and treated as above.

2.8. Cadmium exposure

Juvenile fish maintained in freshwater were exposed to cadmium (Cd) as previously reported (Lu et al., 2012). 16 fish per treatment group (a total of 48 fish) were transferred and sequentially exposed to 5.0 mg/L Cd (as $CdCl_2 \cdot H_2O$) in freshwater for 24 h, and sampled at 4, 8, 12 and 24 h respectively. The control groups in hypo-osmotic challenge experiments were also used here. In the experiments, waterborne Cd^{2+} was kept at a steady concentration. Blood plasma and liver samples were collected and treated as above.

2.9. Quantitative real-time PCR (qPCR) analysis of Pawap65-1 transcripts

Total RNA (1 μg) from each tissue of *P. altivelis* were extracted using RNAiso regents (TaKaRa). After treatment with deoxyribonuclease I (TaKaRa), first-strand cDNA was synthesized in a 20 μL reaction volume. *Pawap65-1* transcripts were examined by qPCR. Specific primers Pawap65-1F: 5′- GAGGCTCGTGACTACTTCAT-3′ and Pawap65-1R: 5′-ACAGCGTCCACGTCA CTATG-3′ were used for amplification of a 230 base pair (bp) fragment from *Pawap65-1* mRNA. As an internal control, pActin2F primers: 5′-TCGTGCGTGACATCAAGGAG-3′ and pActin2R: 5′-CGCACTTCATGATGCTGTTG-3′ were used to amplify a 231 bp fragment of the reference *β-actin* mRNA (Lu et al., 2011). One