Expression of Warm Temperature Acclimation-Related Protein 65-kDa (Wap65) mRNA, and Physiological Changes with Increasing Water Temperature in Black Porgy, *Acanthopagrus schlegeli*

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ABSTRACT   We isolated the warm temperature acclimation-related protein 65-kDa (Wap65) cDNA from the liver of black porgy and investigated the expression by increasing water temperature in black porgy, *Acanthopagrus schlegeli*. Black porgy Wap65 full-length cDNA consists of 1,338 nucleotides, including an open reading frame, predicted to encode a protein of 425 amino acids and showed high homology to pufferfish (79%), Medaka (73%), carp (70%), and goldfish (68%) Wap65. Increase in water temperature ($20°C \rightarrow 30°C$; $1°C$/day) induced the rise of Wap65 mRNA expression in liver of black porgy. Also, the levels of cortisol and glucose in plasma were significantly higher at $30°C$ than at $20°C$. To determine the high water temperature stressor specificity of the induction of Wap65, black porgy were transferred from seawater (SW) to freshwater (FW) for 24 hr. Wap65 expression was not detected when the fish were transferred from SW to FW (in fish transferred from SW to FW), although the levels of cortisol and glucose in plasma were increased. These results suggest that increase in Wap65 gene is related to high water temperature stress and play important roles in high water temperature environment of black porgy. *J. Exp. Zool. 309A:206–214, 2008*. © 2008 Wiley-Liss, Inc.

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Water temperature is one of the most important factors influencing the growth and reproduction of fish. However, in fish farming, rapid changes in water temperature stress fish to the same degree as moving, confinement, sorting, and high stocking density (Schreck et al., '89). Fish are constantly exposed to stressful conditions and have consequently evolved stress response systems at all levels of biological organization. At the organism level, stress activates the nervous system and triggers a cascade of humoral reactions along the hypothalamus–pituitary–interrenal axis that ultimately result in the rapid release of corticosteroid stress hormones (Wendelaar Bonga, '97; Mommsen et al., '99). Corticosteroids are biosynthesized by various microsomal enzymes located in the interrenal cells, and their production is under the strict control of the pituitary gland (Donaldson, '81). Once released into the circulation, corticosteroids can enter a cell by passive diffusion, but recent data suggest that carrier-mediated processes facilitate this uptake (Vijayan et al., '97). The glucocorticoid receptor, a ligand-inducible transcription factor, can activate or repress target genes (Munck et al., '84; Adcock, 2000).

Corticosteroid-mediated gene induction can influence a variety of physiological functions related to metabolism, immunity behavior, osmoregulation, and cardiovascular transport (Wendelaar Bonga, '97; Mommsen et al., '99).

In general, poikilotherms and homeotherms synthesize intracellular heat shock proteins (HSPs) in response to heat shock, allowing the organism to maintain physiological homeostasis. Such mechanisms, including the expression of HSPs, are well known (Schlesinger et al., '92; Choi et al., 2006).

In addition, it was recently reported that warm temperature acclimation-related protein 65 kDa (Wap65) is expressed as the water temperature rises. Wap65 was first identified as an abundant cytosolic protein in eurythermal fish such as goldfish (*Carassius auratus*) and carp (*Cyprinus carpio*) acclimated to 30°C. The expression of Wap65 was markedly increased for both protein and transcript in concert with ambient temperature increasing. Kikuchi et al. ('95) reported that in goldfish kept at water temperatures of 10 and 30°C, Wap65 mRNA was expressed in the hepatopancreas at the higher temperature. In addition, at 30°C, Wap65 was confirmed in the serum of goldfish and carp (Kinoshita et al., 2001). These results suggest that Wap65 plays an important role as water temperature increases. However, the mechanism and function of Wap65 remain unclear yet.

Therefore, our purpose of this study is to isolate the full-length Wap65 cDNA and investigate changes in Wap65 mRNA expression in tissues (brains, livers, gonads, kidneys, and intestines) when the fish were exposed to high water temperature, and we compared the mRNA expression of the Wap65 gene during transfer to freshwater (FW) to determine whether the Wap65 genes are specifically expressed under high water temperature stress. Also, we analyzed changes in the levels of cortisol and plasma to provide basic data on the cellular stress reaction of black porgy exposed to high water temperature.

MATERIALS AND METHODS

Experimental animals and rising temperature

Black porgy (average length 14.3 ± 0.4 cm, weight 51.0 ± 6.0 g) were collected from a fish farm in Pukyung National University and reared in 200 L circulation filter tanks in the laboratory for 2 weeks. The increase in water temperature and transfer to FW of black porgy were performed according to the methods of Choi et al. (2006) and Min et al. (2003), respectively. Fish were divided into three groups; 20°C control group, 30°C experimental group, and FW experimental group (each 10 fish). Before the experiment, water temperature was maintained at 20 ± 1°C, with a 12L:12D photoperiod, and fish were fed a commercial feed (42% protein, 7% fat, 4% fiber, 17% ash, and 2.7% phosphorus) twice a day (3% of body weight for each meal at approximately 09:00 and 16:00). During the high water temperature stress experimental period, except the osmotic stress experiment, the water temperature was increased by 1°C every day from 20 to 30°C (1°C/day) using automatic temperature regulation systems (Johnsam Co., Boocheon, Korea), and no feed was given during the experimental period.

Sampling procedure

Four fish from each treatment group (20°C control group, 30°C experimental group, and FW experimental group) were randomly selected for blood and tissue sampling. Fish were anesthetized with a 200 mg/L solution of tricaine methanesulfonate (Sigma, St. Louis, MO) blood collection. Blood was collected from the caudal vasculature using a 3-mL syringe coated with heparin. Plasma samples were separated by centrifugation and stored at −80°C until analysis. Fish were killed by spinal transection for the collection of the tissues (brains, livers, gonads, kidneys, and intestines). Immediately after collection, the samples were frozen in liquid nitrogen and stored at −80°C until total RNA was extracted.

Isolation of Wap65 cDNA

Total RNA was extracted from various tissues using Total RNA Isolation System (Promega, Madison, WI). Wap65 primers were designed using highly conserved regions, with reference to the known sequences of other species (Table 1).

Reverse transcription and polymerase chain reaction (PCR) amplification were conducted using the AccuPower RT/PCR PreMix (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. A single PCR product of the expected size (963 bp) was obtained. Amplified PCR products were processed by electrophoresis in 1% agarose gels containing ethidium bromide (0.5 μg/μL). The PCR product was purified and then cloned into pGEM-T Easy Vector (Promega, Madison, WI). The colony formed by transformation was cultivated in DH5α, and then plasmid

TABLE 1. Primers used for the RT-PCR of the black porgy Wap65 and β-actin

Gene	Primer	DNA sequence
Wap65	Wap65-F	5′-ACCTGGGACATGTGGATGCT-3′
	Wap65-R	5′-GGCAACCACTACTACCACTT-3′
	3′ RACE-GSP	5′-GGTCCCATTGATGCTGCATTCGTCTGC-3′
	5′ RACE-GSP	5′-TACAGCAGCATCCAGGTGGTCAGGGATT-3′
	Wap65-GSP-F	5′-ACCTGGGCCATGTAGATGCT-3′
	Wap65-GSP-R	5′-GGCAACCACTTCTACCACTT-3′
β-actin	β-actin-F	5′-TCGAGCACGGTATTGTGACC-3′
	β-actin-R	5′-ACGGAACCTCTCATTGCCGA-3′

PCR, polymerase chain reaction.

DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Seoul, Korea) and EcoRI (Fermentas, Hanover, MD). Based on the plasmid DNA, Wap65 cDNA sequence data were analyzed using ABI DNA Sequencer (Applied Biosystems, Foster City, CA).

Rapid amplification of Wap65 cDNA 3′ and 5′ ends (3′ and 5′ RACE)

For the PCR reaction, total RNA was extracted from liver tissue using the total RNA Isolation System (Promega, Madison, WI). With 2 μg of total RNA as a template, 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (Seegene, Seoul, Korea). First-strand cDNA synthesis was conducted using oligo (dT) anchor primer (5′-CTG TGA ATG CTG CGA CTA CGA T(T)$_{18}$-3′) and a CapFishing™ adaptor (Seegene, Seoul, Korea).

Gene-specific primers were selected from the PCR product (963 bp) obtained by RT-PCR. For 3′ RACE PCR, the 50 μL PCR reaction mixture was as follows: 5 μL of 3′ RACE cDNA, 1 μL of 10 mmol/L 3′ target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μL of 10 mmol/L 3′ RACE gene-specific primer (Table 1), and 25 μL of SeeAmp Taq Plus master mix (Seegene, Seoul, Korea). PCR was carried out for 35 cycles as follows: one cycle of denaturation at 94°C, denaturation at 94°C for 45 sec, annealing at 58°C for 45 sec, and extension at 72°C for 90 sec, followed by one cycle of 5 min at 72°C for extension.

For 5′ RACE PCR, the 50 μL PCR reaction mixture was as follows: 5 μL of 5′ RACE cDNA, 1 μL of 10 mmol/L 5′ target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μL of 10 mmol/L 5′ RACE gene-specific primer (Table 1), and 25 μL of SeeAmp Taq Plus master mix. PCR was carried out for 35 cycles as follows: one cycle of denaturation

at 94°C, denaturation at 94°C for 45 sec, annealing at 58°C for 45 sec, and extension at 72°C for 90 sec, followed by one cycle of 5 min at 72°C for extension.

The PCR product was amplified, cloned into a pGEM-T Easy Vector (Promega, Madison, WI), and sequenced. DNA and deduced amino acid sequence data were analyzed using GENETYX-WIN (Software Development Co., Tokyo, Japan).

Phylogenetic analysis

Phylogenetic analysis was performed on the amino acid sequences from full-length Wap65 cDNA from various fishes. Amino acid sequences were aligned using the BioEdit Software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequences used for comparison and their GenBank accession numbers are as follows: black porgy (*Acanthopagrus schlegeli*, ABL74446), European seabass (*Dicentrarchus labrax*, ABL75414), pufferfish (*Takifugu rubripes*, BAD18109), Medaka (*Oryzias latipes*, BAB97303), carp (*C. carpio*, BAB60809), goldfish (*C. auratus*, BAA08928), and green swordtail (*Xiphophorus hellerii*, ABD 14149). A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, '87) and analyzed using Mega 3.1 software package (Kumar et al., 2001). The degree of support for internal branches was inferred using bootstrapping (1,000 replicates) analysis.

Validation of semi-quantitative RT-PCR

Total RNA was extracted using Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's protocol.

To optimize the cycle number used for semi-quantitative PCR analysis, the RT reaction (1 μg) from the brain, liver, kidney, gonad, and intestine was used as template for PCR amplification.

Wap65 gene-specific primers are shown in Table 1. RT-PCR was conducted using SuperScript™ reverse transcriptase (Invitrogen, Carlsbad, CA) with 1 μg of total RNA as a template. A reverse transcript reaction was conducted at 42°C for 50 min, after which PCR was carried out as follows: one cycle of denaturation at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 45 sec, followed by one cycle of 5 min at 72°C for the final extension. The PCR products from different cycles of amplification were visualized on a UV-transilluminator after electrophoresis on 1.0% agarose gel containing ethidium bromide, and the signal intensity was quantitated with the Gel-Doc System and Gelpro 3.1 Software (KBT, Incheon, Korea). The cycle numbers that generate half-maximal amplification were used for subsequent semi-quantitative analysis of gene expression, and they are 33 cycles for Wap65 and 27 cycles for β-actin.

Analysis of plasma parameters

Plasma cortisol was analyzed by radioimmunoassay using a radioimmunoassay kit (Diagnostic System Laboratories, Webster, Texas). Plasma glucose was analyzed using the Biochemistry Auto analyzer (Hitachi Co., Tokyo, Japan).

Statistical analysis

The data from each experiment were tested for significant differences using a one-way analysis of variance (unpaired Student's t-test) with the SPSS statistical package (version 10.0) at a significance level of $P < 0.05$.

RESULTS

Isolation of Wap65 cDNA

A PCR-based cloning strategy (RT-PCR followed by 3′ RACE and 5′ RACE) was used to clone a cDNA encoding a putative Wap65 from black porgy liver. The cDNA included a 1,275 bp open reading frame that began with the first ATG codon at position 61 bp and ended with a TAG stop codon at position 1,338 bp (GenBank accession no. EF134717; Fig. 1). Black porgy Wap65 contained a 23-amino-acid signal peptide and a 402 amino acid mature protein with ten cysteines, and four N-linked glycosylation sites (NES, NSS, NCT, and NDT; Fig. 2).

Identity of Wap65

The amino acid identity of black porgy Wap65 with that of other fish species is as follows: pufferfish (79%; GenBank accession no. AB125932), Medaka (73%; GenBank accession no. AB195240), carp (70%; GenBank accession no. AB052623), and goldfish (68%; GenBank accession no. D50437) (Fig. 2). The five fish species compared for homology have ten cysteines and over two N-linked glycosylation sites in common (Fig. 2).

Molecular phylogeny

A range of seven fishes' Wap65 amino acid sequences were analyzed, including that of bpWap65, to build a phylogenetic tree. A phylogenetic tree is shown in Figure 3. The phylogenetic analysis indicated that there are three groups of Wap65: group 1 (black porgy, European seabass, pufferfish, and Medaka), group 2 (carp and goldfish), and group 3 (green swordtail). The bpWap65 is most closely related to European seabass Wap65 than other fishes (Fig. 3).

Tissue distribution of Wap65 mRNA

The tissue distribution of Wap65 mRNA was investigated by RT-PCR. In control (20°C) and FW experimental groups, the levels of Wap65 mRNA expression were slightly detected in livers. In contrast, in 30°C experimental group, the expression of Wap65 mRNA was observed only in liver and approximately ten times higher than in the control group ($P < 0.05$; Fig. 4).

Plasma parameters

The plasma cortisol levels were 5.3 ± 1.3 ng/mL in control, but increased to 45.0 ± 6.4 and 38.0 ± 6.4 ng/mL in 30°C and FW group, respectively ($P < 0.05$). The plasma glucose levels increased significantly from 53.4 ± 2.5 mg/dL in control to 70.0 ± 2.3 and 63.8 ± 1.9 mg/dL in 30°C and FW group ($P < 0.05$; Fig. 5).

DISCUSSION

In general, many factors trigger a neuroendocrine and cellular stress reaction in fish. These reactions affect osmoregulatory function, reduce the resistance to disease, and suppress reproduction (Ackerman et al., 2000). To overcome such an unstable physiological state, fish require an energy source (glucose) to maintain homeostasis. The neuroendocrine reaction promotes gluconeogenesis

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    1  GATAGGGGACCTTTTGCTCTAGGCCCCTCAAGTCTTTCCTGAACTAGTGAACTGCTCGCC

   61  ATGAAGCTGCTCGCTCACATCCTTTTCCTGGCCCTGGCTGTGGTATTGGCTCAGTGTGAT
       M  K  L  L  A  H  I  L  F  L  A  L  A  V  V  L  A  Q  C  D     20

  121  GAAGATACATCAGCTGTCCCTGACCGTTGTCAAGGCCTTGAGATGGACGCTGTTGCAGTA
       E  D  T  S  A  V  P  D  R  C  Q  G  L  E  M  D  A  V  A  V     40

  181  AATGAAGAATATATACCTTACTTTTTCAAGGGGTGACCATCTGTTCAAGGGCTTCCATGGC
       N  E  E  Y  I  P  Y  F  F  K  G  D  H  L  F  K  G  F  H  G     60

  241  AAAGCAGAGCTGTCTAATGAGTCCTTCGCAGAGTTGGATGACCATCACCACCTGGGCCAT
       K  A  E  L  S  N  E  S  F  A  E  L  D  D  H  H  H  L  G  H     80

  301  GTAGATGCTGCTTTCCGCATGCACTATGAGAACAGCAGCGACCATGACCACATGTTCTTC
       V  D  A  A  F  R  M  H  Y  E  N  S  S  D  H  D  H  M  F  F    100

  361  TTCTTGGACAACAAGGTGTTCAGCTATTACAAACACAAACTTGAGGACGGCTTCCCCAAG
       F  L  D  N  K  V  F  S  Y  Y  K  H  K  L  E  D  G  F  P  K    120

  421  GATACCTCTGAAGTCTTCCCTGGAATCCCTGACCACCTGGATGCTGCTGTAGAGTGTCCC
       D  T  S  E  V  F  P  G  I  P  D  H  L  D  A  A  V  E  C  P    140
                              ← 5'RACE primer

  481  AAGCCAGAGTGTGATGCAGACTCTGTCATCTTCTTCAAGGGAAACGACATCTACCACTTC
       K  P  E  C  D  A  D  S  V  I  F  F  K  G  N  D  I  Y  H  F    160

  541  AATGTCCAAACCAAAGCAGTTGATGAGAAAGAGTTCGAGTCCATGCCAAACTGCACATCT
       N  V  Q  T  K  A  V  D  E  K  E  F  E  S  M  P  N  C  T  S    180

  601  GCTCTCCGCTTTATGGAGCACTATTACTGCTTCCATGGACATATGTTCTCCAAGTTTGAC
       A  L  R  F  M  E  H  Y  Y  C  F  H  G  H  M  F  S  K  F  D    200

  661  CCAAAGACTGGCGAGGTACCTCGCACACACCCCAAAGAGGCACGAGACTACTTCATGAGA
       P  K  T  G  E  V  P  R  T  H  P  K  E  A  R  D  Y  F  M  R    220

  721  TGCTCTAAGTTCAGTGAAGAAAGCGACCACGTAGAGAGAGAGCGCTGCAGTCGTGTTCAC
       C  S  K  F  S  E  E  S  D  H  V  E  R  E  R  C  S  R  V  H    240

  781  CTGGATGCCATCACATCTGACAATGCTGGGAACATGTATGCCTTCAGAGGCCACCATTTC
       L  D  A  I  T  S  D  N  A  G  N  M  Y  A  F  R  G  H  H  F    260

  841  CTCAGTAAAGATGGGAGCAATGACACAATGACGGCAGACACCATCGAAAATGCTTTCAGG
       L  S  K  D  G  S  N  D  T  M  T  A  D  T  I  E  N  A  F  R    280

  901  GAGCTTCACAGTGAGGTGGACGCTGTTTTCACATACCAGGATCACCTTTACATGATCAAG
       E  L  H  S  E  V  D  A  V  F  T  Y  Q  D  H  L  Y  M  I  K    300

  961  GATGACCATCTGTTCCTTTACAAAGTTGGTGAGCCCCACACCCACTTGGATGGTTACCCC
       D  D  H  L  F  L  Y  K  V  G  E  P  H  T  H  L  D  G  Y  P    320

 1021  AAGCCTGTGAAGGAGGAGCTGGGCATTGAGGGTCCCATTGATGCTGCATTCGTCTGCGAG
       K  P  V  K  E  E  L  G  I  E  G  P  I  D  A  A  F  V  C  E    340
                              3' RACE primer →

 1081  GATCATCACATTGCTCACATTATCAAAGGTAATAAGATCTATGATGTGGAATTGAAGGCC
       D  H  H  I  A  H  I  I  K  G  N  K  I  Y  D  V  E  L  K  A    360

 1141  AGCCCTCGTGTTGCAGGCAACGAGCGTACTATTTCCCTCTTCGATAACGTTGATGCTGCC
       S  P  R  V  A  G  N  E  R  T  I  S  L  F  D  N  V  D  A  A    380

 1201  ATGTGCGATGGTACAGGAGTGAAGGTGATCAAAGGCAACCACTTCTACCACTTCATCAGC
       M  C  D  G  T  G  V  K  V  I  K  G  N  H  F  Y  H  F  I  S    400

 1261  ACACATGGGTTTGTTGCTGGCAGGAGCCTGCCTGAGCAGCATAGGGTTTCCTTGGAGCTG
       T  H  G  F  V  A  G  R  S  L  P  E  Q  H  R  V  S  L  E  L    420

 1321  TTTGGCTGTGACCACTAG
       F  G  C  D  H  *                                             425
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Fig. 1. Base sequence of the black porgy Wap65 cDNA clone and deduced amino acid sequence. Nucleotide number is shown on the left and the amino acid residue number is shown on the right. The signal peptide of black porgy Wap65 is boxed. Potential N-linked glycosylation sites are underlined. Shaded regions show a target sequence for 5′ RACE GSP. These sequence data are available from the NCBI/GenBank nucleotide sequence databases with the accession number EF134717.

(Munck et al., '84) and the cellular stress reaction synthesizes protein, the energy source used in gluconeogenesis (Gamperl et al., '94).

In this study, we first isolated full-length Wap65 cDNA from the liver of black porgy. Black porgy Wap65 cDNA is composed of 1,338 nucleotides, including an open reading frame, predicted to encode a protein of 425 amino acids. Black porgy Wap65 contained a 23-amino-acid signal peptide, a 402-amino-acid mature protein with ten cysteines, and four N-linked glycosylation sites (NES, NSS, NCT, NDT; Fig. 2). Kinoshita et al. (2001) reported that three such sites in the goldfish protein and two in the carp. Moreover, the goldfish
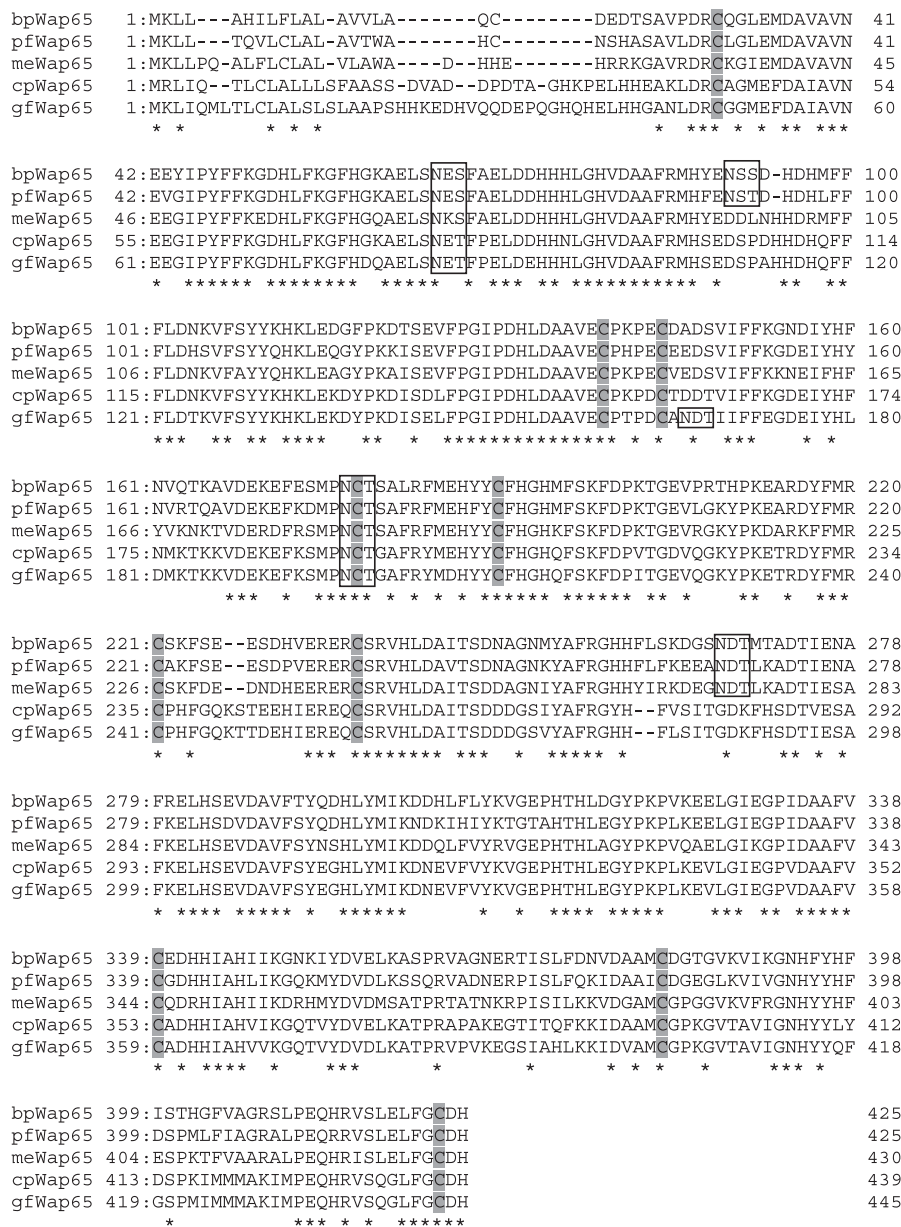
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bpWap65   1:MKLL---AHILFLAL-AVVLA-------QC--------DEDTSAVPDRCQGLEMDAVAVN  41
pfWap65   1:MKLL---TQVLCLAL-AVTWA-------HC--------NSHASAVLDRCLGLEMDAVAVN  41
meWap65   1:MKLLPQ-ALFLCLAL-VLAWA----D--HHE-------HRRKGAVRDRCKGIEMDAVAVN  45
cpWap65   1:MRLIQ--TLCLALLLSFAASS-DVAD--DPDTA-GHKPELHHEAKLDRCAGMEFDAIAVN  54
gfWap65   1:MKLIQMLTLCLALSLSLAAPSHHKEDHVQQDEPQGHQHELHHGANLDRCGGMEFDAIAVN  60
            *  *       * *  *                        *  *** *  * ** ***

bpWap65  42:EEYIPYFFKGDHLFKGFHGKAELSNESFAELDDHHHLGHVDAAFRMHYENSSD-HDHMFF 100
pfWap65  42:EVGIPYFFKGDHLFKGFHGKAELSNESFAELDDHHHLGHVDAAFRMHFENSTD-HDHLFF 100
meWap65  46:EEGIPYFFKEDHLFKGFHGQAELSNKSFAELDDHHHLGHVDAAFRMHYEDDLNHHDRMFF 105
cpWap65  55:EEGIPYFFKGDHLFKGFHGKAELSNETFPELDDHHNLGHVDAAFRMHSEDSPDHHDHQFF 114
gfWap65  61:EEGIPYFFKGDHLFKGFHDQAELSNETFPELDEHHHLGHVDAAFRMHSEDSPAHHDHQFF 120
            * ****** ********  *****  *  * ***  ** *********** *    **  **

bpWap65 101:FLDNKVFSYYKHKLEDGFPKDTSEVFPGIPDHLDAAVECPKPECDADSVIFFKGNDIYHF 160
pfWap65 101:FLDHSVFSYYQHKLEQGYPKKISEVFPGIPDHLDAAVECPHPECEEDSVIFFKGDEIYHY 160
meWap65 106:FLDNKVFAYYQHKLEAGYPKAISEVFPGIPDHLDAAVECPKPECVEDSVIFFKKNEIFHF 165
cpWap65 115:FLDNKVFSYYKHKLEKDYPKDISDLFPGIPDHLDAAVECPKPDCTDDTVIFFKGDEIYHF 174
gfWap65 121:FLDTKVFSYYKHKLEKDYPKDISELFPGIPDHLDAAVECPTPDCANDIIIFFEGDEIYHL 180
            ***   ** ** ****  **  *  ***   * *************** * *  * *** * *

bpWap65 161:NVQTKAVDEKEFESMPNCTSALRFMEHYYCFHGHMFSKFDPKTGEVPRTHPKEARDYFMR 220
pfWap65 161:NVRTQAVDEKEFKDMPNCTSAFRFMEHYYCFHGHMFSKFDPKTGEVLGKYPKEARDYFMR 220
meWap65 166:YVKNKTVDERDFRSMPNCTSAFRFMEHYYCFHGHKFSKFDPKTGEVRGKYPKDARKFFMR 225
cpWap65 175:NMKTKKVDEKEFSMPNCTGAFRYMEHYYCFHGHQFSKFDPVTGDVQGKYPKETRDYFMR 234
gfWap65 181:DMKTKKVDEKEFKSMPNCTGAFRYMDHYYCFHGHQFSKFDPITGEVQGKYPKETRDYFMR 240
            ***   *  ***** * *  * **** ***** ** *      ** *   ***

bpWap65 221:CSKFSE--ESDHVERERCSRVHLDAITSDNAGNMYAFRGHHFLSKDGSNDTMTADTIENA 278
pfWap65 221:CAKFSE--ESDPVERERCSRVHLDAVTSDNAGNKYAFRGHHFLFKEEANDTLKADTIENA 278
meWap65 226:CSKFDE--DNDHEERERCSRVHLDAITSDDAGNIYAFRGHHYIRKDEGNDTLKADTIESA 283
cpWap65 235:CPHFGQKSTEEHIEREQCSRVHLDAITSDDDGSIYAFRGYH--FVSITGDKFHSDTVESA 292
gfWap65 241:CPHFGQKTTDEHIEREQCSRVHLDAITSDDDGSVYAFRGHH--FLSITGDKFHSDTIESA 298
            *  *        *** ******* ***  *  ***** *        *    ** * *

bpWap65 279:FRELHSEVDAVFTYQDHLYMIKDDHLFLYKVGEPHTHLDGYPKPVKEELGIEGPIDAAFV 338
pfWap65 279:FKELHSDVDAVFSYQDHLYMIKNDKIHIYKTGTAHTHLEGYPKPLKEELGIEGPIDAAFV 338
meWap65 284:FKELHSEVDAVFSYNSHLYMIKDDQLFVYRVGEPHTHLAGYPKPVQAELGIKGPIDAAFV 343
cpWap65 293:FKELHSEVDAVFSYEGHLYMIKDNEVFVYKVGEPHTHLEGYPKPLKEVLGIEGPVDAAFV 352
gfWap65 299:FKELHSEVDAVFSYEGHLYMIKDNEVFVYKVGEPHTHLEGYPKPLKEVLGIEGPVDAAFV 358
            * **** ***** *  ******     *   * **** *****   *** ** *****

bpWap65 339:CEDHHIAHIIKGNKIYDVELKASPRVAGNERTISLFDNVDAAMCDGTGVKVIKGNHFYHF 398
pfWap65 339:CGDHHIAHLIKGQKMYDVDLKSSQRVADNERPISLFQKIDAAICDGEGLKVIVGNHYYHF 398
meWap65 344:CQDRHIAHIIKDRHMYDVDMSATPRTATNKRPISILKKVDGAMCGPGGVKVFRGNHYYHF 403
cpWap65 353:CADHHIAHVIKGQTVYDVELKATPRAPAKEGTITQFKKIDAAMCGPKGVTAVIGNHYYLY 412
gfWap65 359:CADHHIAHVVKGQTVYDVDLKATPRVPVKEGSIAHLKKIDVAMCGPKGVTAVIGNHYYQF 418
            * * ****  *    ***      *        *     * * *   *      *** *

bpWap65 399:ISTHGFVAGRSLPEQHRVSLELFGCDH                                 425
pfWap65 399:DSPMLFIAGRALPEQRRVSLELFGCDH                                 425
meWap65 404:ESPKTFVAARALPEQHRISLELFGCDH                                 430
cpWap65 413:DSPKIMMMAKIMPEQHRVSQGLFGCDH                                 439
gfWap65 419:GSPMIMMMAKIMPEQHRVSQGLFGCDH                                 445
            *           *** * *  ******
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Fig. 2. Multiple alignment of black porgy (*Acanthopgrus schlegeli*) Wap65 with several teleosts. The Wap65 sequences used for alignment were black porgy Wap65 (bpWap65, EF134717), pufferfish Wap65 (pfWap65, AB125932), Medaka Wap65 (meWap65, AB195240), carp Wap65 (cpWap65, AB052623), and goldfish Wap65 (gfWap65, D50437). Identical amino acids are indicated by asterisks. Shaded regions indicate five pairs of cysteine residues. Potential N-linked glycosylation sites are boxed.

has more oligosaccharides than the carp. As the black porgy Wap65 includes four N-linked glycosylation sites, it is likely to bind to more oligosaccharides than other fish Wap65. The oligosaccharides attached to this protein promote glycoprotein folding, contribute to protein–protein interactions, regulate other ligand recognition processes, and stabilize the protein, making it resistant to proteolysis (Helenius and Aebi, 2004; Ohtsubo and Marth, 2006). Although

the role of oligosaccharide of glycoprotein is unclear, Satoh et al. ('94) described that N-linked glycosylation is essential to bestow a high affinity of human hemopexin for heme. It is unknown whether or not any difference of activity would exist between Wap65 among fish species (Kinoshita et al., 2001).

Kikuchi et al. ('93) first reported Wap65 of fish as a plasma glycoprotein in the serum of goldfish. As Wap65 of goldfish and carp is 70% identical to

that of rainbow trout and zebrafish hemopexin (a glycoprotein bound to heme), it is postulated that fish Wap65 has an affinity for heme. Recently, Hirayama et al. (2004) reported that Wap65 had a heme-binding ability.

Phylogentic relationship of FW fish and seawater fish appeared. The phylogenetic analysis indicated that there are three groups of Wap65: group 1 (black porgy, European seabass, pufferfish, and Medaka), group 2 (carp and goldfish), and group 3 (green swordtail). The bpWap65 is most closely related to European seabass Wap65 (Fig. 3). Moreover, black porgy and European seabass are perciformes and are more closer than other fish in the taxonomical relation.
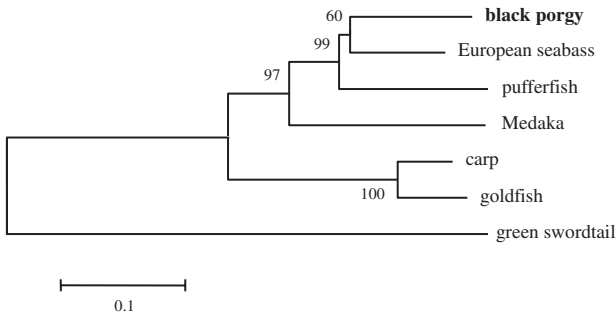
Fig. 3. An unrooted phylogeny showing the most likely relationship between representative Wap65 amino acid sequences. The number associated with each internal branch is the local bootstrap probability. The sequences are black porgy (ABL74446), European seabass (ABL75414), pufferfish (BAD18109), Medaka (BAB97303), carp (BAB60809), goldfish (BAA08928), and green swordtail (ABD14149).

The transcription and expression of Wap65 mRNA were known to increase as water temperature rises (Kinoshita et al., 2001). In this study, we found that Wap65 mRNA increased as the water temperature was increased from 20 to 30°C, in agreement with reports that Wap65 mRNA in goldfish (Kikuchi et al., '95) and carp (Kinoshita et al., 2001) is eight to ten times higher at 30°C than at 10°C. Hirayama et al. (2003) reported higher Wap65 mRNA levels in pufferfish adapted to 25°C than at 15°C. Therefore, we suggested that Wap65 is a protein that reacts to high water temperature.

The tissue expression of Wap65 mRNA in black porgy showed that it was expressed only in the liver. In the carp (Kinoshita et al., 2001), it was expressed only in the hepatopancreas. In goldfish (Kikuchi et al., '97) and pufferfish (Hirayama et al., 2003), Wap65 mRNA was weakly expressed in the brain, eyes, gills, and gonads, although the greatest expression was in the liver. Although the cellular function of Wap65 protein in relation to rising temperature remains unclear, it is thought that this protein serves an important role in maintaining homeostasis by protecting components such as the HSPs produced as a cellular reaction to temperature stress. Also, during osmotic stress, the expression of Wap65 was not increased in the liver and not detected in the other tissues (brains, gonads, kidneys, and intestines). These data indicate that Wap65 mRNA expression is specific to high water temperature stress and does not occur during other common types of stress such as osmotic stress.
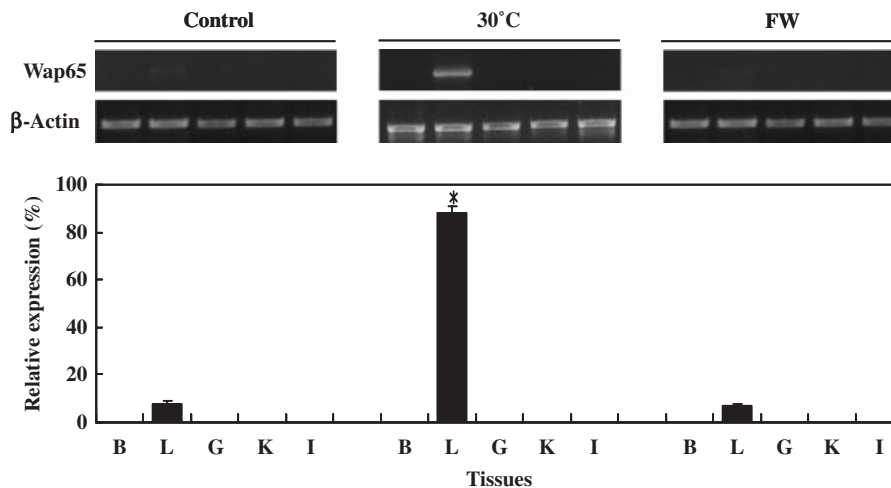

Fig. 4. Tissue-specific expression of Wap65 mRNA in five different tissues from black porgy (A. schlegeli) by RT-PCR (20°C control, 30°C, and freshwater group). Amplification of β-actin gene was used as an internal control. B, brain; L, liver; G, gonad; K, kidney; I, intestine. Value represents a mean ± SD (n = 4). Asterisks indicate significant difference compared with control (P < 0.05).
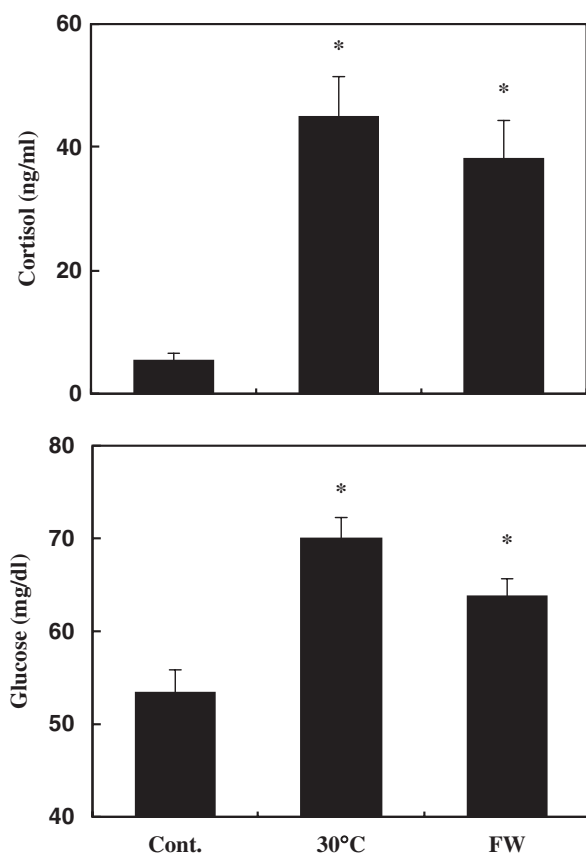
Fig. 5. Level of plasma cortisol and glucose in black porgy (*A. schlegeli*) during rising water temperature or freshwater acclimation. Value represents a mean ± SD (*n* = 4). Asterisks indicate significant difference compared with control ($P < 0.05$).

In this study, plasma cortisol and glucose levels increased significantly as water temperature increased (Fig. 5). This has been reported in a variety of teleosts, including the flounder (*Paralichthys olivaceus*; Chang et al., 2002), sunshine bass (*Morone chrysops* × *Morone saxatilis*; Davis, 2004), and black porgy (Choi et al., 2006, 2007). The increase in plasma cortisol and glucose as water temperature increased suggests that high water temperature stress increases the plasma cortisol and glucose levels in black porgy. A rapid change in water temperature generates stress in fish, primarily by activating the hypothalamus–pituitary–interrenal axis, which results in the secretion of cortisol in the blood (Wendelaar Bonga, '97). Secondary reactions include a water–ion imbalance, increased oxygen and energy consumption, and an increased plasma glucose level (Eddy, '81; Carmichael et al., '84). These reactions are used as typical indices of neuroendocrine reactions. Recently in our study, the phos-

phoenolpyruvate carboxykinase mRNA expression of the liver in black porgy was found to increase with the plasma cortisol and glucose levels when the water temperature was increased (20–30°C; Choi et al., 2007). Therefore, it is thought that high water temperature stress increases levels of plasma cortisol and glucose by activating of hypothalamus–pituitary–interrenal axis. Also, in this study, the levels of cortisol and glucose in plasma were increased significantly during osmotic stress; these results agreed with the previous study (Chang et al., 2002). Therefore, it is suggested that osmotic stress also functions as a stressor in black porgy. In addition, because cortisol levels were increased not only during high water temperature stress experiment but also during osmotic stress experiment, it is evident that the expression of Wap65 mRNA was not by cortisol but by high water temperature.

In fish, cortisol mediates stress-induced hyperglycemia, which is crucial for supporting the increased energy demand associated with stress, via the activation of phosphoenolpyruvate carboxykinase and gluconeogenesis (Mommsen et al., '99; Saplosky et al., 2000). These results suggest that stress-induced increases in cortisol promote gluconeogenesis in the liver. Hyperglycemia via this mechanism supplements the increased energy requirements owing to stress (Vijayan et al., '97).

Also, we observed no significant physiological effects by starvation in our previous experiment using black porgy (Choi et al., 2007). Therefore, we concluded that the increase in plasma cortisol and glucose levels in this study is not by starvation but by water temperature stress.

In conclusion, the expression of Wap65 mRNA was increased in the liver only by high water temperature stress, whereas the levels of cortisol and glucose in plasma were increased in fish for stresses such as osmotic and high water temperature stresses. Hence, we suggested that Wap65 gene is a specific gene related to high water temperature stress. Although its relationship with temperature acclimation mechanisms is unknown, from the results of this study we postulated that Wap65 plays a role in the liver. Further studies on the roles of Wap65 in liver are required.

ACKNOWLEDGMENT

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