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The warm temperature acclimation protein (Wap65) has an important role in the inflammatory response of turbot (*Scophthalmus maximus*)

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ABSTRACT

Wap65 is a molecule similar to the mammalian hemopexin that is a serum glycoprotein produced mainly by the liver with high affinity to heme. Its primary role is participating in iron metabolism scavenging heme that is released into the plasma and transporting it to the liver. It has been reported an important role of hemopexin in the inflammation as an acute-phase protein and its production is up-regulated by pro-inflammatory cytokines. There are also some evidences suggesting this immune-induction in fish Wap65 genes. Most teleost species presents two Wap65 genes but their physiological functions have not been completely elucidated; in fact, the transcriptional patterns of Wap65 genes to stimulatory treatments are variable and contradictory. In the present study two Wap65 genes, Wap65-1 and Wap65-2, have been characterized for the first time in turbot (*Scophthalmus maximus*). Their constitutive expression and differential modulation by thermal treatments, immune challenges (bacterial and viral), as well as iron supplementation, have been investigated. Both genes were mainly expressed in liver, but they were detected in all tested tissues. Whereas Wap65-1 and Wap65-2 were up-regulated by temperature rise and bacterial challenge, VHSV infection inhibited the expression of both genes. Moreover, iron-dextran administration induced only the overexpression of Wap65-1. Interestingly, these induction were observed in head kidney buy not in liver. The effect of Wap65 protein purified from turbot serum by hemin-agarose affinity chromatography was also studied to demonstrate a possible anti-inflammatory role, analyzing its inhibitory effect on leucocytes migration induced by zymosan injection to the peritoneal cavity.

1. Introduction

Hemopexin is a serum glycoprotein produced mainly by the liver and its primary role is to bind free heme and prevent oxidative damage whilst sequestering iron away from bacteria. Hemopexin is not only the plasma transporter for heme to hepatocytes, but also is an acute-phase protein that increases during inflammation [1,2] and its production is up-regulated by pro-inflammatory cytokines [3]. Hemopexin has been identified in mammals, amphibians, and birds. In teleosts, a hemopexin homolog, the 65 kDa protein Wap65 (warm temperature acclimation protein) has been reported. Initially, the expression of the protein was detected in response to raising temperatures and associated to the acclimation of several fish species to warm temperature [4–14]. Besides, Wap65 is specially associated with the acute immune response as an iron scavenger, given the important role of the iron in the bacterial infection. Thus, several studies have reported the up-regulation of Wap65 genes by bacterial or LPS infection [15–20].

Most teleost species presents two Wap65 genes. These two isoforms have undergone subfunctionalization via genome duplication. The functional diversification of the two fish Wap65 genes could have been due to the existence of moderate positive Darwinian selection, indicating an adaptive evolution [17]. Mostly, Wap65-1 is widely and constitutively expressed and slightly modulated by temperature, while Wap65-2 is more strongly regulated by an increase of temperature, bacterial and virus infection or LPS stimulation mainly in liver [16,17,20]. However the transcriptional patterns of Wap65 genes to different stimulatory treatments are variable and contradictory.

In the present study, Wap65 has been characterized for the first time in turbot (*Scophthalmus maximus*) to understand the thermal physiology and innate immunity for this species. Two types of Wap65 were identified and the differential modulation by thermal

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treatments, immune challenges (bacterial and viral), as well as iron supplementation, has been investigated. On the other hand, to our knowledge, it is the first time that the biological effect of purified Wap65 protein was studied to clarify the role of Wap65 as anti-inflammatory mediator in teleosts.

2. Material and methods

2.1. DNA sequence and 3D structure of Wap65-1 and Wap65-2

Several DNA sequences presenting homology to Wap65-1 and Wap65-2 were retrieved from a turbot 454-pyrosequencing performed in our laboratory [21]. Protein sequences were obtained using the Translate tool from Expasy [22] (http://us.expasy.org/tools) and then they were compared with other Wap65 fish sequences through an alignment using ClustalW [23].

Specific primers were designed to confirm the open reading frame (ORF) of both forms (Table 1). The amplifications were performed in a 25 μl total volume containing 10 μl of ultrapure water (Sigma–Aldrich), 12 μl of 2× PCR Master Mix (Fermentas), 1 μl of each specific primer (10 μM) and 1 μl of turbot cDNA. PCR conditions consisted on an initial denaturation for 5 min at 94 °C, 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 1 min and a final extension for 10 min at 72 °C in a GeneAmp® PCR System 2700 thermocycler (Applied Biosystems). Three microliters of PCR product were used to clone both Wap65 forms into pCR® 2.1 plasmid vector (Invitrogen) and then transformed in One Shot® TOP10F′ competent cells (Invitrogen) following the manufacturer instructions. Four positive clones for each form were selected and amplified using M13 vector specific primers (M13F: 5′-GTAAAACGACGGCCAG-3′, M13R: 5′-CAGGAAACAGCTATGAC-3). PCR was performed under the following conditions: 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1.5 min, and then 72 °C for 10 min. cDNA sequencing was conducted using an automated ABI 3730 DNA Analyzer (Applied Biosystems, Inc. Foster City, CA, USA).

Prediction of cleavage sites for the signal peptide was performed using ProP 1.0 server [24]. The molecular weight and isoelectric point of the mature peptide were determined using the *Compute pI/Mw tool* from *ExPASy*. Sequence similarity and identity scores were calculated with the MatGAT program [25]. The protein structure modeling was conducted using I-TASSER [26] selecting the model with the best C-score. The TM-score was used as an indicator of structural similarity.

2.2. Phylogeny

Amino acid sequences of turbot Wap65 were compared with those from other fish and vertebrates (NCBI, National Center for Biotechnology Information, GenBank accession numbers of sequences used are listed in Supplementary data Table 1). Multiple sequence alignment was performed using ClustalW and poorly aligned and divergent regions were eliminated with G-Blocks software [27]. The phylogenetic tree was drawn using Mega 4.0 software [28]. Neighbor-Joining algorithm [29] was used as clustering method. The distances matrix was computed using Poisson correction method and all positions containing alignment gaps and missing data were eliminated. Statistical confidence of the phylogenetic analysis was assessed by performing 1000 bootstrap replicates.

Table 1
Primers used for cloning and real-time PCR.

Gene	Primers	Sequence (5′-3′)	Application
EF-1α	EF1α-F	GGA GGC CAG CTC AAA GAT GG	RT-qPCR
	EF1α-R	ACA GTT CCA ATA CCG CCG ATT T	
Wap65-1	Wap65-1_F1	TTT TCC AGG GCG ACT ATC TG	ORF confirmation
	Wap65-1_R1	AGG GAA ACT CTG TGC TGC TC	
	Wap65-1_F2	AGA TGC TCA AAG TTC AGC G	RT-qPCR
	Wap65-1_R2	GTG TCA TTG CCC TCG TCT TTA	
Wap65-2	Wap65-2_F1	GGG GAG AGT CCA CAT CAG AG	ORF confirmation
	Wap65-2_R1	CCT ACT CCT GAC AGC CCA TC	
	Wap65-2_F2	GTT AGA CGC CAT CAC CAC TG	RT-qPCR
	Wap65-2_R2	CGC ATG TAG ACT GGA CCT GA	

2.3. Experimental animals

Juvenile turbot (average weight 2.5 g) were obtained from a commercial fish farm (Insuiña S.L., Galicia, Spain). Animals were maintained in 500 L fiberglass tanks with re-circulating saline-water system at 15 ± 1 °C and fed daily with standard commercial pellets. A two-week acclimatization period was always carried out prior to the experiments. All animal experiments were reviewed and approved by the CSIC National Committee on Bioethics.

2.4. Constitutive expression of Wap65-1 and Wap65-2

Eight different tissues (kidney, spleen, gill, liver, intestine, heart, brain and muscle including skin) were removed from 20 healthy fish in order to examine the constitutive expression of both Wap65-1 and Wap65-2. Equal amounts of the same tissue from 4 fish were pooled, obtaining 5 biological replicates for each tissue. Total RNA was extracted using TRIzol® (Invitrogen) in accordance with instructions provided by the manufacturer in combination with the RNeasy mini kit (Qiagen) for RNA purification after DNase treatment (RNase-free DNase set, Qiagen). Quantity of the total purified RNA was determined using the spectrophotometer Nanodrop ND-1000. The reverse transcription was performed with the Super-Script II Reverse Transcriptase (Invitrogen), using 0.5 μg of RNA and following the manufacturer indications. Wap65 expression profiles were determined using real-time quantitative PCR. Specific PCR primers (Table 1) were designed using the Primer3 program [30] according to qRT-PCR restrictions. Oligo Analyzer 1.0.2 was used to check for dimer and hairpin formation and efficiency of each primer pair was also analyzed from the slope of the regression line of the quantification cycle versus the relative concentration of cDNA [31]. A melting curve analysis was also performed to verify that only specific amplification occurred and no primer dimers were amplified. If these conditions were not accomplished, new primer pairs were designed. Individual real-time PCR reactions were carried out in 25 μl reaction volume using 12.5 μl of SYBR® Green PCR Master Mix (Applied Biosystems), 10.5 μl of ultrapure water (Sigma–Aldrich), 0.5 μl of each specific primer (10 μM) and 1 μl of five-fold diluted cDNA template. All reactions were performed using technical triplicates in a 7300 Real-Time PCR System thermocycler (Applied Biosystems) with an initial denaturation (95 °C, 10 min) followed by 40 cycles of a denaturation step (95 °C, 15 s) and one hybridization-elongation step (60 °C, 1 min). Relative expression of Wap65-1 and Wap65-2 was normalized using the Elongation factor-1 alpha (EF-1α) as housekeeping gene, which was constitutively expressed and not affected by the experimental treatments, and calculated using the Pfaffl method [31]. Fold-change units were calculated by dividing the normalized expression values of stimulated tissues by the normalized expression values of the controls. For the biological replicates, the average relative level of expression from each replicate was considered as a single point and the mean and standard error calculated.