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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 participate 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.

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Editor

Fish and Shellfish Immunology

Vigo, January 30th 2014

Dear Editor,

Please find as attachment the manuscript "**The warm temperature acclimation protein (Wap65) has an important role in the inflammatory response of turbot (*Scophthalmus maximus*)**" by Diaz-Rosales et al. to be considered for publication in Fish and Shellfish Immunology.

Best wishes,

Sonia Dios

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 participate 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.

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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 proinflammatory cytokines [3]. Hemopexin has been identified in mammals, amphibians, and birds. In teleosts, a hemopexin homologue, 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 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 2x 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 modelling 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.

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 SuperScript 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 normalised expression values of stimulated tissues by the normalised 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.

2.5. Expression of Wap65-1 and Wap65-2 under different stimuli

In order to study the transcriptional induction of turbot Wap65 water temperature increases, bacterial or viral infections and iron overloading were performed.

To analyze the effect of the temperature in the expression of both Wap65, seventy two fish were divided into 2 groups of 36 turbot each. One group was maintained at 15 °C, whereas the other group was kept at 20 °C. Head kidney and liver were sampled from six fish of each tank at 1, 3, 8, 24 and 72 hours as well as at 1 week after temperature challenge. Six individual biological replicates were obtained for each sampling point and treatment.

For the bacterial and viral infections and for iron overloading, fish were divided into five groups of 80 fish each. Turbots belonging to one group were injected intraperitoneally with 50 µl of an *Aeromonas salmonicida* subsp. *salmonicida* (strain VT 45.1 WT) suspension (5.5×10^5 CFU/fish), other group was inoculated with a Viral Haemorrhagic Septicaemia Virus (VHSV- strain UK-860/94) suspension (1.2×10^5 TCID₅₀/fish) and a third group was injected with Iron-dextran (Sigma) (0.1 mg/fish). The Gram-negative bacteria and Iron-dextran were resuspended in phosphate buffer saline (PBS) whereas the viral suspension was resuspended in Eagle's minimum essential medium (MEM, Gibco). Two control groups injected intraperitoneally with 50 µl of PBS or MEM were also used. Head kidney and liver from 20 fish of each experimental group were removed at different sampling points (3, 8, 24 and 72 hours).

For each sampling point and treatment, equal amounts of each tissue from 4 turbot were pooled, constituting five biological replicates (4 fish per replicate) for each tissue.

Total RNA was extracted and cDNA was synthesized as mentioned above and the Wap65-1 and Wap65-2 expression analysis was carried out using the same protocol described above.

2.6. Purification of Wap65

Turbot serum Wap65 was purified using heme affinity chromatography. The protocol for the purification was the result of an adaptation and combination of different protocols already described in the literature for mammals hemopexin purification [32-37] and the only one, reported until now, for teleosts (medaka) Wap65 purification [9].

Briefly, 1 ml of turbot serum was filtrated through 0.22- μ m Millipore membranes and then adjusted to pH 8.0 and mixed with an equivalent volume of cold 1.68% rivanol (6,9-diamino-2-ethoxyacridine lactate, Sigma) solution (pH 8.0). The mixture was stirred gently for 5 h at 4 °C to precipitate and remove rivanol-albumin complex, and then allowed to stand overnight at 4 °C. Free rivanol in the supernatant was then precipitated by centrifugation at 15800 $\times g$ for 30 min. 1% v/v of protease inhibitor cocktail (Sigma) was added and incubated for 15 min by gentle agitation at 4 °C. The sample obtained was dialyzed against PBS overnight at 4 °C. 600 μ l of the dialyzed serum was applied to 1 ml hemin-agarose (Sigma) previously washed with 2 ml of buffer I (10 mmol l^{-1} sodium phosphate, pH 7.4; 0.5 mol l^{-1} NaCl) and then resuspended in 400 μ l of buffer I. The serum and washed hemin-agarose complex was incubated for 1 hour at room temperature. After incubation the mixture was centrifuged at 10000 $\times g$ for 5 min and the supernatant removed. Bound proteins were eluted after incubation at room temperature for 2 min with 600 μ l of elution buffer (0.2 mmol l^{-1} sodium citrate, pH 5.2; 0.5 mol l^{-1} NaCl; 0.02% NaN_3). Elution fractions obtained were concentrated by using Amicon 30 K centrifugal filter units (Millipore) and maintained at -80 °C. Protein concentration was determined by absorbance using the spectrophotometer Nanodrop ND-1000. Total proteins in each elution fraction were separated by SDS-PAGE (BioRad) under denaturing conditions and visualized with Coomassie blue (BioRad).

The proteins purified by PAGE were carbamidomethylated and digested with trypsin. The analytes were detected using a LC-5500 QTRAP linear ion trap mass spectrometer. Scheduled multi-reaction monitoring mode (MRM) was optimized and used. The masses of tryptic peptides and fragments ions from the mass spectrometry were used to

search by MASCOT at the NCBI-nr (National Center for Biotechnology Information Non-redundant) database.

2.7. Wap65 and leucocytes migration

Fish with an average weight of 5 g were injected intraperitoneally either with sterile 100 µl PBS, zymosan (2 mg/ml, 1 ml/50 g body weight, Sigma), or Wap65 protein (5 µg/pez) together with PBS or with zymosan. At 16 hours post-injection, animals were injected intraperitoneally with 1 ml of HBSS-1X (Hank's Balanced Salt Solution) and a peritoneal massage was applied to obtain the peritoneal exudates. Viable peritoneal leucocytes were counted by trypan blue (Sigma) exclusion test using a Neubauer chamber.

2.8. Data analysis

Data were analyzed statistically by one-way analysis of the variance (ANOVA) followed by Tukey's post hoc test for comparison of means, using the computer software package SPSS v. 20. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Characterization of Wap65-1 and Wap65-2

The deduced amino acid sequence of Wap65-1 characterized in the present work consisted of 429 amino acids with a full-length coding sequence of 1290 nucleotides in length (Fig. 1A). Wap65-2 consisted of 436 amino acids with a full-length coding sequence of 1311 bp (Fig. 1B). The nucleotide sequences of turbot Wap65-1 and Wap65-2 cDNA have been deposited into the NCBI GenBank with accession numbers KJ160506 and KJ160507, respectively. The predicted cleavage sites of the Wap65-1 and Wap65-2 was most likely to be located between amino acids 19 and 20 (TWA/DSP) and 22 and 23 (APA/EPQ), respectively. Consequently, the mature processed peptides consisted of 410 amino acids for Wap65-1 and 414 amino acids for Wap65-2. The calculated molecular weight of Wap65-1 and Wap65-2 mature peptide was 46.8 kDa and 46.5 kDa with theoretical isoelectric point values of 5.30 and 5.17, respectively.

The 3D structural analysis of turbot Wap65-1 and Wap65-2 revealed that the best template in the database using X-ray diffraction [38] was the hemopexin from rabbit (*Oryctolagus cuniculus*) (Fig. 2). The tertiary structure of both forms was modelled with a high confidence value (C-score of 1.69 and 1.12 for Wap65-1 and Wap65-2, respectively) and a TM-score of 0.93 for Wap65-1 and 0.920 for Wap65-2, indicating a model of correct topology.

The alignment among both turbot Wap65 with other sequences from teleosts as well as with hemopexins from *Homo sapiens*, *Mus musculus* and *Gallus gallus*, revealed numerous differences in the amino acid sequences, but the cysteine residues were well conserved among vertebrates (Fig. 3). The identity and similarity scores between both Wap65 were 48.4 and 69.7%, respectively (Table 2). Turbot Wap65 shared homology with other fish species at protein level. Specifically, turbot Wap65-1 displayed 98.8/83.3, 89.5/77.4 85.8/72.9 and 86.7/75.1% of similarity/identity values with seabass (*Dicentrarchus labrax*), fugu (*Takifugu rubripes*), medaka (*Oryzias latipes*), pufferfish (*Tetraodon nigroviridis*) Wap65-1 at amino acid level. On the other hand, turbot Wap65-2 displayed 87.4/74.1, 82.4/66.8 79.8/66.1 and 71.6/60% of similarity/identity values with the same species Wap65-2. The sequence similarities of the turbot Wap65 proteins to human hemopexin were 54.1% (Wap65-1) and 57.4% (Wap65-2), and the sequence identities were 31.8% (Wap65-1) and 39.4% (Wap65-2). Despite of these moderate levels of sequence identity, all the fish Wap65 polypeptides, including the turbot Wap65 isoforms, have common features typical of the protein structure of human hemopexin. These include clearly conserved cysteine residues that form disulfide bridges, essential for hemopexin structural integrity (light shaded boxes in Fig. 3), and several conserved aromatic residues that have been defined as important for the structure and stability of the heme-binding pocket (underlined in Fig. 3). Mammalian hemopexins are known to contain two pivotal histidine residues that form the bis-histidyl Fe (III) complex involved in heme axial ligand binding between hemopexin and heme. The turbot Wap65-2 conserved these histidine residues at both locations (His-239 and His-283), as well as the rest of the fish Wap65-2 isoforms examined (highlighted in dark shaded boxes and in bold in Fig. 3). However, in turbot Wap65-1 these histidines were substituted by glutamic acid and lysine. The Wap65-2 proteins were also more homogeneous among the species of the teleost lineage than the Wap65-1 proteins. This suggests that the fish Wap65-2 isoforms are more evolutionarily conserved, with closer orthology to mammalian hemopexin than the

Wap65-1 isoforms. Furthermore, there were three potential N-linked glycosylation sites (highlighted by white text on black in Fig. 3) in Wap65-1, compared to five glycosylation sites for Wap65-2.

3.2. Phylogenetic analysis

A phylogenetic analysis, based on multiple sequence alignment for the full-length deduced amino acid sequences of Wap65s and hemopexins, was conducted in order to analyze the turbot Wap65 proteins in the larger context of vertebrate hemopexin or Wap65 proteins. The phylogenetic tree revealed that turbot Wap65-1 was grouped in a different clade from turbot Wap65-2, although both forms have clustered with teleosts species. On the other hand, hemopexins formed a paraphyletic group (Fig. 4).

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

Both transcripts of Wap65-1 and Wap65-2 were detected in the eight tissues tested in healthy turbot (Fig. 5A and Fig. 5B). Brain was the organ that presented the lower expression for both isoforms, thus the fold-change for each tissue was referenced to this organ. Wap65-1 and Wap65-2 share the same expression pattern, being both genes predominantly expressed in the liver, reaching almost 3000 fold-change value for Wap65-2, and about 2000 fold-change for Wap65-1. In the intestine both transcripts were expressed ten times lower (about 300 fold-change value). The following organs, by expression level were kidney and spleen, with 200 and 100 fold-change values, respectively. Lowest expression levels were obtained in gill, muscle plus skin, heart and brain. Also, the relative proportion of turbot Wap65 isoforms expression in the different tissues showed a higher presence of Wap65-1 mRNA in all the tissues tested, being the ratio of Wap65-1 with regard to its paralogue Wap65-2 around 80% (Fig. 5C).

3.4. Effect of warm temperature on the expression of Wap65-1 and Wap65-2

Water temperature increases (5 °C) affected the expression of both transcripts in a very similar way, although Wap65-1 reached higher expression levels than Wap65-2. Two peaks were detected in the expression pattern during the time course for both transcripts in head kidney (Fig. 6A). The first significant enhancement of Wap65 genes was observed after 3 hours from the beginning of the trial, and then after 3 days the expression was again up regulated significantly. At this point, the expression level for

Wap65-1 reached a high fold-change value (368.5), whereas Wap65-2 fold-change was 81.67. In liver Wap65 expression was not regulated for any of the sampling points (Fig. 6B).

3.5. Expression of Wap65-1 and Wap65-2 after bacterial or viral infection and iron overload

The expression of Wap65-1 and Wap65-2 after bacterial challenge with *Aeromonas salmonicida* subsp. *salmonicida* followed the same pattern along the time (Fig. 7A and Fig. 7B). Although the constitutive expression of Wap65-1 and 2 was predominant in liver, their induction after the pathogenic bacteria infection was only observed in head kidney, being the increase statistically significant for both genes after 8 and 24 hours post infection (p.i.) compared to PBS-injected control (Fig. 7A). Moreover, it was observed a significant up-regulation in head kidney of Wap65-2 at 72 hours p.i. In liver, only a significant down-regulation was observed for Wap65-1 at 24 hours p.i. (0.62 fold-change) (Fig. 7B).

The expression of Wap65-1 and 2 was mainly down-regulated after viral stimulation with VHSV (viral haemorrhagic septicaemia virus). In head kidney, both Wap65 were drastically inhibited at 8 and 24 h p.i (Fig. 8A). Regarding to the liver, only a significant inhibition of Wap65-1 was observed after 72 hours p.i. (Fig. 8B).

Iron overload also affected the expression of Wap65 (Fig. 9A and Fig. 9B). In head kidney, a significant initial inhibition of both transcripts was detected 3 hours after the treatment. However, Wap65-1 suffered a significant up-regulation (45.67 fold-change) in comparison with the control group (animals injected with PBS) after 8 hours. At 24 hours post-treatment, Wap65-1 expression dropped to lower levels than the control group, although the difference was not significant. Surprisingly, after 72 hours, Wap65-1 transcript increased significantly its expression level again, although the fold-change was not as high as it was after 8 hours (8.75) (Fig. 9A). As occurred after bacterial and viral infection, Wap65-1 and 2 expressions were not affected in the liver following iron administration (Fig. 9B).

3.6. Effect of Wap65 on leucocytes migration

Wap65 protein from turbot serum was purified by hemin-agarose column chromatography (Fig. 10A). A proteomic analysis confirmed the existence of both Wap65 isoforms in the sample. The matched peptides for each Wap65 protein sequence

are shown in bold (Fig. 10B); the sequence coverage was 14% for Wap65-1 and 17% for Wap65-2. The purified proteins were then intraperitoneally administrated *in vivo* alone or in combination with zymosan to elucidate the effect of Wap65 protein on leucocytes migration. The intraperitoneal injection of zymosan induced peritoneal inflammation with extensive leucocytes recruitment, in comparison with the control group (fish injected with PBS). However, for those fish injected with a combination of zymosan and 5 µg/fish of the purified Wap65, a significantly decrease of leucocytes number was obtained in the peritoneal cavity with regard to the individuals inoculated only with zymosan (Fig. 10C), suggesting potential anti-inflammatory properties of turbot Wap65.

4. Discussion

The present work reports for the first time, the isolation and sequencing of two warm-associated-acclimation protein 65 kDa genes from turbot (*Scophthalmus maximus*). The expression patterns of Wap65-1 and Wap65-2 genes were determined analyzing their regulation by thermal acclimation, bacterial and viral infections, as well as the possible induction by iron administration. Lastly, the potential anti-inflammatory role of Wap65 protein was suggested.

Wap65 has been described in a large number of fish [5,8-10,13,16-18,20,39-41]. Phylogenetic analysis revealed that the duplication of the whole genome in the teleost radiation, consistent with the 3R hypothesis, led the existence of two copies of Wap65 genes [16,42]. In this sense, turbot Wap65 presented two different isoforms as it was described in other teleosts species [9,16,17,20,40]. The phylogenetic analysis revealed also that each turbot Wap65 isoform grouped tightly with its respective orthologues in the other fish species, showing separate clades for Wap65-1 and 2. The cysteine residues, essentials for hemopexin structural integrity, are very well conserved in all the species, including hemopexin in mammals and avians. In fact, the 3D protein modelling revealed that the best match in the database for the turbot Wap65 was the rabbit hemopexin. Turbot Wap65 sequences showed a relatively high homology with mammalian hemopexin, being Wap65-2 the protein that showed the highest sequence identity to hemopexins, as it was already reported for another teleosts [9,16,17,20,40]. Thus, Wap65-2 contained seven out of eight hydrophobic amino acids, which invariably define the heme pocket in hemopexin sequences [38], whereas Wap65-1 presented only

five. On the other hand, the two conserved histidine residues, which are assumed to serve as heme axial ligands in the binding pockets in mammalian hemopexins [38], were only conserved in turbot Wap65-2. Therefore, the higher identity and similarity of fish Wap65-2 to mammalian hemopexins and the higher homogeneity of this isoform among the species of the teleost lineage seemed to reflect that Wap65-2 is more evolutionary conserved than Wap65-1.

The genome duplication referred above for teleosts is believed to play an important role during evolution, providing opportunities to evolve new functions [42]. The duplicated genes tend to be very susceptible to be lost [43] but if the two copies co-exist in the genome it generally means that both forms could have evolved to gain differential functions, neofunctions or to have spatial and/or temporal functional partitioning [44]. In fact, Sarropoulou et al. [17] demonstrated in sea bass the existence of moderate positive Darwinian selection in the Wap65 domains, which is indicative of adaptive evolution and might be related to the functional diversification of the two paralogues. Wap65 turbot isoforms were abundantly expressed in liver as occurs with mammalian hemopexin, suggesting a pattern of liver specific expression well conserved through evolution. However, Sha et al. [16] reported a drastically different spatial expression pattern between channel catfish Wap65-1 and Wap65-2: Wap65-2 was highly expressed in liver, whilst Wap65-1, although it was most abundantly expressed in liver, it was also expressed in all tissues. In contrast, the results obtained in the present work do not confirm the spatial partitioning of function, since both turbot Wap65s showed the same spatial distribution, although Wap65-1 was the most expressed isoform in all tested tissues.

Given that the tissue expression patterns could be altered by environmental changes and/or infectious process, the role of turbot Wap65 was evaluated under different stimuli. Wap65 was initially described as a temperature acclimation mediator and most of the studies have been focused on this role, finding a strong induction after a temperature rise [4-7,10,13,14,16,20,39]. Nevertheless, some species were not affected by the increase of the temperature, such as fugu [8] or medaka [9]. Some investigations have suggested the importance of liver functions in temperature acclimation of fish [45,46]. Whereas in some species Wap65-1 [20] or Wap65-2 [16] were affected by thermal acclimation at hepatic level, temperature increases did not affect the expression of turbot Wap65 isoforms in this organ, although both genes were highly expressed in liver under basal conditions. In contrast, a strong induction of both Wap65 was

observed in head kidney, reflecting a biphasic response with one peak at 3 hours and a second higher peak after 3 days of temperature increase. These results together seemed to suggest that the transcriptional response of the Wap65 genes to thermal changes varies depending on the organisms, and the isoform specificity to this stimulus varies among species.

Hemopexin belongs to the acute phase reactants, whose synthesis is induced after inflammation [47,48]. The main role of hemopexin is to bind free heme and prevent oxidative damage, as well as to sequester iron away from bacteria [48,49]. Due to this hemopexin function as potential inflammatory mediator, various studies have explored the role of Wap65 in immune response. Previous works in different teleost species have suggested that Wap65 genes could be involved in bacterial and viral pathogenesis [6,15-17,50]. In this sense, there are some evidences that suggest a key role of the Wap65s as immune mediator. Thus, 5'-flanking region and introns of the goldfish Wap65 gene contained enhancer motifs including cytokine (interleukin-6) responsive elements [6] as it has been previously identified in mammals [3,51]. Noteworthy to add the interaction described in ayu between Wap65 and the complement component C3 [52], which suggested that Wap65 might function through the complement activation pathways when microbial infection occurs. In turbot, bacterial infection increased the level of expression of both isoforms with no differences in the expression pattern of Wap65-1 and Wap65-2. This is not in concordance with what was already reported in previous works, where the involvement of Wap65-2 against bacterial infection was more obvious than Wap65-1 [14,16,18,20]. Regarding to virus infection, Sarropoulou et al. [17] observed a differential induction of Wap65s organ-dependent, thus in liver Wap65-2 increases its expression 24 hours after nodavirus infection, whereas in spleen Wap65-1 was induced after 4 and 24 hours. In turbot the response to VHSV was completely the opposite, both isoforms were inhibited after 8 and 24 hours post infection in head kidney, whereas in liver only Wap65-1 was inhibited after 72 hours. Thus, the results seem to suggest that the expression pattern of both isoforms in response to an infection, bacterial or viral, depend on the fish species. Besides, the differences observed between the response to VHSV in turbot and to nodavirus in the European seabass could be related with the group of virus, since some viruses are able to inhibit the expression of certain immune-related genes. It is important to add that in a previous study, based on an oligoarray, turbot from a resistant family to VHSV, showed a high down-regulation of the "hemopexin" expression (now known as Wap65-2) after VHSV infection [50].

Regarding to iron administration, many genes involved in iron homeostasis were found to be highly up-regulated after bacterial infection in several fish species, as occurs in turbot with the hepcidin genes [53]. Considering the heme carrier function of Wap65, several studies have already explored the potential involvement of Wap65 in immune response. Despite of the lack of histidine residues, crucial for heme binding, turbot Wap65-1 was strongly induced in head kidney but not in liver and a biphasic pattern was also observed. On the other hand, no increases were detected in the level of Wap65-2. Surprisingly, Hirayama et al. [9] described that medaka Wap65-1, but not Wap65-2, showed heme-binding ability as revealed by hemin-agarose affinity chromatography, even though Wap65-1 lacked the essential histidine residues. However, medaka Wap65-2, closely related to mammalian hemopexin, cannot bind heme group. It seems that Wap65 orthologues are N-glycosylated possibly allowing interactions with heme group, although the exact role of linked oligosaccharides is still unclear [10,13].

In the recent years, additional biological activities have been proposed for hemopexins, including its role as pro- and anti-inflammatory mediator. Thus, an inhibitory role of hemopexin in neutrophil migration during sepsis in mice was demonstrated recently [54]. According to Mauk et al. [55], the published works until now have reported contradictory results, giving to hemopexin an anti- and pro-inflammatory role. Even though hemopexin could have a dual role, these results are not reliable due to the purification protocol [36,56]. To clarify the role of Wap65 on the inflammatory response in turbot, Wap65 was purified using a hemin agarose column. After mass spectrometry, the existence of both Wap65 isoforms in the elution fraction was confirmed. This result was unexpected, since according to Hirayama et al. [9], only medaka Wap65-1 had the ability to bind to heme. On the other hand, turbot Wap65-1 was strongly overexpressed after iron overload whereas Wap65-2 level was not affected. The intraperitoneal injection of the pool of both proteins *in vivo* confirmed that turbot Wap65s were able to inhibit the migration of leucocytes induced by zymosan to the peritoneal cavity, supporting their role as anti-inflammatory mediators. These results suggest again that the role of Wap65 could depend to a great extent on the species and organisms, since the purified Wap65 from the sting venom of *Cathorops spixii* showed inflammatory properties by increasing the number of leucocytes rolling and adhering to the endothelium [57].

5. Conclusions

In summary, two isoforms of turbot Wap65 have been characterized and their function analyzed for the first time. These two paralogues with high homology to the mammalian hemopexin, showed the same expression pattern in most of the treatments and sampling points tested, except for iron overloading. Both isoforms were induced after bacterial infection and temperature increases, however viral infection inhibited their expression. Iron administration induced a different response between Wap65-1 and Wap65-2, being Wap65-1 strongly induced. Thus, the partitioning of function between both isoforms could be related with iron metabolism, which suggested that Wap65-1 is the isoform more involved in this process. Finally, the anti-inflammatory properties of turbot Wap65s were also demonstrated for the first time.

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Figure legends

Figure 1. cDNA and deduced amino acid sequence of turbot Wap65-1 (A) and Wap65-2 (B). The signal peptide sequences are underlined. Diagrams show the position of the different hemopexin-like repeats that are shaded in the sequences.

Figure 2. Comparison of the predicted tertiary structure of turbot Wap65-1 (A) and Wap65-2 (B) with rabbit hemopexin. Wap65-1 and Wap65-2 are represented in different colour cartoon view, whereas rabbit hemopexin is represented in blue ribbon diagram. The protein structure modelling was conducted using I-TASSER selecting the model with the best C-score.

Figure 3. Multiple aminoacid alignment comparing the Wap65 sequences of turbot with other vertebrates. Asterisks mark the identical aminoacids in all the sequences. Conserved histidines, crucial for heme binding are highlighted in dark shaded boxes and in bold. Conserved cysteines, essential for structural integrity forming disulfide bridges are presented in light shaded boxes. Conserved aromatic residues are underlined. Potential N-linked glycosylation sites are highlighted by white text on black. The conserved residues unique to the fish Wap65-2 proteins and human hemopexin are in italic. (NCBI GenBank accession numbers of sequences used are listed in Supplementary Data Table 1).

Figure 4. Phylogenetic analysis of complete amino acid sequences of turbot Wap65-1 and 2, and different fish species, as well as chicken and mammalian hemopexins (hpx). The tree was drawn from ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2/) and using the Neighbour-Joining algorithm within MEGA 4.0 package. Data were analyzed using Poisson correction and gaps were completely removed. The bootstrapping percentage values (after 1000 replications) are indicated at the nodes. (NCBI GenBank accession numbers of sequences used are listed in Supplementary Data Table 1).

Figure 5. Wap65-1 (A) and Wap65-2 (B) constitutive expression in various tissues of turbot. Fold-change units were calculated by dividing the normalized expression values for each tissue by the normalized expression values obtained in the

organ presenting the lower expression level for each Wap65 (brain, in both cases). (C) Relative proportions of turbot Wap65-1 and 2 in different tissues calculated taking into account the normalized values of each Wap65 in the same tissue.

Figure 6. Expression of Wap65-1 and Wap65-2 in head kidney (A) and liver (B) at 1, 3, 8, 24, 72 h and 7 days after water temperature increase (20 °C). The relative expression of the Wap65 genes was normalized to the expression of EF-1 α and the normalized gene expression level of the stimulated groups was related to normalized gene expression of control groups (15 °C), which was defined as “1”. Data are shown as mean $\pm$ error of six fish. Symbol * denotes statistically significant differences ($P < 0.05$) with respect to the control group.

Figure 7. Expression of Wap65-1 and Wap65-2 in head kidney (A) and liver (B) at 3, 8, 24 and 72 h after *Aeromonas salmonicida* subsp. *salmonicida* challenge. The relative expression of the Wap65 genes was normalized to the expression of EF-1 α and the normalized gene expression level of the stimulated groups was related to normalized gene expression of control groups (PBS-injected), which were defined as “1”. Data are shown as mean $\pm$ error of five biological replicates. Symbol * denotes statistically significant differences ($P < 0.05$) with respect to the control group.

Figure 8. Expression of Wap65-1 and Wap65-2 in head kidney (A) and liver (B) at 3, 8, 24 and 72 h after Viral Haemorrhagic Septicaemia Virus (VHSV) challenge. The relative expression of the Wap65 genes was normalized to the expression of EF-1 α and the normalized gene expression level of the stimulated groups was related to normalized gene expression of control groups (MEM+2% FBS+P/S-injected), which was defined as “1”. Data are shown as mean $\pm$ error of five biological replicates. Symbol * denotes statistically significant differences ($P < 0.05$) with respect to the control group.

Figure 9. Expression of Wap65-1 and Wap65-2 in head kidney (A) and liver (B) at 3, 8, 24 and 72 h after iron-dextran administration. The relative expression of the Wap65 genes was normalized to the expression of EF-1 α and the normalized gene expression level of the stimulated groups was related to normalized gene expression of control groups (PBS-injected), which was defined as “1”. Data are shown as mean $\pm$

error of five biological replicates. Symbol * denotes statistically significant differences ($P < 0.05$) with respect to the control group.

Figure 10. (A) SDS-PAGE of three elution fractions after the hemin-affinity chromatography. M: molecular mass standards kDa (PreStained SDS-PAGE Standards Broad Range, Bio-Rad); Lane 1: elution fraction 1 (pH 5.2); Lane 2: elution fraction 2 (pH 5.2); Lane 3: elution fraction 3 (pH 5.2). Wap65 bands are indicated with white arrows. (B) The matched peptides for each turbot Wap65 obtained after mass spectrometry analysis of the purified proteins are shown in bold. (C) Viable leucocytes number in the peritoneal cavity of fish after 16 hour post injection of sterile PBS, 2 mg/ml of zymosan, 5 mg/fish of purified Wap65 with and without zymosan. Data are shown as mean $\pm$ error of five fish. Symbol * denotes statistically significant differences ($P < 0.05$) with respect to the control group (PBS) and double asterisk shows statistically significant differences ($P < 0.05$) between the treatment with Wap65 + zymosan respect to zymosan alone.

Table 1 Primers used for cloning and real-time PCR

Gene	Primers	Sequence (5'-3')	Application
EF-1α	EF1 α -F	GGA GGC CAG CTC AAA GAT GG	RT-qPCR
	EF1 α -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	

Table 2 Amino acid identities (top right) and similarities (bottom left) of turbot Wap65-1 and Wap65-2 with other vertebrates including fish, mammals and avians. The NCBI GenBank accession numbers of the protein sequences are listed in Supplementary table 1.

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. Turbot Wap65-1		48.4	83.3	50.5	77.4	46.5	72.9	48.5	75.1	42.7	31.8	31.6	31.3
2. Turbot Wap65-2	69.7		49.5	74.1	48.9	66.8	48.2	66.1	50.0	60.0	39.4	37.5	35.9
3. Seabass Wap65-1	92.8	70.6		49.5	81.7	45.9	76.3	48.8	79.9	42.6	31.9	30.5	30.3
4. Seabass Wap65-2	71.1	87.4	69.9		48.6	71.6	48.8	70.7	49.5	62.5	38.1	36.5	36.8
5. Fugu Wap65-1	89.5	68.8	91.8	68.3		44.6	71.9	48.2	82.6	41.0	32.8	31.0	30.7
6. Fugu Wap65-2	66.5	82.4	66.1	82.4	63.6		44.6	65.2	46.4	72.9	40.0	37.9	36.1
7. Medaka Wap65-1	85.8	67.7	88.6	68.3	84.7	64.3		46.8	71.7	41.9	32.4	31.3	32.0
8. Medaka Wap65-2	67.8	79.8	69.3	82.4	68.1	77.1	66.5		47.8	57.5	38.2	38.6	36.1
9. Pufferfish Wap65-1	86.7	69.0	90.4	69.2	91.1	66.5	83.0	68.1		41.7	33.3	31.7	30.6
10. Pufferfish Wap65-2	60.6	71.6	60.9	72.7	58.6	79.4	59.1	68.1	59.7		35.6	35.8	31.4
11. Human hpx	54.1	57.4	54.1	56.5	53.2	58.4	53.0	57.8	53.9	52.2		74.2	38.2
12. Mouse hpx	53.7	56.7	52.6	56.1	51.7	55.0	50.9	55.9	53.0	50.0	85.5		37.6
13. Chicken hpx	51.7	53.9	54.1	53.9	52.7	52.0	51.4	53.4	52.6	52.2	54.1	53.7	

Highlights

1. Two Wap65 genes were described in turbot and they were mainly expressed in liver
2. Temperature rise and bacterial challenge induced an overexpression of both genes
3. Viral challenge inhibited the transcription of both Wap65
4. Only Wap65-1 was responsive to iron-dextran administration
5. Wap65s showed an anti-inflammatory role *in vivo* inhibiting leukocyte migration

Figure 2
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Wap65-1

Wap65-2

Figure 4
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Figure 5
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