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The occurrence of two types of hemopexin-like protein in medaka and differences in their affinity to heme

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Summary

Full-length cDNA clones encoding two types of hemopexin-like protein, mWap65-1 and mWap65-2, were isolated from the HNI inbred line of medaka *Oryzias latipes*. The deduced amino acid sequence of mWap65-2 resembled mammalian hemopexins more closely than that of mWap65-1. Histidine residues required for the high affinity of hemopexins for hemes were conserved in mWap65-2, but not in mWap65-1. Surprisingly, mWap65-1, but not mWap65-2, showed heme-binding ability as revealed by hemin–agarose affinity chromatography, even though mWap65-1 lacked the essential histidine residues. Furthermore, RT-PCR analysis of different tissues demonstrated that the transcripts of *mWap65-2* were restricted to liver, whereas those of *mWap65-1* were found in various tissues including liver, eye, heart and brain. Quantitative RT-PCR revealed that transcripts of *mWap65-2* were expressed earlier than those of *mWap65-1* during ontogeny. However, the accumulated mRNA levels of both *mWap65-1* and *mWap65-2* did not differ significantly in fish acclimated to either 10°C or 30°C for 5 weeks. These characteristics suggest that the two proteins have different physiological functions and that mWap65-2 is not a hemopexin.

Key words: eurythermal fish, medaka, *Oryzias latipes*, temperature acclimation, mWap65-1, mWap65-2, heme-binding ability, hemopexin.

Introduction

Environmental temperature markedly influences the physiology and behaviour of poikilotherms, including fish. Seasonal temperature changes take place over weeks or months, and physiological reorganization to compensate for such changes is often referred to as an acclimation response (Hazel and Prosser, 1974; Johnston and Temple, 2002). Eurythermal temperate fish such as goldfish *Carassius auratus* and common carp *Cyprinus carpio* are able to survive over a wide range of water temperature ranging from near zero to over 30°C. There have been numerous studies on the biochemical mechanisms of temperature acclimation responses in teleost fish (Hazel and Prosser, 1974; Johnston and Temple, 2002). For example, it has been found that in goldfish a 65 kDa protein (Wap65) is induced in the liver following warm acclimation (Watabe et al., 1993; Kikuchi et al., 1993). Moreover, the accumulated mRNA levels of Wap65 in goldfish acclimated to 30°C are higher than those in fish acclimated to 10°C, demonstrating regulation in the expression of *Wap65* at the gene level (Kikuchi et al., 1995). Recently, a similar expression pattern of *Wap65* has been shown in carp (Kinoshita et al., 2001a). Interestingly, *Wap65* was also expressed in goldfish following intra-administration of the bacterial pathogen, lipopolysaccharide (LPS) (Kikuchi et al., 1997), which is a potent inducer of immune responses. This result is consistent with the presence of cytokine responsive elements in the 5′-flanking region of the goldfish *Wap65* gene, suggesting that Wap65 has functions in self defense mechanisms as well as acclimation to warm temperature.

It has been claimed that the deduced amino acid sequences of goldfish and carp Wap65 resemble those of mammalian hemopexins (Kikuchi et al., 1995; Kinoshita et al., 2001a). Hemopexin is a serum glycoprotein that transports heme from hemolysis to the liver (Müller-Eberhard, 1970, 1988; Müller-Eberhard and Bashore, 1970; Delanghe and Langlois, 2001). X-ray crystallography demonstrated that His-213 and His-266 are essential for the high affinity of rabbit hemopexin for hemes (Paoli et al., 1999). However, these histidine residues are substituted by other amino acids in goldfish and carp Wap65, suggesting the possibility that in these fish the Wap65 molecule has different functions from those of mammalian hemopexins. On the other hand, rainbow trout *Onchorhynchus mykiss* hemopexin-like protein, in which histidine residues essential to heme-binding are also substituted by other amino acids, capable of binding hemes (De Monti et al., 1998). Thus,

structure and function relationships of Wap65 are still under debate and, furthermore, the physiological significance of Wap65 expression in temperature acclimation remains unclear.

Medaka *Oryzias latipes* is a temperate eurythermal species like goldfish and carp, which it was thought might also express Wap65 or a related protein following warm acclimation. Furthermore, medaka is a model fish where transgenic techniques can be easily employed. In this study, we isolated cDNA clones encoding two types of Wap65, mWap65-1 and mWap65-2, from medaka and determined their primary structures. In addition, the heme-binding abilities of mWap65-1 and mWap65-2 were examined using specific antibodies and hemin–agarose affinity chromatography. Possible changes in the expression profile during development and temperature acclimation were investigated for *mWap65* transcripts as well as the effect of LPS administration and their tissue distribution.

Table 1. *Embryos used for quantitative RT-PCR at various developmental stages*

Hours post fertilization (h.p.f.)	Stage	Remarks
25	20	4 somite stage
39	24	16 somite stage (start of heat beating)
46	25	18-19 somite stage
58	28	30 somite stage
63	30	35 somite stage (blood vessel development)
77	32	Somite completion stage
98	35	Visceral blood vessels formation stage
110	36	Heart development stage
122	37	Pericardial cavity formation stage
146	38	Spleen development stage
156	39	Hatching stage

Embryos were grown at 27°C.

Data are taken from Iwamatsu (1994).

Materials and methods

Materials

Adult medaka *Oryzias latipes* Temmink and Schlegel of the HNI inbred strain (Hyodo-Taguchi and Sakaizumi, 1993) were acclimated to either 10°C or 30°C for a minimum of 5 weeks in the laboratory (14 h:8 h L:D). This acclimation period was chosen on the basis of experiments with goldfish and carp (Watabe et al., 1993; Kikuchi et al., 1993; Kinoshita et al., 2001a). All fish were fed commercial pellets daily *ad libitum*.

The cDNA clone of OLe05.03f (accession number AU179198 in the DDBJ/EMBL/GenBank databases) was kindly supplied by the Medaka EST Project, The University of Tokyo.

The *Escherichia coli* expression vector pGEX-2T (Amersham Bioscience, Buckinghamshire, UK) and pET-39b (Novagen, Madison, WI, USA), which are designed for expression of glutathione S-transferase (GST) and DsbA fusion proteins, respectively, were used for preparation of recombinant mWap65-1 and mWap65-2, respectively.

Wild adult medaka reared at 25°C were used to prepare a cytosolic protein fraction and blood samples for immunoblotting. The cytosolic protein fraction was prepared from the whole individuals with buffer I containing 10 mmol l^{-1} sodium phosphate (pH 7.4), 0.5 mol l^{-1} NaCl, 3 µg leupeptin and 1 mmol l^{-1} phenylmethanesulfonyl fluoride (PMSF). For preparation of blood samples, 20 individuals were bled through the tail vein, and the blood pooled and made up to 500 µl with physiological saline.

Hemin–agarose (Sigma-Aldrich, St Louis, MO, USA) was used for hemin–agarose affinity chromatography to determine the heme-binding ability of mWap65-1 and mWap65-2.

Samples were also collected during ontogeny from wild medaka at 27°C. Approximately 100 embryos were collected at each of the developmental stages listed in Table 1. The samples were rapidly frozen in liquid N_2 and stored at –80°C until used for quantitative reverse transcriptase polymerase chain reaction (RT-PCR).

cDNA cloning

Total RNA was extracted from the visceral part of HNI medaka reared at 30°C using the ISOGEN system (Nippon Gene, Tokyo, Japan). cDNAs were synthesized from 5 µg of total RNA using TimeSaver cDNA Synthesis Kit (Amersham Bioscience). After inserting synthesized cDNA into λ ZAPII (Stratagene, La Jolla, CA, USA), *in vitro* packaging was performed to construct a cDNA library using Ready-To-Go Lambda Packaging Kit (Amersham Bioscience).

In order to clone *mWap65-1*, the cDNA library was screened with a DNA probe containing 54–1264 nucleotides (nt) of carp *Wap65* amplified by PCR as described in Kinoshita et al., (2001a). Labelling of the probe obtained by PCR and subsequent screening were carried out using DIG DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Prehybridization and hybridization were performed at 58°C for 20 min and 16 h, respectively. Then, positive plaques were selected and subjected to *in vivo* excision into pBluescript II SK^- plasmid for sequencing inserted DNAs.

Determination of the DNA nucleotide sequence of *mWap65-1* subclones was performed for 5′- and 3′-strands with a Dye Deoxy Terminator Cycle Sequencing Kit using an ABI PRISM model 373 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Homology search was performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

The cDNA clone of OLe05.03f encoding mWap65-2 did not contain the initiation codon. Therefore, 5′-rapid amplification of cDNA ends (5′RACE) was employed using the 5′RACE System version 2.0 (Invitrogen, Carlsbad, CA, USA) and total RNA prepared as a template according to the manufacturer's instructions. Gene-specific antisense primer mWapL-R1 (5′-GGGATAATTACCAGTCACCTCG-3′), first nested primer mWAPL-R2 (5′-GCCATGTCTTGGTCTTCAC-3′), and second nested primer mWAPL-R3 (5′-AAAGCGG-

AATCTCCATCAC-3′) were designed from the sequence of clone OLe05.03f. The first nested PCR was initiated by adding 0.1 μg of cDNA synthesized from total RNA to a 100 μl solution containing 40 pmol primers, 10 μl of 10× *Taq* DNA polymerase buffer (500 mmol l^{-1} KCl, 15 mmol l^{-1} $MgCl_2$, 0.01% gelatin and 100 mmol l^{-1} Tris-HCl, pH 8.3) (Applied Biosystems), 20 nmol dNTP mixture, and 5 U *Taq* DNA polymerase (Applied Biosystems). The reaction consisted of 30 cycles under denaturation at 94°C for 30 s, annealing at 60°C for 30 s, polymerization at 72°C for 1 min, and the final extension step at 72°C for 5 min. The second nested PCR was performed by the same method, except that 0.1 μl of a solution containing the first nested PCR products was used as a template. The PCR products obtained were subcloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced.

To evaluate the structural features of mWap65-1 and mWap65-2, the deduced amino acid (aa) sequences of mWap65s were compared with those of related proteins from fish and mammalian hemopexins using CLUSTAL W (Thompson et al., 1994). SignalP (http://www.cbs.dtu.dk/services/SignalP/) and PROSITE (http://us.expasy.org/prosite/) programs were used to predict signal peptide regions and *N*-glycosylation sites in mWap65-1 and mWap65-2, respectively. A phylogenetic tree was constructed on paired alignment of different fish Wap65s and mammalian hemopexins with human vitronectin as outgroup using CLUSTAL W and neighbor-joining method on MEGA2.1 software (http://megasoftware.net/).

Preparation of recombinant proteins

cDNA encoding mWap65-1 was amplified by PCR with oligonucleotide primers mWAP-GSTF (5′-CG**GGATCC**GG-AGCTGTCCGCGACCGC-3′) and mWAP-GSTR (5′-CG**GGATCC**TTAGTGGTCGCAGCCAAACAA-3′), which contained linkers with *Bam*HI restriction site (boldface nucleotides). cDNA encoding mWap65-2 was amplified by PCR with oligonucleotide primers mWAPn-pET-F (5′-CG**GGTACC**<u>GATGACGACGACAAG</u>ACACGGGCAGCC-CCAT-3′) and mWAPn-pET-R (5′-CG**GGTACC**CTAAT-CTTCACAGCCCAC-3′), which contained linkers with the *Kpn*I restriction site (boldface nucleotides). Primer mWAPn-pET-F further contained a linker with the nucleotide sequence encoding an enterokinase cleavage site (underlined nucleotides). These primers were designed to amplify the sequences encoding predicted mature mWap65-1 and mWap65-2 by comparing hydrophobic plots of mWap65s with those of goldfish and carp Wap65s (see Fig. 1). The amplified *mWap65-1* fragment of 1212 bp and *mWap65-2* fragment of 1237 bp, which spanned 112–1323 nt of *mWap65-1* and 138–1375 nt of *mWap65-2*, were digested with *Bam*HI and *Kpn*I, respectively. The two fragments were inserted into pre-digested *E. coli* expression vector pGEX-2T (Amersham Bioscience) and pET-39b (Novagen) to construct pGEX-2T/mWap65-1 and pET-39b/mWap65-2, respectively.

Recombinant mature proteins of mWap65-1 (rWap65-1) and mWap65-2 (rWap65-2) were produced in *E. coli* BL21 and BL21 (DE3) cells, respectively, following the manufacturer's instructions. rWap65-1 accumulated in the inclusion body was solubilized in 8 mol l^{-1} urea containing 50 mmol l^{-1} Tris-HCl (pH 8.0), 1 mmol l^{-1} DTT and 1 mmol l^{-1} EDTA, and refolded by dialysis stepwise against decreasing concentrations of urea. Solubilized rWap65-1 and rWap65-2 were purified using GSTrap and HiTrap columns (Amersham Bioscience), respectively, following the manufacturer's instructions.

SDS-PAGE was performed by the method of Laemmli (1970) using a 12.5% polyacrylamide gel. The protein concentration of the samples was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.

Immunological procedures

Rabbits (*N*=2) and mice (*N*=5) were immunized at 2-week intervals with 50 μg of rWap65-1 and rWap65-2 per animal, respectively, following emulsification in Freund's complete (primary) or incomplete (boosters) adjuvant (Sigma-Aldrich). Antisera were collected from the animals 50 days after the primary immunization.

Immunoblotting analyses were performed as follows. rWap65 isoforms, the cytosolic protein fraction and blood preparations were dissolved in an equal amount of sample buffer (2% SDS, 2% 2-mercaptoethanol, 20 mmol l^{-1} Tris-HCl, pH 6.8, 40% glycerol, 4 mmol l^{-1} EDTA and 0.015% Bromophenol Blue), electrophoresed on a 12.5% SDS polyacrylamide gel and transferred onto Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were incubated at room temperature for 2 h in a solution containing rabbit anti-rWap65-1 or mouse anti-rWap65-2 antisera at 1:1000 dilution in a blocking solution containing 0.1% blocking reagent (Roche Diagnostics) in Tris-buffered saline (TBS) containing 25 mmol l^{-1} Tris (pH 7.4), 137 mmol l^{-1} NaCl and 2.68 mmol l^{-1} KCl, followed by incubation at room temperature for 2 h in a solution containing horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) antibody (Sigma-Aldrich) at 1:5000 dilution in the blocking solution. The colour development was performed using 0.2 mg ml^{-1} 3,3′-diaminobenzidine tetrahydrochloride and 0.6% H_2O_2. The IgGs in rabbit and mouse antisera were purified using HiTrap Protein A HP and HiTrap Protein G HP (Amersham Bioscience), respectively, following the manufacturer's instructions, and their concentrations determined using BCA Protein Assay Kit (Pierce, Rockford) as described above.

Hemin–agarose chromatography

Hemin–agarose binding was performed according to Tsutsui and Mueller (1982) and Lee (1992). Briefly, 1 ml of hemin–agarose (Sigma-Aldrich) was washed three times with buffer I containing 10 mmol l^{-1} sodium phosphate (pH 7.4), 0.5 mol l^{-1} NaCl, 3 μg leupeptin and 1 mmol l^{-1} PMSF. Washed agarose was suspended in 300 μl of buffer I. A 200 μl

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mWap65-1 ---MKLLP-QALFLCLALVLAWADHHEHR---------------RKGAVRDRCKGIEMDAVAVNEEGIPY   51
mWap65-2 ----MA.TFK.A..A.M.A.TR.APL.DS-----------AAGDGDS.LP..GA...F..ITPDDK.QTF   55
goldfish ---...IQMLT.C.A.S.S..APS..KEDHV-QQDEPQGHQHELHH..NL...G.M.F..I.........   66
carp     ---.R.IQ--T.C.A.L.SF.ASSDVADD-----PDTAGHKPELHHE.KL...A.M.F..I.........   60
trout    ---..P.S-.T.C...V.A.SH.H..AGHQGGEDEGHEGHDHGHHE.LLL...Q.........T......   66
rabbit   MVKASGI.IALGVWG.CWS..TVNSVPLTSAHGNVTEGESGTKPEADVIEQCSD.WSF..TTLDDN.TML   70
rat      MARTVVALNILVL.G.CWS..V.NPLP--AAHETVAKGENGTKPDSDVIEHCSDAWSF..TTMDHN.TML   68
human    MARVLGA.VALGLWS.CWS..I.TPLPPTSAHGNVAEGET--KPDPDVTERCSD.WSF..TTLDDN.TML   68

mWap65-1 FFKEDHLFKGFHGQAELSNKSFAELDDHHHLGHVDAAFRMHYEDDLNHHDRMFFFLDNKVFAYYQHKLEA  121
mWap65-2 ...G..VW...E.D.QP.SQY.K..N----.........NPENQGD..HIYL...D...S.FE.T..E  120
goldfish ...G........D......ET.P...E.................S..SPA...HQ.....T...S..K....K  136
carp     ...G.........K.....ET.P.......N..............S..SPD...HQ.........S..K....K  130
trout    ...GG.V.......K.....E........................L..FP.KPTE..HI..M..T...S..K.Q..T  136
rabbit   ...DEFVW.SHR.IR..ISERWKNFIG-----P......H-------G.TSVYLIKGD..WV.TSE.N.K  128
rat      ...GEFVWR.HS.IR..ISERWKNPVT-----S......--------GP.SV.LIKED..WV.PPE.K.N  125
human    ...GEFVW.SHKWDR..ISERWKNFPS-----P......Q-------G.NSV.LIKGD..WV.PPE.K.K  126

mWap65-1 GYPKAISEVFPGIPDHLDAAVECPKPECVEDSVIFFKKNEIFHFYVKNKTVDERDFRSMPNCTSAFRFME  191
mWap65-2 ....E.Q.D...V.A.........G...T...L...GPDVHVYDIVT..KTKTWPHL.A...V..WL.  190
goldfish D...D...L...............T.D.AN.TI...EGD..Y.LDM.T.K...KE.K......G...Y.D  206
carp     D...D..DL..................D.TD.T.....GD..Y..NM.T.K...KE.K......G...Y..  200
trout    .F..D............V..A.D.E..A.....GD..Y.YN..T.K.E.KK.EG...........  206
rabbit   V...SLQDE.....FP.......HRG..QDEGIL..QG.RKWFWDLTTG.KK..SWPAVG.....L.WLG  198
rat      ....LFQ.ES....YPP......HRG..QSEG.L..QG.RKWFWDFATR.QK..SWPAVG...A.L.WL.  195
human    ....LLQDE.....SP.......HRG..QAEG.L..QGDREWFWDLATG.MK..SWPAVG..S..L.WLG  196

mWap65-1 HYYCFHGHKFSKFDPKTGEVRGKYPKDARKFFMRCSKFDE------------DNDHEERERCS-RVHLDA  248
mWap65-2 ........N.TR.Q.V....T.N......RY....PD.GH-------------GGERTTLK..-DFKM..  246
goldfish ........Q......I....Q.....ET.DY....PH.GQ----------KTTDE.I...Q..-......  265
carp     ........Q......V..D.Q.....ET.DY....PH.GQ----------KSTEE.I...Q..-......  259
trout    ........Q..........H.R...E..DY..K....GD------------TT..I.......-......  263
rabbit   R....Q.NQ.LR.N.VS...PPG..L.V.DY.LS.PGRGHR-----SSHRNSTQHGH.ST..DPDLV.S.  263
rat      R....Q.N..LR.N.V....PPR..L...DY.IS.PGRGHGKLRNGTAHGNSTHP--MHS..NADPG.S.  263
human    R....Q.NQ.LR...VR...PPR..R.V.DY..P.PGRGHGHR-NGTGHGNSTHHGP.YM...PHLV.S.  265

mWap65-1 ITSDDAGNIYAFRGHHYIRKDEGNDTLKADTIESAFKELHSEVDAVFSYNSHLYMIKDDQLFVYRVGEPH  318
mWap65-2 ..T..T.RM.M.K.SN.M.L.THR.G.H.FP.TTSW...TNG........DRI.L..G..VYI.KA.AHF  316
goldfish .....D.SV.......FLSITG--.KFHS..................EG.......NEV...K.....  333
carp     .....D.S......Y.FVSITG--.KFHS..V...............EG.......NEV...K.....  327
trout    ...............FLEQ.A....WA......D............T...EN....V...KVYI.K..DS.  333
rabbit   MV..NH.AT.V.S.S..W.L.TNR.GWHSWP.AHQWPQGP.T...A..WEDK..L.Q.TKVY.FLTKGGY  333
rat      LL..HR.AT...S.S..W.L.SSR.GWHSWP.AHHWPQGP.A...A..WDEKV.L.QGT.VY.FLTKGGN  333
human    L...NH.AT...S.T..W.L.TSR.GWHSWP.AHQWPQGP.A...A..WEEK..LVQGT.VY.FLTKGGY  335

mWap65-1 THLAGYPKPVQAELG-----IKGPIDAAFVCQDRHIAHIIKDRHMYDVDMSATPRTATNKRPISILKKVD  380
mWap65-2 .LIE....TLKE..N-----.E.QV.....PGQRT...QG.KITYINLA....EI.LDA.LP-FGDI.  383
goldfish ...E.....LKEV..-----.E..V......A.H....VV.GQTV....LK....VPVKEGS.AH...I.  398
carp     ...E.....LKEV..-----.E..V......A.H....V..GQTV...ELK....APAKEGT.TQF..I.  392
trout    ...D.S...LKEV..-----VE...........H....V..GQTV....LK.S.PVPVKEGSFTLFN...  398
rabbit   .LVN....RLEK...SPPVISLEAV......PGSSRL..MAG.RLWWL.LKSGAQATWTEL.WP-HE...  402
rat      NLVS....RLEK...SPPGISLDT.....S.PGSSKLYVTSG.RLWWL.LKSGAQATWAELSWP-HE...  402
human    .LVS....RLEK.V.TPHGI.LDSV....I.PGSSRL..MAG.RLWWL.LKSGAQATWTEL.WP-HE...  404

mWap65-1 GAMCGPG------------GVKVFRGNHYYHFESPKTFVAARALPEQHRISLELFGCDH            430
mWap65-2 A.F.SSD------------.I.I.Q.SN...YD..MLL.MS.IA.IPLKVTSAMV..ED            427
goldfish V....K------------..TAVI.....Q.G..MIMMM.KIM.....V.QG......            445
carp     A....K------------..TAVI.....LYD...IMMM.KIM.....V.QG......            439
trout    A....E------------...L.K....F..Q.V.VMLM.K.I..E.KTA.......            445
rabbit   ..L.MEKPLGPNSCSTSGPNLYLIH.PNL.CYRHVDKLN..KN..QPQ.V.R-.L..T.            460
rat      ..L.LEKSLGPYSCSSNGPNLFFIH.PNL.CYS.IDKLN..KS..QPQKVNS-IL..SQ            460
human    ..L.MEKSLGPNSCSANGP.LYLIH.PNL.CYSDVEKLN..K...QPQNVTS-.L..T.            462
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Fig. 1. Comparison of the amino acid sequences of two types of medaka Wap65, mWap65-1 and mWap65-2, with those of fish Wap65s and mammalian hemopexins, including goldfish Wap65 (Kikuchi et al., 1995), carp Wap65 (Kinoshita et al., 2001), rainbow trout hemopexin-like protein (Miot et al., 1996) and hemopexins of rabbit (Morgan et al,. 1993), rat (Nikkilä et al., 1991) and human (Altruda et al., 1985; Takahashi et al., 1985). Amino acids identical to those of mWap65-1 are shown as dots; gaps introduced to maximize the alignment are represented by hyphens. Lightly shaded boxes indicate conserved cysteine residues, and heavily shaded boxes indicate conserved histidine residues that are thought to serve as heme axial ligands (Paoli et al., 1999). Conserved histidine residues in all Wap65s are shown by blue boxes, and conserved histidine residues in all except for mWap65-2 by green boxes. Black arrowheads in Wap65s and hemopexins (not mWap65-2) indicate cleavage sites for secreted proteins when data are available. The predicted signal peptide regions of mWap65-1 and mWap65-2 were defined using SignalP program and are underlined. White arrowheads in mWap65-1 and mWap65-2 indicate predicted cleavage sites used for the construction of expression vectors for recombinant mWap65-1 and mWap65-2. Potential *N*-glycosylation sites predicted using PROSITE are shown by red boxes. The numbers in the right margin represent those of amino acids from the N-terminal methionine in premature proteins.

solution containing blood from medaka, as described above, was added to the washed agarose suspension and the mixture incubated at room temperature for 1 h. The reaction mixture was centrifuged at 10 000 ***g*** for 5 min and the supernatant removed. Hemin–agarose was washed ten times as described above, and bound proteins were eluted after incubation at room temperature for 2 min with 400 µl of an elution buffer containing 0.2 mol l^{-1} sodium citrate (pH 5.2), 0.5 mol l^{-1} NaCl and 0.02% NaN_3. Proteins eluted from the hemin–agarose were obtained as supernatant by centrifugation at 10 000 ***g*** for 5 min and the eluting procedure was performed three more times. The protein fractions obtained were analyzed by SDS-PAGE and immunoblotting by the same method as described above.

N-terminal amino acid sequencing

The N-terminal amino acid sequence was determined by the method of Matsudaira (1987). Briefly, serum proteins separated on SDS-PAGE were electrically transferred onto an Immobilon PVDF membrane and stained with Coomassie Brilliant Blue (CBB) R250. Parts of the membrane carrying the blotted protein, which was eluted from hemin–agarose by the elution buffer described above, were cut out with a clean razor and subjected to an Applied Biosystems model 492HT protein sequencer.

Reverse transcription-polymerase chain reaction

In order to demonstrate tissue distribution for the transcripts encoding mWap65-1 and mWap65-2, first strand cDNA was

synthesized from 5 μg total RNA extracted from liver, heart, gill, eye, ovary, brain, skin and muscle from three individuals acclimated to 10°C by the method described in Kinoshita et al. (2001b).

Primers used for PCR amplification of *mWap65-1* were mWAP-3′F (5′-CGGCACATGTACGATGTAGA-3′) and mWAP-3′R (5′-GCACGAAAGGACCACAGACT-3′), their sequences being located at 1096–1116 and 1514–1534 nt of the full-length cDNA clone, respectively. Primers for *mWap65-2* were mWAPn-in-F (5′-GATGATACCGGCAGAATGTA-3′) and mWAPn-in-R (5′-TTCTAATCTTCACATCCCAC-3′), their sequences being located at 838–857 and 1357–1376 nt of the full-length cDNA clone, respectively. The nucleotide sequences of the full-length cDNA clones encoding mWap65-1 and mWap65-2 are registered in the DDBJ/EMBL/GenBank databases, accession numbers AB075198 and AB075199, respectively. PCR using 0.1 μg of a given cDNA as template was performed by the same method as for 5′RACE for *mWap65-2,* except that 40 cycles were employed. Medaka *β-actin* cDNA, which is a housekeeping gene, was used as the internal standard in RT-PCR, where primers mACT-F2 (5′-AACTCATTGGCATGGCTTC-3′) and mACT-R2 (5′-TAGTCAGTGTACAGGTTTGGC-3′) were synthesized from 1305–1323 and 1784–1804 nt in the reported sequence, respectively (Takagi et al., 1994). PCR was performed by the same method as adopted for amplifying *mWap65-2* in 5′RACE except that 25 cycles were employed.

For quantitative RT-PCR of the transcripts encoding mWap65-1 and mWap65-2, first strand cDNA was synthesized from 5 μg total RNAs of various samples during ontogeny. Subsequent PCR was performed by the same method as described above except that the PCR cycle was shortened to 32. Amplified products were subjected to 1% agarose gel electrophoresis, stained with ethidium bromide, and quantified by using the Electrophoresis Documentation and Analysis System 120 (Eastman Kodak, New Haven, CT, USA).

Northern blot analysis

Total RNAs were extracted from liver tissues of 12 individuals acclimated to either 10 or 30°C for a minimum of 5 weeks. The 12 fish were separated into four groups each containing three fish, and 20 μg of total RNAs from each group were subjected to northern blot analysis. Northern blot analysis was performed as described in our previous report (Kinoshita et al., 2001a) except that the DNA fragments containing 1096–1534 nt of *mWap65-1* cDNA and 838–1376 nt of *mWap65-2* cDNA were used as probes. Signals on the hybridized membranes were analyzed using a Fujix BAS 1000 computerized densitometer scanner (Fuji Photo Film, Tokyo, Japan). *mWap65-1* and *mWap65-2* mRNA levels were standardized against the signal intensity of 18S rRNA, quantified using the Electrophoresis Documentation and Analysis System 120 (Eastman Kodak) from the same samples and statistically analyzed using Student's *t*-test.

Lipopolysaccharide administration to medaka

The effects of LPS administration on medaka were examined as described for goldfish (Kikuchi et al., 1997). HNI medaka reared at 25°C were anesthetized with 168 μg ml^{-1} 3-aminobenzoic acid ethylester. A solution of 10 μl saline containing 50 μg of LPS from *Salmonella typhimurium* (Sigma-Aldrich) was injected intraperitoneally (i.p.), and control fish received a similar volume of saline solution. On day 2 or 4 after the treatment, nine fish each from both groups were killed and separated into three groups, each containing three fish. Liver tissues were collected from the three fish of each group, mixed together and total RNAs extracted. The RNAs were then subjected to northern blot analysis, performed by the same method as described above.

Results

Nucleotide sequences of medaka Wap65 isoform cDNAs

Screening of the medaka cDNA library probed with a fragment of carp *Wap65* cDNA gave several positive plaques, resulting in the isolation of the full-length cDNA of *mWap65-1*. It was composed of 1534 bp in total together with polyadenylation signals of 1437 and 1510 nt and a poly(A)$^+$ tail of 1535 nt. The open reading frame contained 1290 bp encoding 430 amino acid residues.

The full-length cDNA of *mWap65-2* obtained from cDNA clone of OLe05.03f and 5′RACE in this study comprised 1485 nt, encoding 427 amino acids. Codon ATG, which started from 91 nt, was contained in the Kozak sequence (A/C)NN<u>ATG</u>G conserved in eukaryotes (Kozak, 1981), and judged to be the initiation codon for *mWap65-2* cDNA.

Comparison of the deduced amino acid sequences of mWap65-1 and mWap65-2 with those of fish and mammalian hemopexins

The deduced amino acid sequence of mWap65-1 showed 66–68% identity with those of goldfish Wap65 (Kikuchi et al., 1995), carp Wap65 (Kinoshita et al., 2001a) and rainbow trout hemopexin-like protein (Miot et al., 1996), and 29–31% identity to those of rabbit (Morgan et al., 1993), rat (Nikkilä et al., 1991) and human hemopexins (Altruda et al., 1985; Takahashi et al., 1985) (Table 2). The mWap65-2 sequence showed 43–46% identity with those of fish Wap65s and related proteins (Kikuchi et al., 1995; Miot et al., 1996; Kinoshita et al., 2001a), and 37% identity with those of mammalian hemopexins (Altruda et al., 1985; Takahashi et al., 1985; Nikkilä et al., 1991; Morgan et al., 1993) (Table 2).

Cysteine residues essential to the structural integrity of hemopexin (Takahashi et al., 1985) were conserved in mWap65-1 and mWap65-2, as in other fish species (Fig. 1). His-213 and His-266 residues, which are essential for the high affinity of mature rabbit hemopexin for heme (Paoli et al., 1999), were conserved in mWap65-2, but substituted by glutamic acid and lysine residues, respectively, in mWap65-1. Furthermore, there were four potential *N*-glycosylation sites mWap65-1, compared to only one in mWap65-2 (Fig. 1).

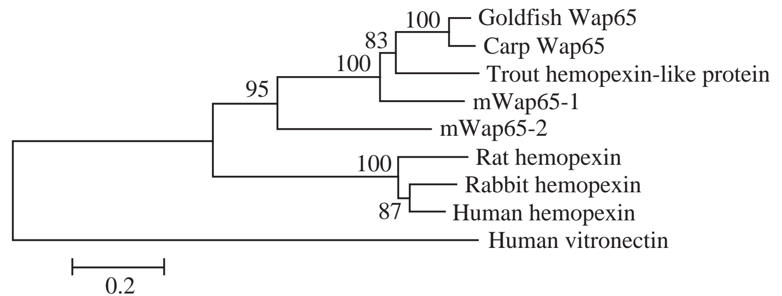

Fig. 2. Phylogenetic analysis of fish Wap65s and mammalian hemopexins. The neighbor-joining method with Poisson distance matrix was used to infer the tree topology. Bootstrap values (%) are shown at each branch point of the tree. The scale at the bottom is in units of amino acid substitutions per site. Data are cited for goldfish Wap65 (Kikuchi et al., 1995), carp Wap65 (Kinoshita et al., 2001) and rainbow trout hemopexin-like protein (Miot et al., 1996), and hemopexins of rabbit (Morgan et al,. 1993), rat (Nikkilä et al., 1991) and human (Altruda et al., 1985; Takahashi et al., 1985).

There were twice as many histidine residues in mWap65-1 compared to mWap65-2. Predicted signal peptide regions were found in both mWap65-1 and mWap65-2 and their lengths were similar to those of other fish Wap65s and mammalian hemopexins.

The phylogenetic tree representing the multiple sequence alignment for the full-length deduced amino acid sequences of Wap65s and hemopexins is shown in Fig. 2. The tree clearly shows that mWap65-1 is clustered together with known teleost Wap65s and its related protein, forming a separate clade from mWap65-2. Moreover, teleost Wap65s, including rainbow trout hemopexin-like protein and tetrapod hemopexins, formed a paraphyletic group.

Preparation and immunological specificity of recombinant proteins of mWap65-1 and mWap65-2

Initially, the recombinant protein rWap65-1 was prepared as a GST-fusion protein using the *E. coli* expression vector pGEX-2T. When the purified rWap65-1 was digested with thrombin to remove the GST portion, non-specific degradation of rWap65-1 was observed (data not shown). Therefore, rWap65-2 was prepared using the expression vector pET-39b,

Table 2. *Percentage identities of deduced amino acid sequences of fish Wap65s and mammalian hemopexins*

	Wap65				Hemopexin		
	Medaka-2	Goldfish	Carp	Trout	Rabbit	Rat	Human
Medaka-1	45	66	65	68	31	29	31
Medaka-2		43	46	43	37	37	37
Goldfish			87	68	32	30	31
Carp				69	33	30	30
Trout					30	30	31
Rabbit						71	80
Rat							75

The two types of medaka Wap65 are mWap65-1 (medaka-1) and mWap65-2 (medaka-2).

Data are cited for goldfish Wap65 (Kikuchi et al., 1995), carp Wap65 (Kinoshita et al., 2001) and rainbow trout hemopexin-like protein (Miot et al., 1996), and hemopexins of rabbit (Morgan et al., 1993), rat (Nikkilä et al., 1991) and human (Altruda et al., 1985; Takahashi et al., 1985).

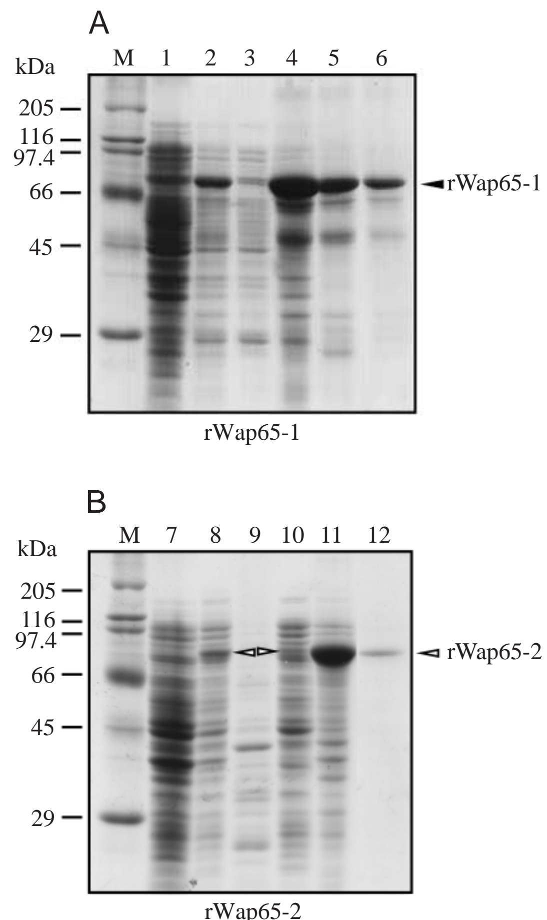

Fig. 3. SDS-PAGE of expressed and purified recombinant proteins of the two types of medaka Wap65, mWap65-1 and mWap65-2. (A) Purification of recombinant mWap65-1 (rWap65-1). Lane 1, control total cell lysates of *Escherichia coli* BL21 used as host cell; lane 2, total cell lysate after induction; lane 3, soluble fraction; lane 4, insoluble fraction; lane 5, the fraction containing solubilized rWap65-1; lane 6, eluate after GSTrap affinity purification. (B) Purification of recombinant mWap65-2 (rWap65-2). Lane 7, control total cell lysates of *E. coli* BL21 (DE3) used as host cell; lane 8, total cell lysate after induction; lane 9, periplasm fraction; lane 10, soluble fraction; lane 11, insoluble fraction; lane 12, eluate after HiTrap affinity purification. 10–20 μg of protein were loaded in lanes 1–8, 10 and 11, and 5 μg in lanes 9 and 12. M, molecular mass standards (kDa). The positions of rWap65-1 and rWap65-2 in SDS-PAGE gels are marked by black and white arrowheads, respectively.

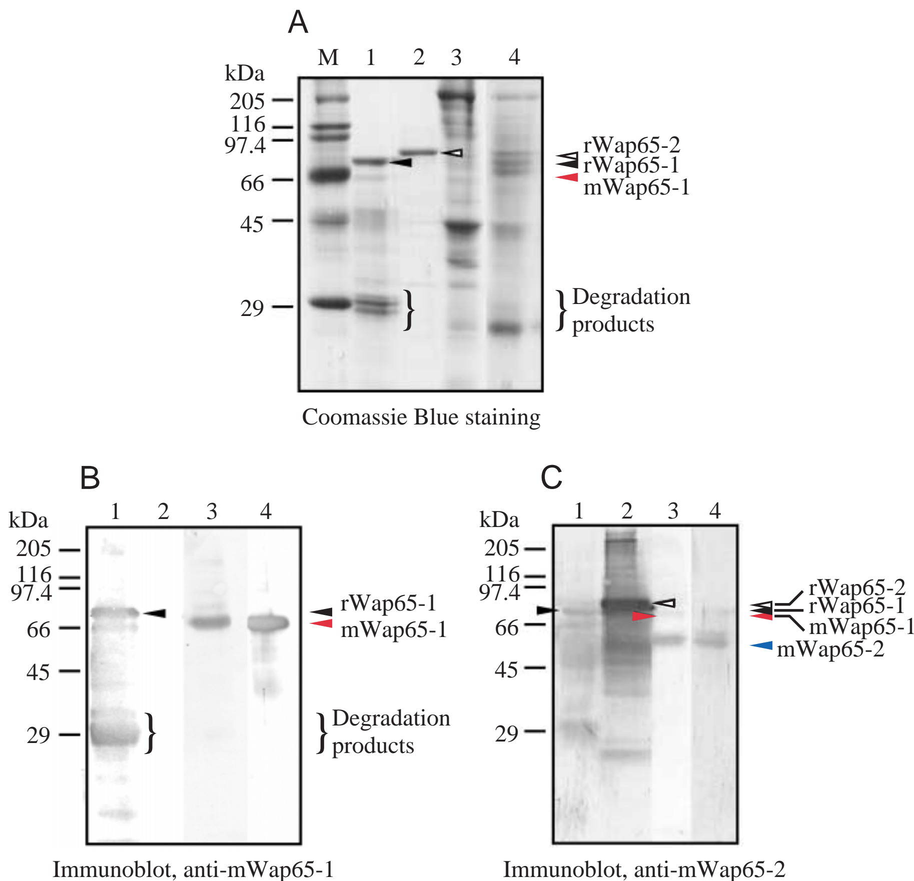

Fig. 4. SDS-PAGE (A) and immunoblotting (B,C) patterns for recombinant proteins mWap65-1 (rWap65-1) and mWap65-2 (rWap65-2), and cytosolic protein fraction (8.0 mg ml^{-1}) and blood solution (2.8 mg ml^{-1}) from adult medaka. 10 μg of proteins were applied per lane and specific anti-rWap65-1 rabbit antiserum (B) and anti-rWap65-2 mouse antiserum (C) were used for immunoblotting. Lanes 1, rWap65-1; lanes 2, rWap65-2; lanes 3, a cytosolic protein fraction extracted from whole individuals of medaka; lanes 4, a blood solution obtained from adult medaka. M, molecular mass standards (kDa). The marks for rWap65-1 and rWap65-2 are as in Fig. 3 and the positions of intact mWap65-1 and mWap65-2 in SDS-PAGE gels are marked by red and blue arrowheads, respectively.

which contained sequences encoding DsbA and the cleavage site for enterokinase, with high substrate specificity. Although the purified rWap65-2 was digested with enterokinase, the yield of rWap65-2 free of DsbA was quite low (data not shown). We assumed that intact fusion proteins would be suitable for preparation of specific antisera, and used them without digestion with specific proteases.

The induced rWap65-1 was localized only in the insoluble fraction (lane 4 in Fig. 3A). By contrast, some rWap65-2 was found in the soluble fraction, although the major part was localized in the insoluble fraction (lanes 10 and 11 in Fig. 3B). Therefore, rWap65-1 and rWap65-2 were purified from the insoluble and soluble fractions, respectively. While rWap65-2 was of high purity, rWap65-1 showed a few lower-molecular-mass protein bands, which were probably produced by degradation during the purification procedure (lanes 6 and 12 in Fig. 3). The apparent molecular masses of rWap65-1 and rWap65-2 fused to GST and DsbA, respectively, were calculated as 72 000 and 74 000, respectively, based on the aa sequences. These values were in good agreement with the molecular masses determined by SDS-PAGE (Fig. 3), i.e. 72 and 74 kDa for rWap65-1 and rWap65-2, respectively.

Purified rWap65-1 and rWap65-2 were used for preparation of antisera. The specificity of antisera raised against rWap65-1 and rWap65-2 was determined by immunoblotting (Fig. 4). The antiserum raised against rWap65-1, diluted to a final IgG concentration of 4.8 μg ml^{-1}, reacted with a 72 kDa protein band of rWap65-1 (lane 1 in Fig. 4B), whereas no cross-reactivity was observed against rWap65-2 (lane 2 in Fig. 4B). The antiserum also reacted with low molecular mass protein bands of 28–30 kDa, which were probably degradation products of rWap65-1 during storage (lane 1 in Fig. 4B). The antiserum raised against rWap65-2, diluted to a final IgG concentration of 2.3 μg ml^{-1}, showed strong cross-reactivity with a homologous antigen (lane 2 in Fig. 4C). Although very weak cross-reactivity was observed against rWap65-1 (lane 1 in Fig. 4C), it was apparent that the antiserum against rWap65-2 had high specificity.

Cytosolic and serum proteins obtained from adult medaka yielded several bands in SDS-PAGE (lanes 3 and 4 in Fig. 4A). When these proteins were transferred to PVDF membranes and subjected to immunoblotting, a single band of 67 kDa showed cross-reactivity with anti-rWap65-1 antiserum (lanes 3 and 4 in Fig. 4B), whereas one major band of 55 kDa together with a minor band of 67 kDa were observed with anti-rWap65-2 antiserum (lanes 3 and 4 in Fig. 4C). Since the molecular mass of the minor band reacting with anti-rWap65-2 antiserum was the same as that of the single band reacting with anti-rWap65-1 antiserum, this protein was identified as intact mWap65-1. Correspondingly, the protein band of 55 kDa, which was the major component to have cross-reactivity with anti-rWap65-2 antiserum, was judged to be intact mWap65-2.

When processed after secretion, intact mWap65-1 and mWap65-2 would have molecular masses of 47 600 and 45 900 kDa, respectively, assuming that the cleavage sites of mWap65-1 are located between Asp-20 and His-21 and of mWap65-2 between Ala-20 and Pro-21 by SignalP program (see Fig. 1). However, the molecular masses of intact mWap65-1 and mWap65-2 were determined by SDS-PAGE to

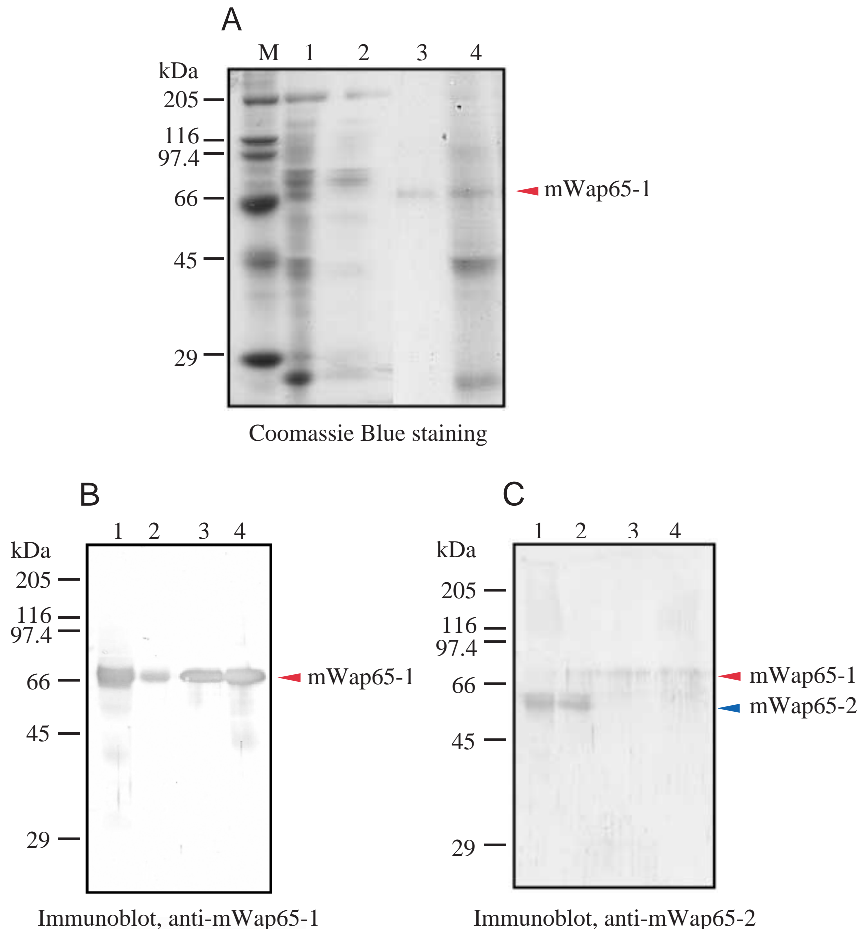

Fig. 5. SDS-PAGE (A) and immunoblotting (B,C) patterns of blood solution fractions obtained by hemin–agarose affinity chromatography. Immunoblotting used antisera specific to mWap65-1 (B) and to mWap65-2 (C). Lanes 1, blood solution of 2.8 mg ml^{-1} obtained from adult medaka; lanes 2, supernatant; lanes 3, eluted fraction; lanes 4, proteins still bound to hemin–agarose after elution; M, molecular mass markers (kDa). 10 µl solution/lane. Arrowheads, see Fig. 4.

be 67 and 55 kDa, respectively, as described above. Since mWap65-1 has four predicted glycosylation sites and mWap65-2 has only one (see Fig. 1), the differences between the molecular masses calculated from the deduced aa sequences and those determined by SDS-PAGE were considered to be the result of such glycosylation after the synthesis of nascent proteins.

Heme-binding ability of mWap65-1 and mWap65-2

Medaka Wap65 isoforms were examined for heme-binding ability using the fractions separated by hemin–agarose chromatography and subsequent immunoblotting analyses using the specific antisera raised against rWap65-1 and rWap65-2 (Fig. 5). When hemin–agarose treated with the serum proteins was washed with buffer I containing 10 mmol l^{-1} sodium phosphate (pH 7.4), 0.5 mol l^{-1} NaCl, 3 µg leupeptin and 1 mmol l^{-1} PMSF, an unbound protein fraction containing both mWap65-1 and mWap65-2 (lane 2 in Fig. 5) was obtained, suggesting that excess amounts had been applied over the capacity of hemin–agarose. Interestingly, the 67 kDa component bound to hemin–agarose was released by the elution buffer containing 0.2 mol l^{-1} sodium citrate (pH 5.2), 0.5 mol l^{-1} NaCl and 0.02% NaN_3 as shown in lane 3 of Fig. 5A. This protein had a strong cross-reactivity with anti-rWap65-1 antiserum, but hardly any with anti-rWap65-2 antiserum. Furthermore, the N-terminal aa sequence of this component was determined to be DHHEHRRKGAVRD, which was consistent with the sequence of Asp-20–Asp-32 for precursor mWap65-1 (see Fig. 1). These results indicate that the 67 kDa component released from hemin–agarose by the elution buffer was intact mWap65-1 and that its cleavage site is genuinely located between Ala-19 and Asp-20 (Fig. 1). By contrast, the 55 kDa protein band was not detected in the eluate by either SDS-PAGE (lane 3, Fig. 5A) or immunoblotting using anti-rWap65-2 antiserum (lane 3, Fig. 5C). As shown in lane 4 of Fig. 5A, some protein bands including that of mWap65-1 were observed in the fraction that remained bound to hemin–agarose resin. Similar results were obtained in other experiments using the cytosolic protein fraction from the whole body of adult medaka (data not shown).

Expression patterns of mRNAs encoding mWap65-1 and mWap65-2 in various tissues of adult fish and during development

In a preliminary experiment with northern blot analysis, the transcripts of *mWap65*-1 and *mWap65*-2 were detected only in liver (data not shown). Using RT-PCR, however, *mWap65-1* mRNA was found to be expressed in liver, eye, heart and brain, whereas *mWap65-2* mRNA was expressed only in liver (Fig. 6). The accumulated mRNA levels were apparently highest in the liver, followed by heart, eye and brain. These differences in expression pattern were significant when compared to those of β-actin, the expression level of which was almost the same in all the tissues examined.

Quantitative RT-PCR was then performed to demonstrate changes in the accumulated mRNA levels of *mWap65*-1 and *mWap65*-2 in the whole embryo during ontogeny (Fig. 7). The levels of β-actin used as the internal standard were almost constant at the various developmental stages. By contrast, transcripts of *mWap65*-1 were first detected in embryos 39 h post-fertilization (h.p.f.) at the start of heart beating and increased rapidly after 98 h.p.f. (Fig. 7). Transcripts of *mWap65*-2 were expressed even at the beginning of the

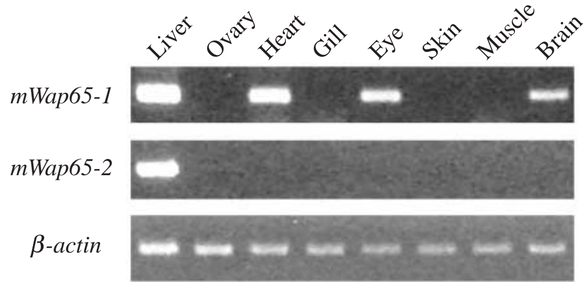

Fig. 6. RT-PCR analysis of mRNAs encoding mWap65-1 and mWap65-2, and β-actin, from different tissues of adult medaka. After PCR, amplified products were separated on agarose gels and stained with ethidium bromide.

experiments (25 h.p.f.) and gradually increased thereafter, though the levels did sometimes decrease (Fig. 7). In addition, the *mWap65-2* signals were apparently stronger than those of *mWap65-1* during the early stages of ontogeny.

Effects of acclimation temperature on transcription levels of mWap65-1 *and* mWap65-2 *in adult fish*

Since *mWap65-1* and *mWap65-2* transcripts were only expressed together in the liver of adult medaka, this tissue was examined by northern blot analysis to detect any changes in their expression with temperature acclimation. The signal intensities of *mWap65-2* were almost constant irrespective of samples examined, whereas those of *mWap65-1* showed large individual variations (Fig. 8). These signals were quantified with a computerized densitometer scanner (Fujix BAS 1000), and compared with those of 18S rRNA quantified with the Electrophoresis Documentation and Analysis System 120 after staining with ethidium bromide. We found no significant difference in the accumulated mRNA levels of either *mWap65-1* or *mWap65-2* in samples acclimated to 10°C or 30°C.

Effects of LPS on mRNA levels of mWap65-1 *and* mWap65-2

The accumulated *mWap65-1* and *mWap65-2* mRNA levels were not significantly changed at different water temperatures, so we examined the effects of LPS on *mWap65-1* and *mWap65-2* mRNA levels in adult medaka. However, northern blot analysis revealed no increase in mRNA levels of either *mWap65-1* or *mWap65-2* on days 2 and 4 after LPS injection (Fig. 9), in contrast to goldfish, which showed more than twofold increase in the levels of Wap65 on day 4 after the injection (Kikuchi et al., 1997).

Discussion

The present study demonstrates that two types of fish hemopexin-like protein, mWap65-1 and mWap65-2, are present in one species as homologs of mammalian hemopexin. mWap65-2 showed a higher sequence identity to hemopexins than mWap65-1 and Wap65s from goldfish and carp. In addition, the histidine residues, His-213 and His-266, which are required for the high affinity of hemopexin for heme, were conserved in mWap65-2, but not in mWap65-1. It was therefore thought that mWap65-2 would have functions similar to those of mammalian hemopexins, whereas mWap65-1 might not. Unexpectedly, hemin–agarose affinity chromatography

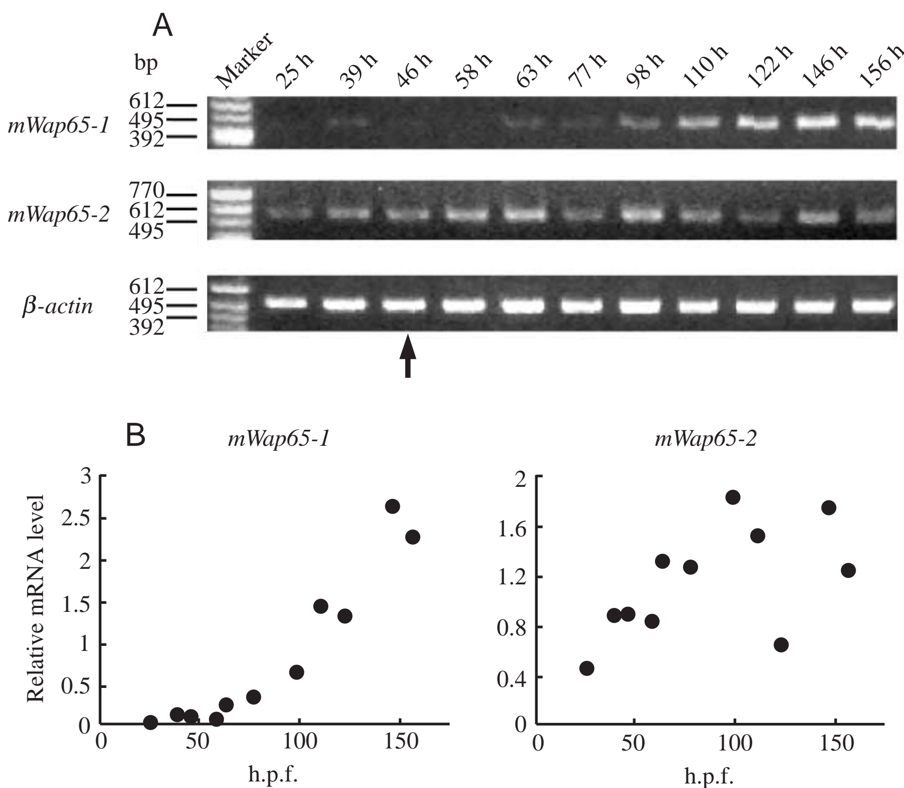

Fig. 7. Quantitative RT-PCR analyses on mRNAs encoding mWap65-1 and mWap65-2, and β-actin from embryos at various developmental stages (h.p.f.). (A) Agarose gel electrophoresis patterns for amplified products obtained by RT-PCR. Total RNAs were isolated from embryos at different developmental stages (see Table 1). The arrow indicates the stage at which the liver begins to develop. After PCR, amplified products were separated on agarose gels and stained with ethidium bromide. Numbers in the left margin represent base pairs of ϕX174 DNAs digested with *Hinc*II. (B) Changes in relative transcription levels of *mWap65-1* and *mWap65-2* during ontogeny. The signal intensities of each transcript in agarose gels stained with ethidium bromide were quantified, using Electrophoresis Documentation and Analysis System 120, relative to those of *β-actin* at the same stage.

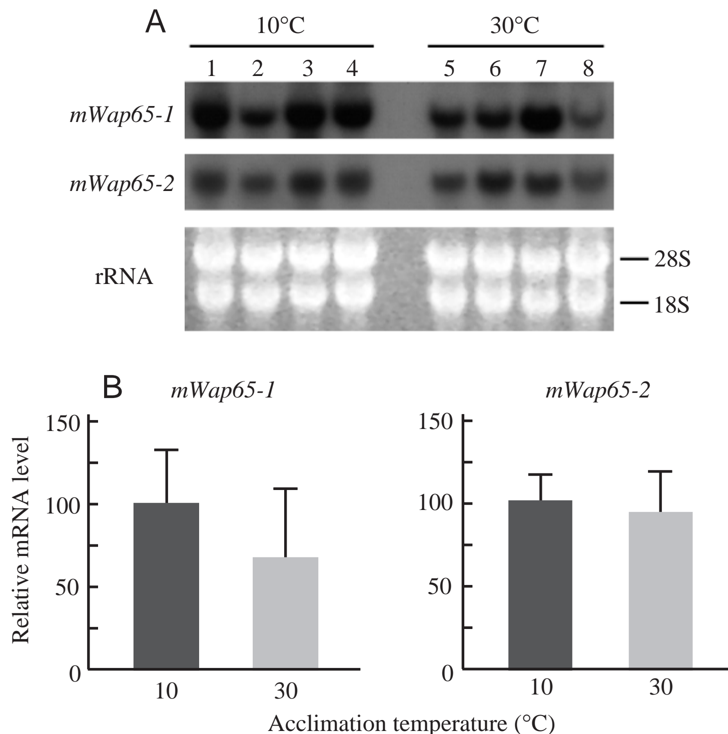

Fig. 8. Accumulated mRNA levels of *mWap65-1* and *mWap65-2* in livers from adult medaka acclimated to 10 and 30°C. (A) Northern blot analysis was performed for four groups each containing three individuals acclimated to either 10 or 30°C. Lanes 1–4 contained 20 μg of total RNAs from the liver of fish acclimated to 10°C, and lanes 5–8 contained those from each group of fish acclimated to 30°C. (B) The transcriptional levels of *mWap65-1* and *mWap65-2* relative to those of 18S rRNA. RNA blots for *mWap65-1* and *mWap65-2* were quantified using a Fujix BAS 1000 densitometer, and signal intensities of 18S rRNA in agarose gels stained with ethidium bromide were quantified by using the Electrophoresis Documentation and Analysis System 120. Relative mRNA levels are compared to those of 18S rRNA for the same sample; values are means ± S.D.

demonstrated that mWap65-1 had an ability to bind to heme, but mWap65-2 did not. De Monti et al. (1998) reported that a recombinant rainbow trout hemopexin-like protein, artificially prepared by the baculovirus expression system, could bind to heme, despite lacking the two histidine residues required for mammalian hemopexins to bind heme. This is similar to the situation found with mWap65-1 in the present study. Consequently, it is predicted that fish Wap65s bind to heme in a different manner from that of mammalian hemopexins. Satoh et al. (1994) demonstrated using a recombinant human hemopexin expressed in a baculovirus system that glycosylation contributes critically to the heme-binding property of hemopexin. In fact, predicted *N*-glycosylation sites are abundant in mammalian hemopexins, mWap65-1 and trout hemopexin-like protein, which all have heme-binding ability. Phylogenetic analysis suggested that mWap65-1 and mWap65-2 are paralogues and fish orthologs of mammalian hemopexins. We found two Wap65 isoforms from the databases for other fish, including pufferfish *Fugu rubripes* (Fugu genomic sequence assembly data version 3.0, scaffolds 175 and 725), zebrafish *Danio rerio* (AI588537 and BM095588), rainbow trout *Oncorhynchus mykiss* (Z68112 and AF281339) and channel catfish *Ictalurus punctatus* (BM438553 and BM438613). No isoforms have been found in mammalian hemopexins, however. Accordingly, these results imply that the ancestral gene might have been duplicated at an early period after teleosts and mammals diverged.

Mammalian hemopexins are not only synthesized in the hepatic parenchymal cells (Thorbecke et al., 1973), but also in other cell types including neurons of the peripheral nervous system, retinal photoreceptor and ganglion cells, and ovary as minor sites of synthesis (Swerts et al., 1992; Hunt et al., 1996; Chen et al., 1998). Transcripts of goldfish *Wap65* have been reported to be present in hepatopancreas, ovary, eye, gill and muscle (Kikuchi et al., 1997). By contrast, the transcripts of *mWap65*-2 were expressed only in liver, whereas those of *mWap65-1* were found in liver, eye, heart and brain (see Fig. 6). Gene duplication is believed to play an important role during evolution, providing opportunities to evolve new functions that can lead to novel morphological and physiological characters (Ohno, 1970). While mWap65-1 has an ability to bind heme, mWap65-2 may have different functions, which would be important to maintain fish cellular integrity.

While the accumulated mRNA levels of *mWap65-1* were first observed in embryos 39 h.p.f., those of *mWap65-2* were found in embryos from the earliest stage studied (25 h.p.f.) (see Fig. 7). Iwamatsu (1994) reported that in medaka the liver appears in the embryo at the time the blood circulation starts, which was 46 h.p.f. in this study. These results again imply that the physiological functions of mWap65-1 are different from those of mWap65-2, as suggested by sequence analysis, tissue distribution and heme-binding ability. In addition, these expression patterns were in a marked contrast to those of rat and chicken hemopexins, which are only expressed immediately before birth (Grieninger et al., 1986; Nikkilä et al., 1991).

Gracey et al. (2001) reported that goby *Gillichthys mirabilis* increases the expression levels of hemopexin-like protein mRNA during hypoxia. Hypoxia is a stress on goby, and it is possible that extensive changes in environmental water temperature are also stressful to goldfish and carp. It has been reported that goldfish and carp increase the quantities of Wap65 and their accumulated mRNA levels following warm temperature acclimation (Kikuchi et al., 1995; Kinoshita et al., 2001a). In the present study, northern blot analysis demonstrated that the levels of both *mWap65-1* and *mWap65-2* mRNA accumulated were not statistically significant in cold- and warm-acclimated fish. Interestingly, White et al. (1983) reported that in plaice *Pleuronectes platessa* the expression of C-reactive protein, a major acute phase protein, changed seasonally as a result of unknown factor(s) that did not include

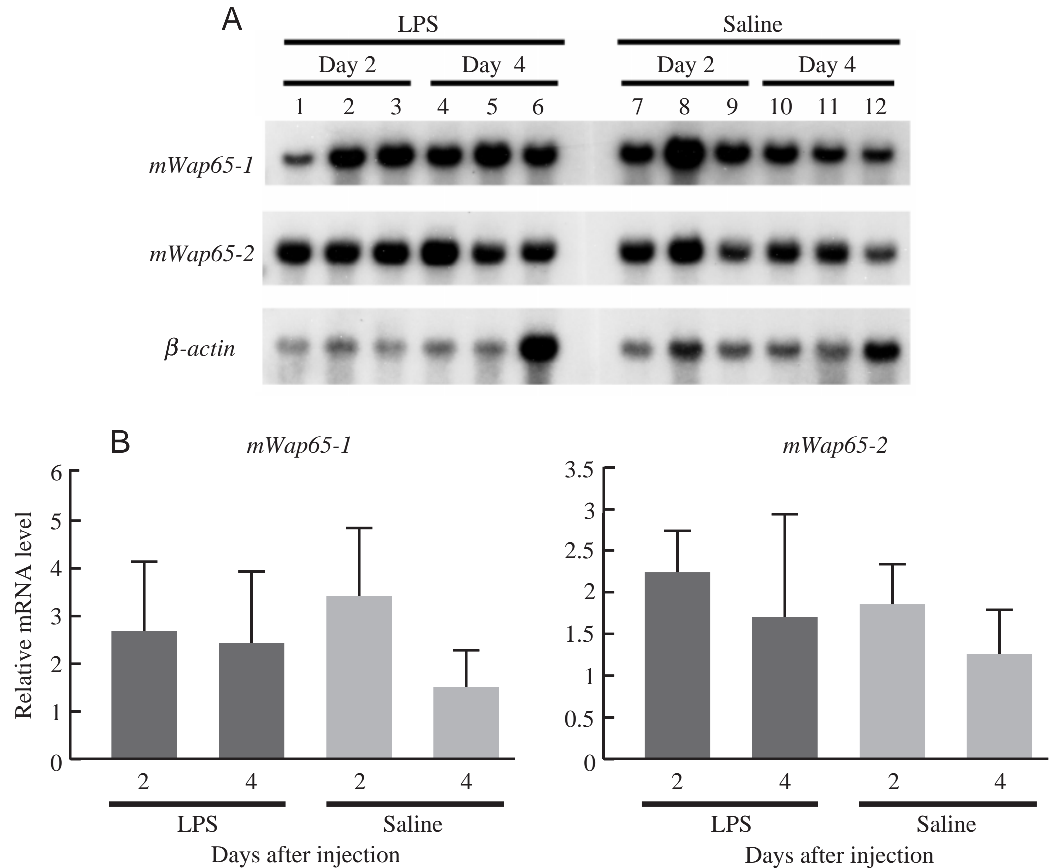

Fig. 9. Changes in *mWap65-1* and *mWap65-2* mRNA levels in liver from adult medaka following i.p. injection of LPS. (A) Northern blot analysis for three groups each containing three individuals following injection of either LPS or saline. Lanes 1–3 and 4–6 contained 20 μg of total RNAs from fish on days 2 and 4 after LPS injection, respectively, whereas lanes 7–9 and 10–12 contained those on days 2 and 4 after saline injection, respectively. (B) The transcriptional levels of *mWap65-1* and *mWap65-2* relative to those of *β-actin*. RNA blots were quantified using a Fujix BAS 1000 densitometer. Relative mRNA levels are compared to those of *β-actin* for the same sample; values are means ± S.D.

temperature. Therefore, factors unrelated to water temperature, such as hypoxia and bacterial infection, may induce expression of medaka Wap65 isoforms. Interestingly, hypoxia increased IL-6, a potent inducer for several acute-phase genes, in endothelial cells of human (Pearlstein et al., 2002). The transcripts of goldfish *Wap65* were rapidly and markedly increased after administration of bacterial LPS (Kikuchi et al., 1997), which induces expression of IL-6, as does hypoxia in human. This result suggests that the goldfish response occurs through reactions with NF-IL6 and IL6-RE consensus sequences in the promoter region (Kikuchi et al., 1997). Human hemopexin plasma levels also increase during acute infections, suggesting that its biosynthesis is subject to control mechanisms responsible for the acute-phase reaction (Baumann et al., 1983; Poli and Cortese, 1989). Thus, goldfish Wap65 could be considered as an acute-phase protein, like hemopexins. We examined changes in transcriptional levels of *mWap65-1* and *mWap65-2* mRNA after the administration of LPS, using the method of Kikuchi et al. (1997), but found no significant difference in accumulated levels of either *mWap65-1* or *mWap65-2* mRNA in fish injected with LPS from those not injected (see Fig. 9). In fact, different levels of plasma hemopexin have been observed after an inflammatory event in human and rodent: the increase is only slight in humans, while plasma hemopexin rises severalfold in rodent, which was thought to be due to differences in the promoters of these genes (Tolosano and Altruda, 2002).

In conclusion, we have characterized two isoforms of the medaka protein Wap65, mWap65-1 and mWap65-2. mWap65-1 had a heme-binding ability, but mWap65-2 did not, and each had different expression profiles. However, the accumulated mRNA levels of these genes were not dependent on water temperature or LPS administration. Further investigation, such as promoter assay or exploration of substances that might bind to mWap65-2, are required to elucidate the different features of medaka Wap65 isoforms.

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