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Molecular Identification and Expression Analysis of Tumor Necrosis Factor Receptor-associated Factor 2 in Grass Carp *Ctenopharyngodon idella*

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Abstract Tumor necrosis factor receptor-associated factor 2 (TRAF2) is a crucial component of almost the entire tumor necrosis factor receptor superfamily signaling pathway. In the present study, a *TRAF2* gene has been cloned from grass carp (*Ctenopharyngodon idella*) by reverse transcription-polymerase chain reaction and rapid amplification of cDNA ends. The full-length cDNA is 3162 bp, including a 60 bp 5′ untranslated region (UTR), a 1611 bp open reading frame, and a 1491 bp 3′ UTR. The polyadenylation signal (AATAAA) and the mRNA instability motifs (ATTTTA, ATTTA) were followed by a poly(A) tail in the 3′ UTR. No signal peptide or transmembrane region has been found in the putative amino acids of grass carp TRAF2 (gcTRAF2). Phylogenetic tree analysis clearly showed that gcTRAF2 is nearest to the *TRAF2* gene of goldfish. The identity of gcTRAF2 with its homologs in other vertebrates ranges from 56% to 97%. It is characterized by one RING-type signature at the N-terminus, one zinc finger in the middle part, and one conserved TRAF domain consisting of a C-proximal (TRAF-C) subdomain and a N-proximal (TRAF-N) subdomain. The identity of TRAF-C among all TRAF2 homologs in vertebrates varies from 78% to 97%, whereas the identity of TRAF-N ranges from 56% to 100%. The recombinant gcTRAF2 has been expressed in *Escherichia coli* using pET-32a expression vector. The rabbit anti-gcTRAF2 polyclonal antibody was obtained. The expression of gcTRAF2 in different organs was examined by real-time quantitative polymerase chain reaction and Western blot analysis. It was widely distributed in heart, head kidney, thymus, brain, gill, liver, spleen, and trunk kidney. This is the first report of a TRAF2 homolog molecule in fish.

Keywords TRAF2; grass carp; cDNA; expression; organ distribution

The tumor necrosis factor (TNF) and TNF receptor (TNFR) superfamilies comprise a group of secreted or membrane-bound ligands and their receptors, respectively, with diverse and widespread physiological functions, transmitting signals in regulating apoptosis, osteoclastogenesis, and immune system regulation [1,2]. TNFR-associated factors (TRAF) have emerged as the major signal transducers for TNF signaling [3–5].

TRAF molecules were originally identified on the basis of their ability to bind to the cytoplasmic tail of TNFR2. They were consequently designated as TRAF1 and TRAF2 [6,7]. Six different proteins of this family have been identified in mammals, three in *Drosophila melanogaster* (DTRAF1, DTRAF2, and DTRAF6), and one in *Caenorhabditis elegans* [8–18]. The TRAF family is defined by several distinct structural features. First, the TRAF proteins are characterized by a conserved domain at the C-terminal, the TRAF domain, which is further subdivided into a more divergent N-proximal (TRAF-N) and a highly conserved C-proximal (TRAF-C) subdomain. The TRAF domain is responsible for homodimerization and heterodimerization of the TRAF proteins, as well as for

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their direct and indirect interactions with cognate surface receptors or other signaling molecules [6,9,19]. Second, all six known mammalian TRAFs, except TRAF1, contain N-terminal RING finger domains, followed by five or seven zinc finger motifs, critical for downstream effector functions [20]. TRAFs have no intrinsic enzymatic activity and are assumed to transmit signals by acting as adapter molecules or scaffold proteins, being involved in a wide range of biological functions, such as adaptive and innate immunity, embryonic development, stress response, and bone metabolism [21].

TRAF2 was the first identified and well characterized member of the larger TRAF family. TRAF2 shares its overall architecture with all the other TRAFs found in vertebrates with the exception of TRAF1. The transcripts have been detected in almost every tissue [6], making TRAF2 the most widely expressed TRAF family member. Various transfection and overexpression experiments have suggested that TRAF2 is a crucial component of almost the entire TNFR superfamily signaling pathway [22]. The mechanism might be that TRAF2 can interact directly with the cytoplasmic tail of receptors, such as TNFR2, that do not contain death domains, and indirectly with death domain-containing receptors such as TNFR1 through association with their death domain adaptor proteins [23, 20]. It is noteworthy that TRAF2 plays a key role in mediating TNFR1-induced activation of nuclear factor (NF)-κB and jun N-terminal kinase. Moreover, recent studies suggest that TRAF2 represents an integration point for pro- and anti-apoptotic signals [4].

Interestingly, a splice variant of TRAF2, known as TRAF2A, was found in murine. The TRAF2A differs from TRAF2 by an additional seven amino acids in the RING finger domain [24]. Functionally, TRAF2A differs from TRAF2 by its ability to selectively activate the jun N-terminal kinase, but not the NF-κB signaling pathway [24]. However, the splice variant of TRAF2 has not been found in other species, such as human or rat [25]. The TRAF2 in *D. melanogaster* (DTRAF2) was identified by the expressed sequence tag through DTRAF1. However, the characteristics of DTRAF2 were far distinct from TRAF2 in mammals, with high identity with TRAF6 in mammals [17]. Additionally, there is no report about the characterization of TRAF2 in other species.

Although TRAF2 is the extensively studied member of the TRAF family, little is known about it in fish. In the present study, the molecular characterization and expression analysis of the *TRAF2* gene from the grass carp, *Ctenopharyngodon idella*, is the first such report. The results obtained add to the knowledge about the *TRAF2* gene in fish, and they will help to explore its function in fish innate immunity.

Materials and Methods

Total RNA isolation for amplifying the sequence of TRAF2

Total RNA from the spleen of the healthy grass carp was extracted with TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Then the SMART cDNA was synthesized and amplified using a Clontech SMART PCR cDNA Synthesis Kit (Clontech, Carlsbad, USA) following the supplier's protocol.

Cloning TRAF2 cDNA sequences by rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR)

Based on the conserved sequences of the *TRAF2* gene in other vertebrates (GenBank accession Nos. CF661402, AJ297860, XM_678539, and XM_702048), one pair of degenerate primers was designed to obtain the internal region of the grass carp TRAF2 (gcTRAF2). All primers used in this paper are listed in **Table 1**. The PCR cycle conditions were 1 cycle of 94 ºC for 5 min, 9 cycles of 94 ºC for 30 s, 68 ºC for 30 s and 72 ºC for 90 s, 29 cycles of 94 ºC for 30 s, 64 ºC for 30 s and 72 ºC for 90 s, followed by 1 cycle of 72 ºC for 10 min.

The product size of cloned cDNA sequences was 528 bp. The resultant products were isolated using the Omega agarose purification kit (Omega, Doraville, USA), and cloned into pMD-18 vector (TaKaRa, Shiga, Japan) following the manufacturer's instructions. Putative clones were screened by PCR using the above primers under the same cycle conditions, and the selected clones were sequenced using the dideoxy chain-termination method on an automatic DNA sequencer (Model 377; Applied Biosystems, Foster City, USA).

To recover the full-length cDNA sequence, 3′ RACE and 5′ RACE were carried out using the gene-specific primers and adaptor primer (UPM). All the primers used are listed in **Table 1**, and the strategy is displayed in **Fig. 1**. The PCR cycle conditions were 1 cycle of 94 ºC for 5 min, 9 cycles of 94 ºC for 30 s, 68 ºC for 30 s and 72 ºC for 90 s, 29 cycles of 94 ºC for 30 s, 64 ºC for 30 s and 72 ºC for 90 s, followed by 1 cycle of 72 ºC for 10 min.

Sequence analysis

Protein prediction was carried out using software at

Table 1 Primers used for the grass carp tumor necrosis factor receptor-associated factor 2 (TRAF2) cloning and expression analysis in this study

Name	Sequence (5′→3′)	Application
JBTF	GGGGCTGGAGAGTT(G)GCAGGAAGA	Conserved region cloning
JBTR	AAAGAACAGA(G)GA(G)AAG(A)GTGAG(A)TGCC	Conserved region cloning
UPM	Long (0.4 μM): CTAATACGACTCACTATAGGGCAAGCC-AGTGGTATCAACGCAGAGT; short (2 μM): CTAATAC-GACTCACTATAGGGC	Adaptor for RACE
3′ Out_1	AGACTCTACCTGAATGGTGACGG	3′ RACE first-round PCR
3′ In_1	GCACTCACCTTCCTCTGTTCTT	3′ RACE second-round PCR
3′ Out_2	GGACCAGAACAACAGAGAGCACA	3′ RACE first-round PCR
3′ In_2	AAAGCTCGTATCTGAGAGATGAC	3′ RACE second-round PCR
5′ Out	GACTGCTCGGATTCTGCTAACTG	5′ RACE first-round PCR
5′ In	GCATTATGGGCGGCAGCATTAGG	5′ RACE second-round PCR
$Traf_2$-F	GAAGGTACCATGGGCACTGCGGGTAAATG	Expression in *E. coli*
$Traf_2$-R	CCGAAGCTTCTAAGACTGCTCGGATTCTG	Expression in *E. coli*
RT_2-F	CTCTACCTGAATGGTGACGG	Real-time PCR primer used in expression study
RT_2-R	CACTGATGGGTCTCTGGAAG	Real-time PCR primer used in expression study
ActinF	CCTTCTTGGGTAGGAGTCTTG	Real-time PCR control
ActinR	AGAGTATTTACGCTCAGGTGGG	Real-time PCR control

E. coli, *Escherichia coli*; F, forward; PCR, polymerase chain reaction; R, reverse; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; UPM, universal primer.

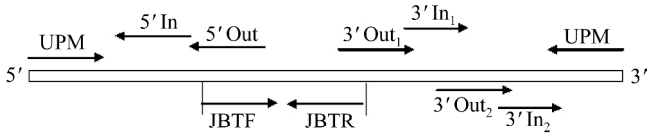

Fig. 1 Polymerase chain reaction strategy for cloning full-length cDNA sequence of grass carp tumor necrosis factor receptor-associated factor 2

The blank box represents the cDNA fragment. The arrows mark the direction in which the primers amplified. JBTF and JBTR, one pair of degenerate primers to obtain the internal region of the gcTRAF2; UPM, universal primer.

the ExPASy Molecular Biology Server (http://expasy.pku.edu.cn). The putative open reading frames were analyzed for the presence of signal peptides using the algorithms in SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). Multiple alignments were generated by the CLUSTAL 1.8 program (http://www.ebi.ac.uk/Tools/clustalw/index.html). Identities between the full length of gcTRAF2 and other TRAF2 proteins were determined using the software at the website http://www.ch.embnet.org/software/BOX_form.html. A phylogenetic tree was constructed based on the deduced amino acid sequences using the neighbor-joining algorithm within PHYLIP version 3.66 (http://www.molecularevolution.org/software/phylip/). Reliability of the tree was assessed by 100 bootstrap repetitions.

RNA extraction and cDNA synthesis for mRNA expression analysis

Three grass carps, weighing approximately 100 g, obtained in January 2006 in Niushan Lake (Wuhan, China), were cultured for approximately 1 week under same conditions. Then the total RNA was extracted using TRIzol reagent from tissues of interest, such as brain, gills, heart, head kidney, trunk kidney, liver, spleen, and thymus from each. After treatment with RNase-free DNase (TaKaRa), 50 μg RNA from different tissues was obtained, and approximately 5 μg RNA was reverse-transcribed with Power-Script Reverse Transcriptase (MBI Fermentas, Vilnius, Lithuanian) according to standard protocol.

The primers that performed best for specific TRAF2 and the housekeeping *β-actin* gene in both reverse transcriptase (RT)-PCR and real-time amplification were designated as RT_2-F, RT_2-R, ActinF, and ActinR, as listed in **Table 1**.

Real-time quantitative RT-PCR

TRAF2 and β-actin cDNA fragments were generated

by RT-PCR, and each amplicon was purified using the DNA Gel Extraction Kit (Promega, Madison, USA) and cloned using pMD18-T vector (TaKaRa) according to standard protocol. Cloned amplicon sequences were confirmed by sequencing. Plasmid DNA was obtained by using the A7100 Wizard Plus Minipreps DNA Purification System (Promega). The cDNA plasmid concentrations were measured at OD_{260} and the corresponding copy numbers were based on the formula that 1 μg of 1000 bp DNA is equivalent to 9.1×10^{11} molecules.

Serial 10-fold dilutions of the resulting plasmid clones, for example, ranging from 10^9 down to 10^4 input cDNA copies, were used as a standard curve in each PCR run. Quantitative real-time PCR was carried out on a Chromo4 Real-Time Detection System (MJ Research, Waltham, USA). Amplifications were carried out at a final volume of 20 μl, containing 1.0 μl DNA sample, 10 μl SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan), 1.0 μl each primer, and 7.0 μl ddH_2O. The reaction carried out without template was used as the blank. PCR amplification was carried out in triplicate wells using the following conditions: 5 min at 94 ºC, followed by 45 cycles consisting of 20 s at 94 ºC, 20 s at 62 ºC, and 30 s at 72 ºC.

Statistical analyses

After the completion of PCR amplification, data were obtained with Opticon Monitor software version 2.03 (MJ Research). The exact copy number of constitutively transcribed mRNA in each organ was derived from each threshold value according to the standard curve.

The data are presented as the fold change in *gcTRAF2* gene expression in tissue normalized to β-actin using that of trunk kidney as a calibrator of 1 (which has the lowest value).

Expression of TRAF2 in *Escherichia coli* and preparation of antibody

The expression fragment located in 6–376 amino acids of TRAF2 was obtained by one pair of primers, $Traf_2$-F and $Traf_2$-R. PCR amplifications were carried out for one cycle of 5 min at 94 ºC, 33 cycles of 30 s at 94 ºC, 30 s at 64 ºC, 60 s at 72 ºC, with a final extension step of 10 min at 72 ºC. Purified fragment was digested with *Kpn*I and *Hin*dIII, ligated into the pET-32a expression vector for construction of recombinant proteins, and transformed into DH5α competent cells. After sequencing the positive clones to ensure in-frame insertion, the pET-32a-gcTRAF2 construct was transformed into *E. coli* BL21(DE3) strain for protein expression.

After sequencing the positive clones to ensure the insertion, the fusion protein was expressed by IPTG induction, and analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) every 2 h until 8 h.

IPTG was added at a final concentration of 1 mM when the culture reached OD_{600}=0.6. After culturing at 37 ºC for 6 h, the cells were harvested by centrifugation, and the recombinant protein was purified by affinity chromatography in a column of Ni^{2+}-charged resin (Novagen, San Diago, USA). Briefly, the bacterial pellet was resuspended in ice-cold 1×Ni-NTA binding buffer, lysed by three consecutive freeze-thaw (–35 ºC-room temperature) cycles, and then sonicated on ice (20 s intervals, 10 min). The bacterial lysate was then centrifuged at 13,000 *g* for 30 min, and the supernatant was added to the Ni-NTA HisBind slurry and mixed gently by shaking for 1 h. After washing the column, the protein was eluted with different concentration imidazole elution buffer according to manufacturer protocols. Purity of the recombinant protein was assessed on 12% SDS-PAGE gel, and the concentration was determined by Bradford method. Recombinant His_6-gcTRAF2 protein was eluted from the resin with 6 M urea and 20 mM Tris-HCl/pH 7.9, containing 200 mM imidazole/0.5 M NaCl. Then the gel-purified protein was used to immunize one rabbit.

The specificity of polyclonal antibody was evaluated by Western blot analysis. The samples from the gel-purified protein were divided into two parts: control and positive. The control was designed to determine polyclonal antibody specificity. In the control, the gcTRAF2 antiserum was replaced by the antiserum pre-adsorbed with pure fusion protein for 16 h at 4 ºC [26]. In brief, the gel-purified protein was subjected to Western blot detection by pre-immune rabbit serum as the negative control and anti-TRAF2 rabbit serum as the positive control.

Western blot analysis

For identifying the protein expression of TRAF2, brain, gill, heart, head kidney, trunk kidney, liver, spleen, and thymus extracts of grass carp were each lysed in electrophoresis sample buffer. Protein concentrations were determined using the Bradford protein assay (Bio-Rad, Hercules, USA). Each sample, equivalent to 10 mg total protein, was run on 10% SDS-PAGE and subsequently transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, USA). The blotting membrane was blocked with 5% dry milk for 1 h, then incubated in TTBS buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween-20) containing 1% milk with antiserum (1:50) from rabbit at 4 ºC overnight. After washing three times for

approximately 30 min, the membrane was further incubated for 1 h with 1:200 diluted alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G. After washing three times for approximately 10 min each in TBST buffer, detection was carried out using BCIP/NBT staining.

Results

cDNA sequence of gcTRAF2

The gcTRAF2 cDNA sequence (GenBank accession No. DQ455748) is 3162 bp in length, including a 60 bp 5′ untranslated region (UTR), a 1611 bp open reading frame, and a 1491 bp 3′ UTR. Two polyadenylation signals (AATAAA) and several mRNA instability motifs (ATTTTA, ATTTA) were found followed by a poly(A) tail in the 3′ UTR (**Fig. 2**). The putative TRAF2 in grass carp is predicted to be 536 amino acids long, with a calculated molecular mass of 60.08 kDa and an isoelectric point of 6.69. Analysis of the putative amino acids sequence by the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html) suggests that gcTRAF2 is not a transmembrane protein. Analysis using the SignalP-NN program (http://

Fig. 2 cDNA sequence of grass carp tumor necrosis factor receptor-associated factor 2 (TRAF2)

The nucleotide sequence (upper) and the deduced amino acid sequence (lower) are numbered (GenBank accession No. DQ455748). The potential N-glycosylation sites are boxed. The putative N-terminal of the TRAF domain is underlined with continuous dot lines; the C-terminal of the TRAF domain is highlighted in gray. The broken underline indicates the predicted zinc finger TRAF-type profile. The RING signature is indicated by wavy lines. The start and stop codons of the open reading frame are in bold. The mRNA instability motif is highlighted in italic. The double underline indicates the position of the polyadenylation signal.

www.cbs.dtu.dk/services/SignalP/) showed that TRAF2 has no signal peptide. The TRAF2 protein obtained was predicted to be unstable with the instability index of 44.63. According to k-NN prediction (http://psort.ims.u-tokyo.ac.jp/form2.html) and previous study [5,6], the protein is probably located in the cytoplasmic region.

Using the Scanprosite programs in the PROSITE database (http://ca.expasy.org/prosite), the putative TRAF2 protein of grass carp contains: two potential N-glycosylation sites at 97–100 and 351–354; 10 protein kinase C phosphorylation sites at 128–130, 157–159, 264–266, 353–355, 364–366, 376–378, 397–399, 418–420, 436–438, and 462–464; and some other modified sites, such as casein kinase II phosphorylation sites (21–24, 128–131, 157–160, 250–253, 315–318, 364–367, and 376–379), five N-myristoylation sites (122–127, 134–139, 305–310, 435–440, and 439–444), and one amidation site (203–206). Importantly, the putative protein has a conserved domain, named TRAF/MATH, at the C-terminus of gcTRAF2 ($gcTRAF2_{386–531}$), together with one zinc finger domain in the middle part ($TRAF2_{188–235}$) (**Fig. 2**), and one zinc finger RING-type signature at the N-terminus ($TRAF2_{45–84}$), consistent with the results by BLAST analysis of the gcTRAF2 protein at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/).

When compared with TRAF2 of other vertebrates, the identity varies from 56% (rat similar to TRAF2, GenBank accession No. XP_001077727) to 97% (goldfish TRAF2, GenBank accession No. CAC82653). Based on the previous reports about the structure analysis of TRAF in mouse and human [25,27], together with the biological information analysis, the TRAF domain (TD) of gcTRAF2 was obtained, with approximately 146 amino acids in the TD-C terminus (TD-C) and 76 amino acids in the TD-N terminus (TD-N) (**Fig. 2**). The identity of the TD, TD-C terminus, and TD-N terminus of gcTRAF2 was compared with those in other vertebrates (**Table 2**). The results showed that the identity of the complete TD domain varies from 72% to 95%; the identities of TD-C of gcTRAF2 with higher vertebrates were almost the same at 80%, whereas the identities of TD-C in fish were all above 90%. The identity for the TD varies too much: it varies from 56% to 63% with higher vertebrates, but is more than 82% compared with fish. Surprisingly, the TD-N of TRAF2 between grass carp and goldfish was up to 100% (**Table 2**). Consistently, multiple sequence alignments of gcTRAF2 and mammalian TRAF2 sequences (**Fig. 3**) also indicate that most of the conserved residues are found at the C-terminal half of the polypeptide within a 150 amino acids TRAF region.

In addition, BLAST analysis of the TRAF domain of gcTRAF2 protein showed it also had high identity with another protein, which was designated as similar to

Table 2 Amino acid sequence identities between grass carp tumor necrosis factor receptor-associated factor 2 (TRAF2) and TRAF2-related proteins from other verterbrates

Animal	Protein	GenBank accession No.	Identity			
			Full length (%)	TRAF domain (%)	TRAF-C (%)	TRAF-N (%)
Zebrafish	Similar to vertebrate TRAF2	CAM15136	92	95	97	92
Rainbow trout	TRAF2	CAD69021	79	90	94	82
Monkey	Similar to TRAF2	XP_001092078	58	73	80	60
Opossum	Similar to TRAF2	XP_001366851	58	72	80	59
Mouse	TRAF2	NP_033448	57	74	80	63
Dog	Similar to TRAF2	XP_537792	57	72	80	57
Chicken	Similar to TRAF2	XP_415560	59	73	80	61
Goldfish	TRAF2	CAC82653	97	94	91	100
Rat	Similar to TRAF2	XP_001077727	56	73	78	63
Human	TRAF2	NP_066961, NP_663770	57	72	80	59
Cow	Similar to TRAF2	XP_874100	58	72	80	56
Chicken	Similar to EBV-induced protein	XM_415406	39	57	66	42
Cow	Similar to EBV-induced protein	XP_613776	43	59	67	43
Opossum	Similar to EBV-induced protein	XP_001369540	43	55	62	42

EBV, Epstein-Barr virus; TRAF-C, C-proximal subdomain; TRAF-N, N-proximal subdomain.

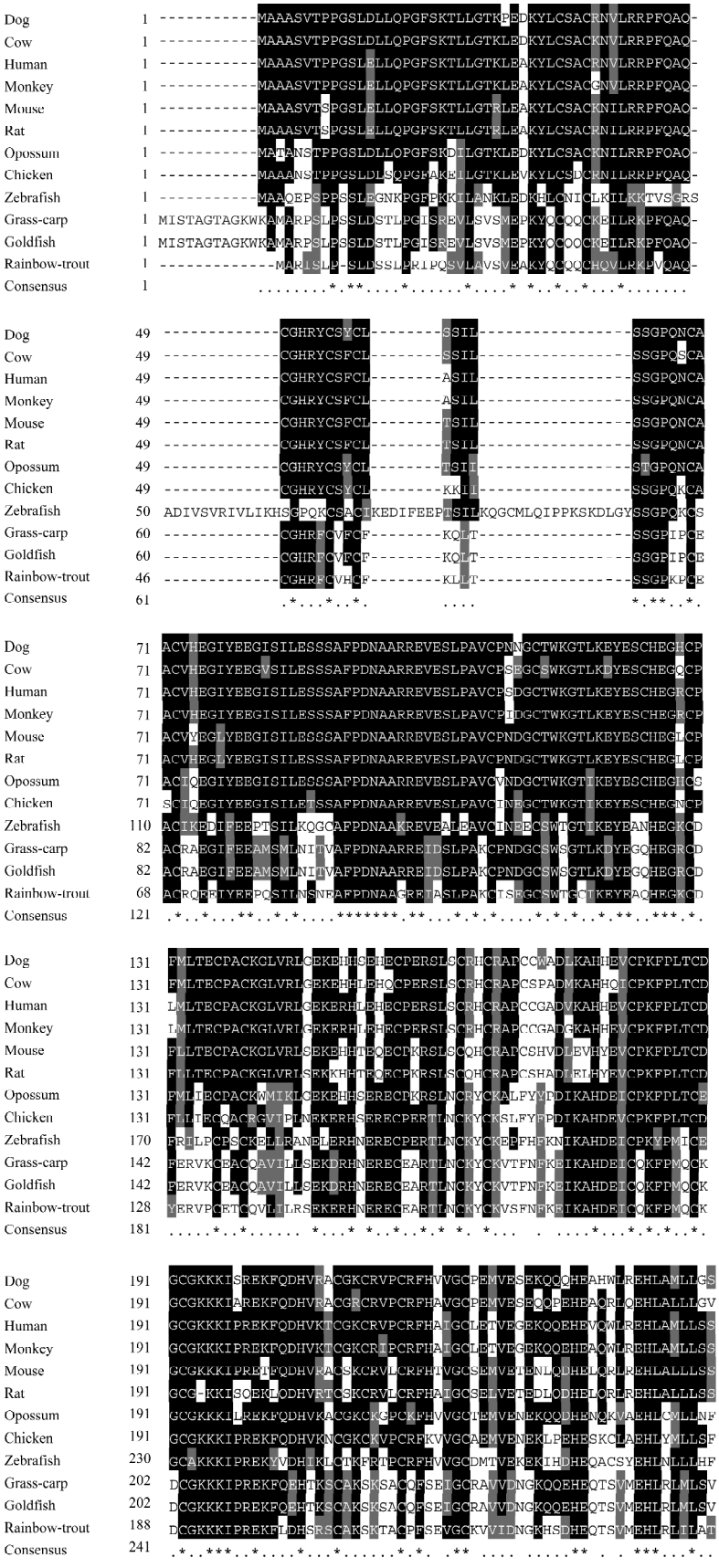

Fig. 3 to be continued

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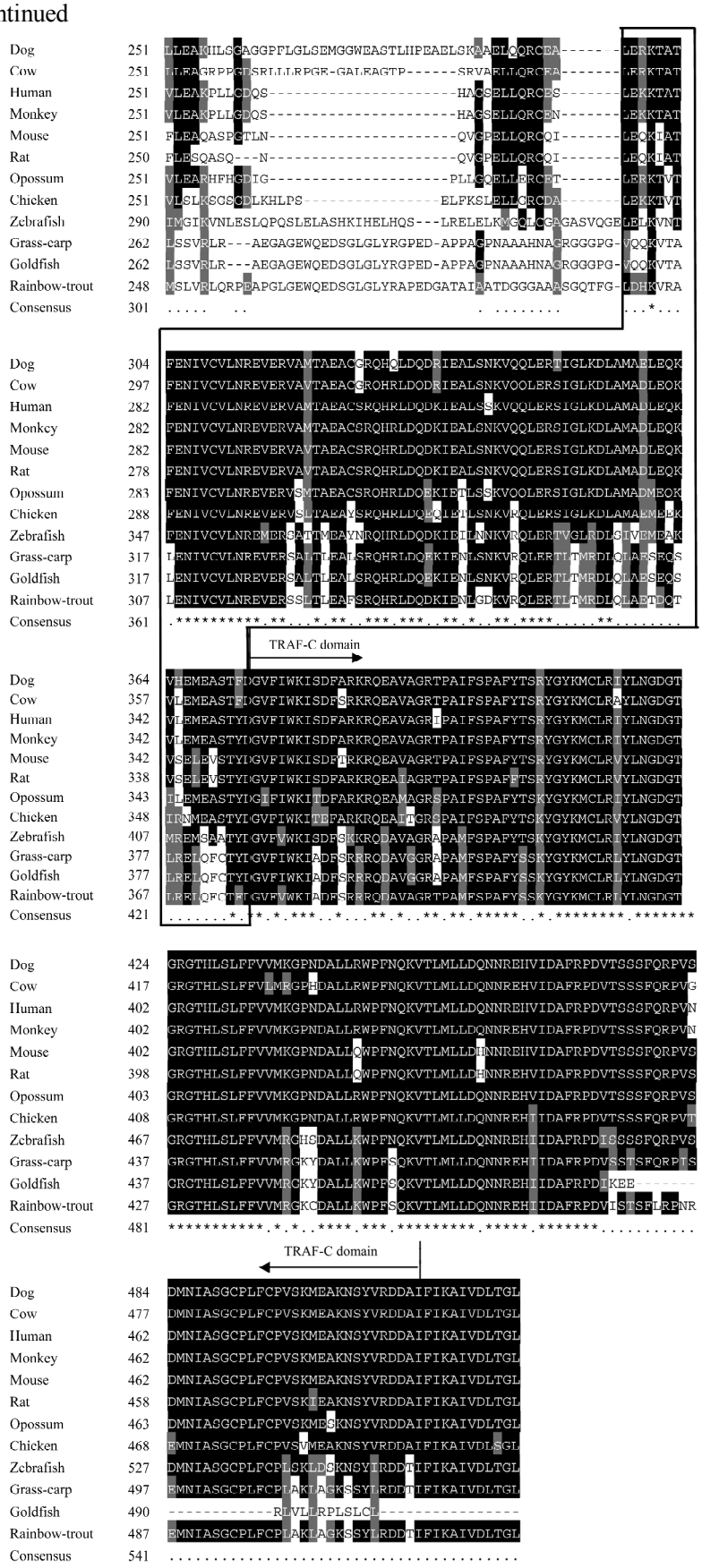

Fig. 3 Alignment of deduced amino acid sequences of human tumor necrosis factor receptor-associated factor 2 (TRAF2; GenBank accession No. NP_066961)

Monkey similar to TRAF2 (XP_001092078); rat similar to TRAF2 (XP_001077727); mouse TRAF2 (NP_033448); dog similar to TRAF2 (XP_537792); grass carp TRAF2 (ABE99696) and zebrafish similar to TRAF2 (XP_683230); cow similar to TRAF2 (XP_874100); chicken similar to TRAF2 (XP_415560); rainbow trout TRAF2 (CAD69021); goldfish TRAF2 (CAC82653); gray short-tailed opossum similar to TRAF2 (XP_001366851). The identical amino acids are indicated with an asterisk (*) and shaded in black, and conservative substitutions with dots (·). The similar amino acids are shaded in gray. The arrow toward indicates the TRAF/MATH domain. The N-terminus of the TRAF domain is boxed. TRAF-C, TRAF C-proximal subdomain.

Epstein-Barr virus (EBV)-induced protein. The identity of the TD of gcTRAF2 with that of EBV-induced protein in chicken (GenBank accession No. XP_415406), cow (XP_613776), and opossum (XP_001369540) was 57%, 59%, and 55%, respectively. The identity of the TD-C between them was 67%, 66%, and 62%, respectively, with the relatively low identity of approximately 42% for TD-N (**Table 2**).

Phylogenetic analysis

In a phylogenetic tree based on amino acid sequences from 13 TRAF2-related sequences from fish and other vertebrates, gcTRAF2 is nearest to the *TRAF2* gene of goldfish (**Fig. 4**).

mRNA expression of gcTRAF2 in different tissues

The mRNA expression of gcTRAF2 in different tissues was examined by real-time PCR. Just as shown in **Fig. 5**, the gcTRAF2 was widely distributed in all the tissues, with the highest level in heart, then in head kidney, brain, gill, thymus, spleen, liver, and lowest in trunk kidney.

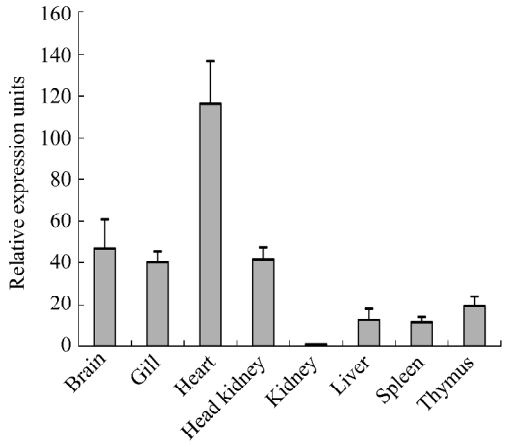

Fig. 5 Expression characterization of grass carp tumor necrosis factor receptor-associated factor 2 in organs, detected by real-time polymerase chain reaction

The total RNA was extracted from grass carp brain, gill, heart, head kidney, trunk kidney, liver, and thymus.

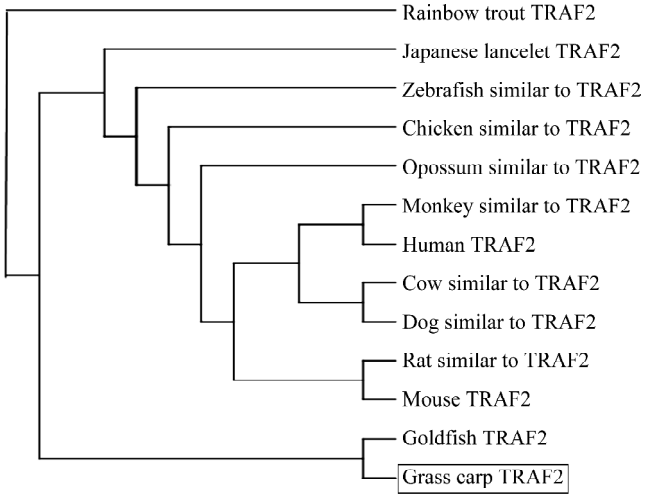

Fig. 4 Phylogenetic tree showing the relationship of tumor necrosis factor receptor-associated factor 2 (TRAF2) from fish and higher vertebrates

Full-length amino acid sequences were aligned using the CLUSTAL 1.8 program within DNASTAR and the phylogenetic tree was constructed based on the deduced amino acid sequences using the neighbor-joining algorithm within PHYLIP version 3.66. Reliability of the tree was assessed by 100 bootstrap repetitions. GenBank accession numbers: human TRAF2 (NP_066961); monkey similar to TRAF2 (XP_001092078); rat similar to TRAF2 (XP_001077727); mouse TRAF2 (NP_033448); dog similar to TRAF2 (XP_537792); grass carp TRAF2 (ABE99696); zebrafish similar to TRAF2 (XP_683230); cow similar to TRAF2 (XP_874100); chicken similar to TRAF2 (XP_415560); rainbow trout TRAF2 (CAD69021); goldfish TRAF2 (CAC82653); gray short-tailed opossum similar to TRAF2 (XP_001366851); and Japanese lancelet TRAF2 (ABN04151).

Expression of the recombinant gcTRAF2 in *E. coli*

The recombinant gcTRAF2s expressed in *E. coli* as inclusion body, observed after Coomassie Brillant Blue R-250 staining by SDS-PAGE. The highest expression level was induced by IPTG after approximately 6 h when cultured at 37 ºC (**Fig. 6**).

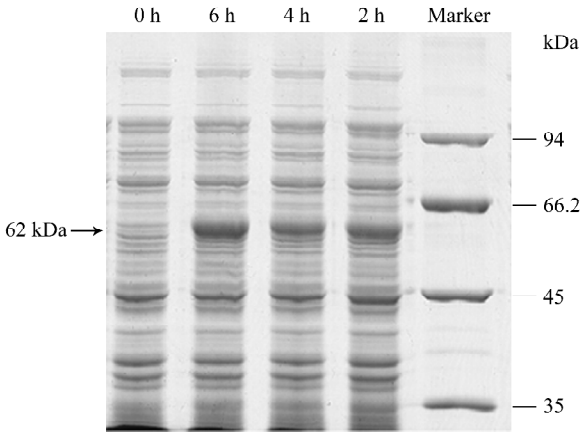

Fig. 6 Expression of the recombinant grass carp tumor necrosis factor receptor-associated factor 2 in *Escherichia coli* at different time points after induction by IPTG

Antibody specificity

The specificity of the expressed protein antibody was confirmed by Western blot analysis. The specific 62 kDa protein polypeptide band was recognized from the gel-purified protein, as shown by **Fig. 7**. However, the 62 kDa protein could barely be detected by the pre-immune rabbit serum (**Fig. 7**). Therefore, we conclude that the gcTRAF2 antibody is specific.

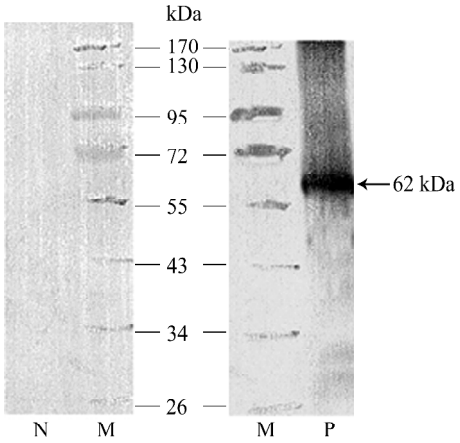

Fig. 7 Specificity confirmation of grass carp tumor necrosis factor receptor-associated factor 2 (TRAF2) antibody by Western blot analysis

The purified recombinant protein was subjected to Western blot detection by pre-immune rabbit serum as the negative control and anti-TRAF2 rabbit serum as the positive control. M, marker; N, negative control; P, positive control.

Western blot detection of tissue expression

Distribution of gcTRAF2 protein in different organs was examined by Western blot analysis. As shown in **Fig. 8**, an immunoreactive band at approximately 59 kDa was detected in heart, thymus, liver, brain, gill, head kidney, and spleen, whereas the gcTRAF2 protein was barely detected in trunk kidney.

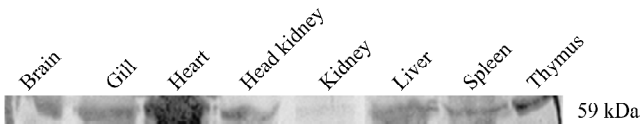

Fig. 8 Expression characterization of grass carp tumor necrosis factor receptor-associated factor 2 (gcTRAF2) in organs, detected Western blot analysis

Extracts from organs were subjected to 10% sodium dodecyl sulfate-polyacrilamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, blotted with the anti-gcTRAF2 antiserum, and detected by BCIP/NBT staining.

Discussion

TRAFs constitute a family of adapter proteins that link a wide variety of cell surface receptors or other signaling molecules, with an important role in regulating cell death, survival, and cellular responses to stress [3,5].

TRAF2 is the classical member of the TRAF family. In the present study, the full-length cDNA of gcTRAF2 was successfully obtained by RT-PCR and RACE. The putative amino acids of gcTRAF2 share identity with that in other vertebrates, ranging from 56% to 97%. Phylogenetic tree analysis revealed the gcTRAF2 is clustered with TRAF2 proteins of fish, being closest to the goldfish TRAF2 protein.

The gcTRAF2 protein contains one conserved TRAF domain, one zinc finger domain and one RING finger domain. In addition, the TD consisted of one TD-C subdomain and one TD-N subdomain. The structure is the main architecture of the TRAF family, as well as the homologs of gcTRAF2 in mammals [6,27]. In comparison with homologs of other vertebrates, the TD-C domain is the most conserved part of the molecule with the high degree ranging from 80% in higher vertebrates to 97% in fish. However, the identity of the TD-N domain varies too much, ranging from 56% in higher vertebrates to 100% in goldfish. This is consistent with the characterization of high conservation of TD-C and more divergence of TD-N for the TRAF family [6,13,21]. To our knowledge, the highly conserved TD-C has been shown to mediate both homodimerization and heterodimerization of TRAF proteins and interactions of the adapter proteins with distinct cell surface receptors that are capable of recruiting TRAFs [9,20,27]. The variable TD-N is predicted to mediate homotrimerisation with a coiled-coil configuration [19]. It is noteworthy that the identity of TD-N for TRAF2 between grass carp and goldfish is up to 100%, which suggests the high variation of TD-N is only for different species and the conservation of TD-N among the same species is very high.

Interestingly, another protein, named EBV-induced protein, was found to be similar to gcTRAF2 with the TRAF domain. This EBV-induced protein was found to exist in chicken, cow, and opossum. The protein also has one TD and one finger motif, just as TRAF2. In addition, the TD can also be divided into two parts, TD-C and TD-N. Instructively, the conservation of the TD-C between

them is very high, varying from 62% to 67%. The conservation suggests that the *gcTRAF2* gene has some relationship with EBV. Several studies showed that EBV latent membrane protein 1 could interact with TRAF2 of human to activate NF-κB directly through its carboxy-terminal activating region 1 [28–31]. The relationship between gcTRAF2 and EBV is the point of interest for further research.

In the present study, real-time quantitative PCR revealed that the mRNA expression of TRAF2 in grass carp displayed a constitutive expression, which is consistent with the wide distribution of TRAF2 in mouse, reported previously [6]. However, gcTRAF2 has the most abundant expression level in heart, whereas the highest expression level was observed in spleen of mouse. The difference might be due to the difference of species.

To further study the tissue distribution of TRAF2, gcTRAF2 was examined in different organs at the protein level by Western blot analysis. Interestingly, gcTRAF2 protein was also detected in all the organs examined. The expression is almost consistent with the mRNA distribution with the exception of head kidney, which is slightly more at the protein level. Perhaps the discrepancy between mRNA and surface protein expression suggests complex post-transcriptional regulatory mechanisms for TRAF2 expression [32]. Taken together, TRAF2 is widely distributed in grass carp, suggesting that gcTRAF2 plays an important role in the physiological process [3].

Interestingly, TRAF2A, the splice variant of TRAF2 in mouse, has been reported to be ubiquitously expressed in mRNA, but its expression relative to TRAF2 varies from very low in brain, lung and heart, to almost equivalent levels in spleen [24]. However, the mRNA expression has only been detected in cells from three different mouse strains, C57BL/6, Balb/c, and B10.BR [24], not in humans or rats, suggesting that TRAF2A is not evolutionarily conserved [25]. No splice variant of TRAF2 was found in grass carp, either.

In the present study, the structure and the expression of TRAF2 protein has been described for the first time in fish. Its main function in fish will be the focus of further research.

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