Short communication

Isolation and characterization of tumor necrosis factor receptor-associated factor 6 (TRAF6) from grouper, Epinephelus tauvina

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\textbf{A B S T R A C T}

Tumor necrosis factor receptor-associated factor 6 (TRAF6) is one of the key adapter molecules in Toll-like receptor signal transduction that triggers downstream cascades involved in innate immunity. In the present study, a TRAF6 (named as Et-TRAF6) was identified from the marine fish grouper, \textit{Epinephelus tauvina} by RACE PCR. The full-length cDNA of Et-TRAF6 comprised 1949 bp with a 1713 bp open reading frame (ORF) that encodes a putative protein of 570 amino acids. Similar to most TRAF6s, Et-TRAF6 includes one N-terminal RING domain (78aa-116aa), two zinc fingers of TRAF-type (159aa-210aa and 212aa-269aa), one coiled-coil region (370aa-394aa), and one conserved C-terminal meprin and TRAF homology (MATH) domain (401aa-526aa). Quantitative real-time PCR analysis revealed that Et-TRAF6 mRNA is expressed in all tested tissues, with the predominant expression in the stomach and intestine. The expression of Et-TRAF6 was up-regulated in the liver after challenge with Lipoteichoic acid (LTA), Peptidoglycan (PGN), Zymosan, polyinosine-polycytidylic acid [Poly(I:C)] and Polydeoxyadenylic acid-Polythymidylic acid sodium salt [Poly(dA:dT)]. The expression of Et-TRAF6 was also up-regulated in the liver after infection with \textit{Vibrio alginolyticus}, Singapore grouper iridovirus (SGIV) and grouper nervous necrosis virus (GNNV). Recombinant Et-TRAF6 (rEt-TRAF6) was expressed in \textit{Escherichia coli} BL21 (DE3) and purified for mouse anti-Et-TRAF6 serum preparation. Intracellular localization revealed that Et-TRAF6 is distributed in both cytoplasm and nucleus, and predominantly in the cytoplasm. These results together indicated that Et-TRAF6 might be involved in immune responses toward bacterial and virus challenges.

\section*{1. Introduction}

As pattern-recognition receptors (PRRs), toll-like receptors (TLRs) play a crucial role in the innate immune system by recognizing conserved pathogen-associated molecular patterns (PAMPs) and triggering the signaling pathways [1]. After the PAMPs recognition, TLRs can recruit adapter molecules Myeloid differentiation factor 88 (MyD88) and Tumor necrosis factor receptor-associated factor 6 (TRAF6) for signal transduction to activate nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) [2]. The most likely scenario is that once recruited, MyD88 interacts with IL-1 receptor-associated kinase-4 (IRAK-4) via their respective death domains. IRAK-4 then recruits IRAK-1 to the complex, leading to its phosphorylation and activation [3]. IRAK-1 and IRAK-4 then dissociate from the complex and interact with TRAF6 in mammals [4]. TRAF6 counterparts were also identified in other vertebrates and invertebrates, such as fish [5-8], scallop [9-11], shrimp [12] and sea cucumber [13]. Their connection with pathogen challenges (bacteria or virus) have been also investigated, suggesting the existence of a Toll-signaling pathway mediated innate immunity in each species.

Groupers, \textit{Epinephelus} sp., are widely cultured in China and Southeast Asian countries. The emergence of bacterial and viral pathogens, including \textit{Vibrio alginolyticus}, iridovirus and nervous necrosis virus, caused heavy economic losses in grouper aquaculture [14-16]. For example, the gram-negative bacterium,
2.3. Challenge experiments

Cell lines of grouper spleen (GS) and fathead minnow (FHM) were propagated by the recommended methods with Leibovitz’s L15 and M199 culture medium with 10% fetal calf serum at 28 °C. The cells were treated with PBS as external control. For each group divided with one of six groups of TLRs ligands, including Lipopolysaccharide (LPS) (50 μg/ml), Lipoteichoic acid (LTA) (5 μg/ml), Zymosan (100 μg/ml), Polyinosine-polycytidylic acid [Poly(I:C)] (1 μg/ml) and Polydeoxyadenylic acid Polythymidylic acid sodium salt [Poly(dA:dT)] (1 μg/ml) from Escherichia coli or challenge experiments. The virus challenge sample was injected subcutaneously (s.c.), intraperitoneal injection (i.p.) with a live bacterial suspension of V. alginolyticus (5 × 10^8 CFU/ml), which belonged to family Enterobacteriaceae [14,17]. V. alginolyticus also caused mass mortality in grouper fry in many countries [15].

2.4. RNA isolation and cDNA synthesis

2.5. Cloning and sequence analysis of E. tauvina TRAF6 (Et-TRAF6)

2.5.1. Degenerate primers design and initial PCR cloning

Multiple-sequence alignment of TRAF6 nucleotide sequences from a variety of species were performed with the Clustal X multiple-alignment software. Degenerate primers were designed based on the conserved nucleotides of three fish TRAF6 sequences reported before, including Donio rerio (accession no. AAT37634.1), Cyprinus carpio (accession no. AD566512.1), and Tetrodon nigroviridis (accession no. CA999941.2). Two fragments of Et-TRAF6 gene were amplified from grouper liver cDNA by two pairs of degenerate primers DF1 (5’-CAAGCCTATGATGTAGAGTTTGACCCTCCA-3’), DF2 (5’-ATCTGCCGTTTCGTGCACATGCACG-3’), DR1 (5’-TCGTITGGCAAAGGTATCAGGGGAAGTTG-3’) and DR2 (5’-CACA-TACCCGAATCTCCTGGGTTGCG-3’) using the LaTaq polymerase (Takara, Japan). PCR was performed with an initial denaturation step of 5 min at 94 °C, and then 35 cycles were run as follows: 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s.

2.5.2. Rapid amplification of 5’ and 3’ cDNA ends (RACE) of Et-TRAF6 cDNA

Based on the first partial sequences amplified by the degenerate primers DF1 and DR2, the 5’ and 3’ ends of Et-TRAF6 cDNA were obtained using the RACE approaches. The first-strand cDNA was synthesized from the total liver RNA with the SMART™ RACE cDNA amplification kit (Clontech) for 3’ RACE and 5’ RACE. Two primers F1 (5’-ACCATCGCGGCGGGGCGATC-3’) and R1 (5’-GAGACTCTGGATCGGAATCC-3’) were designed based on the first partial sequence PCR. PCR was performed with 10 μM F1 or R1 and 500 nM of Nested Universal Primer A (NUP, Clontech). Denaturation was performed at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s.

2.6. TA cloning, sequencing and database analysis

PCR products were analyzed on 1% agarose gels, extracted with an Axyprep DNA gel extraction kit (AxysGEN), and then ligated into pMD18-T vectors (TaKaRa) and transformed into competent Escherichia coli DH5 cells. Positive colonies were screened by PCR and at least two recombinant plasmids were sequenced.

Sequences were analyzed based on nucleotide and protein databases using the BLASTN and BLASTX program (http://www.ncbi.nlm.nih.gov/BLAST/). The protein and its topology prediction were performed using software at the ExPaSy Molecular Biology Server (http://expasy.boku.edu.cn). Multiple sequence alignment of the Et-TRAF6 was performed with the Clustal X multiple-alignment
software. MEGA 4.0 was also used to produce the phylogenetic tree. Neighbor-joining (NJ) method was used in MEGA 4.0 was used for phylogenetic analysis. The robustness of bifurcations was estimated with bootstrap analysis and bootstrap percentages were obtained with 1000 replicates.

2.7. Analysis of Et-TRAF6 mRNA expression profiles

qRT-PCR was employed to detect the Et-TRAF6 expression profiles using β-actin as a reference gene. The qRT-PCR primers, F3 (5'-CTTACGCCCTCGCTTGCCT-3')/R3 (5'-ACAGCCGGACATTGAGGATGAT-3'), and actin-F (5'-TACAGCTGCTCTGACGCA-3')/actin-R (5'-GGCTGTGATCTTCTTGTGCA-3'), were designed based on the full-length cDNA of Et-TRAF6 and β-actin. qRT-PCR was performed on Roche LightCycler 480 Real-time PCR system (Roche, Switzerland) using the 2× SYBR Green Real-time PCR Mix (TOYOBO, Japan). PCR amplification was performed in triplicate wells, using the cycling parameters: 94 °C for 5 min, followed by 40 cycles of 5 s at 94 °C, 10 s at 60 °C and 15 s at 72 °C. Relative gene expression was analyzed by the comparative Ct method (2^-ΔΔCt method). Target Ct values were normalized to the endogenous gene β-actin. Results for each treated sample were expressed as N-fold changes in target gene expression relative to the same gene target in the calibrator sample, both normalized to the β-actin gene. All samples were analyzed in three duplications and all data were given in term of relative mRNA expression level as means ± SD, and then subjected to Student's t-test. Differences were considered significant at p < 0.05 or p < 0.01.

2.8. Expression and purification of recombinant Et-TRAF6 and preparation of antiserum

Et-TRAF6 specific primers F4 (5'-CGCCGGATCC-GATGGCTGCTTGCAGTAAATAA-3') and R4 (5'-GGCTGCCTGACGTCCTGAAAACACTCAGGCTC-3') were used to amplify the mature sequence encoding the mature peptide of Et-TRAF6. The target PCR product was digested with BamH I and Sal I (Takara), and then subcloned into the BamH I/Sal I sites of expression vector pET-21b. The recombinant plasmid (pET-Et-TRAF6) was transformed into E. coli BL21 (DE3) and subjected to nucleotide sequencing. Positive clone was incubated in 200 ml LB medium (containing 100 mg/ml ampicillin) at 37 °C with shaking at 220 rpm. The parent vector without an insert fragment was used as negative control. When the culture medium reached OD_600 of 0.5–0.7, the cells were incubated for 4 additional hours with the induction of IPTG at the final concentration of 0.5 mmol/l. The recombinant Et-TRAF6 protein (designated as rEt-TRAF6) was purified by affinity chromatography with Ni-nitrilotriacetic acid-agarose (Qiagen, Germany) according to the manufacturer’s instruction. The resulting protein was analyzed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Coomassie brilliant blue R-250. The concentration of recombinant fusion protein was determined using Bradford method. The polyclonal antibody against rEt-TRAF6 produced by immunizing BALB/c mice according to the conventional method [25]. The specificity of the antisera was detected with western blot.

2.9. Intracellular localization

Intracellular localization of Et-TRAF6 was performed by EGFP fusion protein expression and immunofluorescence. Et-TRAF6 specific primers F5 (5'-GCCGATCCCTTCTGCTTGCAGTAAATAA-3') and R5 (5'-GCGCTCCCTCGCTTGAACCTACGCGCCTCGT-3') were used to amplify the DNA fragment encoding the mature peptide of Et-TRAF6. The target PCR product was digested with EcoR I and BamH I (Takara), and then subcloned into the EcoR I/BamH I sites of expression vector pEGFP-N3. The recombinant plasmid (pEGFP-Et-TRAF6) was transformed into E. coli DH5α and subjected to nucleotide sequencing. After transfection with pEGFP-Et-TRAF6 or pEGFP-N3 for 48 h, cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min, and then stained with 6-diamidino-2-phenylindole (DAPI) for 15 min. Finally, cells were mounted with 50% glycerol, and observed under fluorescence microscopy (Leica, Germany). For immunofluorescence localization, GS cells were seeded onto coverslips (10 mm × 10 mm) in a 6-well plate. After allowing the cells to adhere for 24 h, the cells were fixed with 4% paraformaldehyde and then the coverslips were blocked using 2% bovine serum albumin (BSA) at room temperature for 30 min. Cells were incubated either with anti-Et-TRAF6 serum (1:100) or preimmune mice serum (1:100) for 1 h, rinsed with PBS three times for 10 min and then incubated with FITC-conjugated goat anti-mouse antibodies (Pierce, USA) for a further hour. Finally, cells were stained with 6-diamidino-2-phenylindole (DAPI) (1 μg/ml) and observed under fluorescence microscopy (Leica, Germany).

3. Results

3.1. Sequencing analysis of grouper TRAF6 cDNA

The partial cDNA sequences amplified by the degenerated primers of DF1/DR1, DF2/DR2, and DF1/DR2 from E. tauvina liver cDNA were 216 bp, 195 bp, and 1322 bp in size, respectively. Sequencing and Blastx analysis of the 1322 bp fragment showed that it shares high similarity to other reported TRAF6 genes. Based on the partial cDNA sequences of TRAF6, the Et-TRAF6-specific primers of F1 and R1 were designed for the rapid amplification of 3′ and 5′ cDNA (RACE), and the full-length cDNA of Et-TRAF6 was amplified. The full-length cDNA of Et-TRAF6 was 1949 bp (Genbank accession no. JK000381) and contained a 1713 bp open reading frame (ORF) coding for a protein of 570 amino acid (aa), and had a 60 bp of 5′-UTR and a 176 bp of 3′-UTR including a putative polyadenylation consensus signal (AATAAA) and a poly (A) tail (Fig. 1A). Similar to most TRAF6s, Et-TRAF6 includes one N-terminal RING domain (78aa–116aa), two zinc fingers of TRAF-type (159aa–210aa and 212aa–269aa), one coiled-coil region (370aa–394aa), and one conserved C-terminal meprin and TRAF homology (MATH) domain (401aa–526aa) (Fig. 1B).

The deduced amino acid sequence of Et-TRAF6 protein was remarkably conserved. It shared significant homology with TRAF6s from other fish species (Table 1). Multiple sequence alignments were carried out using the Clustal X multiple-alignment software. The Neighbor joining tree of TRAF6 amino acid sequences showed that the greasy grouper TRAF6 was clustered with orange-spotted grouper TRAF6, stickleback TRAF6 and fugu TRAF6. The grouping of TRAF6 proteins was well-supported by bootstrapping and in accordance with the assumed evolutionary trend of the species (Fig. 2).

3.2. Expression profiles of Et-TRAF6

To investigate the tissue expression profiles of Et-TRAF6 in healthy E. tauvina, qRT-PCR was used to analyze its expression levels in various tissues. The Et-TRAF6 expression was detected in all tested tissues, with the predominantly expression in the stomach and intestine (Fig. 3A).

Liver is a unique immune organ with many resident innate and adaptive immune cells, which induce various complex and sophisticated immune responses. The liver in a fish is considered to be one important organ that has a different number of functions for...
Fig. 1. Sequences and domain topology of Et-TRAF6. (A) The nucleotide (upper row) and deduced amino acid (lower row) sequences of the ORF and UTR regions are shown and numbered on the left. The RING domain (amino acids 78–116) and two TRAF domains (amino acids 159–210 and 212–269) were marked by the single and double underline. The coiled-coil region (amino acids 370–394) was boxed, and the MATH domain (amino acids 401–526) was shaded. The bold letters indicated the polyadenylation signal sequence (AATAAA). (B) Schematic representation of the domain topology of Et-TRAF6.
Table 1
Comparisons of the deduced amino acid sequence of Et-TRAF6 with TRAF6s from other known fish species.

<table>
<thead>
<tr>
<th>Fish species</th>
<th>Accession number</th>
<th>Identity (%)</th>
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<td>Epinephelus coioides</td>
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<td>Oreochromis niloticus</td>
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<td>Takifugu rubripes TRAF6</td>
<td>XP_003969671.1</td>
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<td>Tetraodon nigroviridis</td>
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<td>Cyprinus carpio</td>
<td>ADI56561.2</td>
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<tr>
<td>Danio rerio</td>
<td>AAT37634.1</td>
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</tbody>
</table>

The recombinant Et-TRAF6 protein (designated as rEt-TRAF6) was purified by affinity chromatography with Ni-nitrilotriacetic acid agarose (Qiagen, Germany) according to the manufacturer’s instruction (Fig. S1 lane 3).

To identify the biological properties of Et-TRAF6, the recombinant protein was used to immunize mouse to prepare immune serum. The specificity of anti-TRAF6 serum was examined by western blot. The rEt-TRAF6 protein (5 μg) was specifically recognized by polyclonal antibody against Et-TRAF6 (Fig. S1 lane 4), while no bands were found detected by negative control serum (Fig. S1 lane 5), indicating that the anti-TRAF6 antibody specifically recognized the purified Et-TRAF6 protein.

3.4. Intracellular localization of Et-TRAF6

Intracellular localization of Et-TRAF6 was determined by pEGFP-Et-TRAF6 fusion protein expression and immunofluorescence assay with anti-Et-TRAF6 serum. Firstly, a recombinant plasmid, pEGFP-Et-TRAF6, was constructed and transfected into FHM cells. The green fluorescence signal in pEGFP-Et-TRAF6 transfected cells was distributed in both the cytoplasm and nucleus, and predominantly in the cytoplasm, whereas the signal was detected in both the cytoplasm and nucleus in the control cells (Fig. 4A).

Secondly, the intracellular localization of Et-TRAF6 in GS cells was determined by immunofluorescence assay. In GS cells, the FITC green fluorescence was distributed in both the cytoplasm and nucleus, and predominantly in the cytoplasm, while no green fluorescence signal was detected in samples detected by the preimmune mice serum (Fig. 4B).

4. Discussion

TRAF6 is one of the crucial factors in the TLR signaling pathway, the ubiquitinated TRAF6 activates TAK1, which subsequently phosphorylates IKK and MAPK, leading to NF-kβ and AP-1 activation, respectively [26,27]. Previous studies have shown that TRAF6 contains four conserved domains, including a RING domain, a series of zinc fingers, a coiled-coil region and a MATH domain [5,7,28]. In mammals, the N-terminal RING domain and zinc fingers coordinate TRAF6 auto-ubiquitination and its interaction with Ubc13 [29,30]. The coiled-coil region is essential for auto-ubiquitination and activation of the NF-kβ signaling pathway [31]. Furthermore, the conserved MATH domain links TRAF6 to upstream molecules [32]. Similar to the TRAF6s of mammals and other species, Et-TRAF6 also contains the classical domains, including one RING domain, two zinc fingers, one coiled-coil region, and one MATH domain. The neighbor joining tree showed that the evolutionary trend of TRAF6 was in accordance with that of the species. High similarity of gene sequence and structural domains suggests that Et-TRAF6 may have a function similar to that of other mammalian and teleost TRAF6s.

In E. tauvina, Et-TRAF6 was expressed in all examined tissues, indicating an ubiquitous and constitutive expression of Et-TRAF6, which is similar to expression pattern of teleost fish TRAF6 genes. TRAF6 has been reported in other teleost fish, such as D. rerio, C. carpio, Cirrhinus mirgala and Ctenopharyngodon idella. TRAF6 was found to be predominantly expressed in the gill of D. rerio [7], liver of C. carpio [5], kidney of C. mirgala [33], and in the main immune-related tissues (head kidney, posterior kidney, and spleen) of C. idella [8]. In our study, Et-TRAF6 expression was high in the main immune-related tissues (stomach, intestine, brain, and spleen) and low in the liver, skin, and muscle. The discrepancies among species in expression patterns might be a result of species variation and differences in developmental stage. Once pathogen invading, the host could trigger the immune response through TRAF6 via the TLR signaling pathway. In mammals, TRAF6 has been shown to...
participate in activation of the TLR signaling pathways in response to some bacterial and viruses. For example, grass carp TRAF6 (CiTRAF6) was significantly up-regulated during Ichthyophthirius multifiliis infection. In zebrafish embryos and adults, both SHRV and Edwardsiella tarda induced zfTRAF6 upregulation. Orange-spotted grouper TRAF6 (EcTRAF6) increased after infection with Cryptocaryon irritans. We detected that the expression Et-TRAF6 can be induced by bacterial and virus infection. In the liver of grouper, Et-TRAF6 was significantly up-regulated not only after challenging with PGN, Zymosan, Poly(I:C) and Poly(dA:dT), but also after V. alginolyticus, SGIV and GNNV infections. Taken together, our work will help to elucidate the functions of TRAF6 for better understanding of grouper innate immunity and ultimately development of disease control strategies.

Localizations of the components in TLR signaling pathways are very important for our understanding of the protein function and molecular organization [35]. In this study, Et-TRAF6 was located in the cytoplasm and nuclear in GS cell, which is similar with Litopenaeus vannamei TRAF6 (LvTRAF6) in S2 cells. The cytoplasmic localization of TRAF6 is consistent with its putative function in

![Graphs](image-url)

**Fig. 3.** Expression of Et-TRAF6 mRNA in healthy and immune-challenged groupers. (A) Tissue distribution of Et-TRAF6 in healthy groupers by qRT-PCR analysis. The expression of Et-TRAF6 in muscle was set to 1.0. (B) Quantification of mRNA expression upon TLRs ligand induction. Six groups of TLRs ligands LPS (10 μg/ml), LTA (5 μg/ml), PGN (20 μg/ml), Zymosan (100 μg/ml), Poly(I:C) (1 μg/ml) and Poly(dA:dT) (1 μg/ml) were i.p. injected into adult groupers at six time points (3, 6, 12, 24, 48 and 72 h). Total RNA was extracted by Trizol and reverse-transcribed into cDNA. qRT-PCR was conducted using the primers. Graphs of mRNA induction fold were presented. (C) Temporal expression of Et-TRAF6 in liver after V. alginolyticus, SGIV and GNNV challenge. Expression values were normalized to those of β-actin, using relative standard curve method. qRT-PCR was carried out three replicates per sample. Data are expressed as the mean fold change (means ± S.E., n = 3) from the PBS challenged group. Statistical significance was calculated by Student’s t-test. Bars with “a” at p < 0.05 or “b” at p < 0.01 indicate statistical differences.
Fig. 4. Intracellular localization of Et-TRAF6. (A) Subcellular localization of Et-TRAF6 by fluorescence microscopy. FHM cells were transfected with pEGFP-N3 (upper row) or pEGFP-Et-TRAF6 (lower row). The location of the nucleus was shown by DAPI staining. (B) Expression of Et-TRAF6 in GS cells. After allowing the cells to adhere for 48 h in 6-well plates, indirect immunofluorescence staining of Et-TRAF6 was performed using mice anti-Et-TRAF6 serum and Goat anti-mouse FITC secondary antibody (lower row). Preimmune mouse serum was used as control (upper row). Blue images show the location of the nucleus, stained by DAPI. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
forming a receptor complex when the TLR signaling pathway is activated by PAMPs [36–38]. The nuclear localization of TRAF6 has been reported in human [39,40]. It has been found that TRAF6 negatively regulated c-Myb in the nuclei of B-lymphocytes that maintained cell homeostasis and immune surveillance [40].

In conclusion, the full-length cDNA encoding TRAF6 was isolated from goby group, E. taurota. It was expressed in all tested tissues, and the expression in the liver was not only up-regulated after challenge with viral/bacterial agents, but also after infections with bacteria and virus. These results indicated that ET-TRAF6 might be involved in immune response toward bacterial and virus challenge.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2014.04.022.

References


