Fish & Shellfish Immunology 35 (2013) 1200–1208

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Molecular cloning, subcelluar location and expression profile of signal transducer and activator of transcription 2 (STAT2) from turbot, *Scophthalmus maximus*

Na Wang [a,1], Xian-Li Wang [b,1], Chang-Geng Yang [c], Song-Lin Chen [a,*]

[a] Yellow Sea Fisheries Research Institute, Chinese Academy of Fisheries Sciences, 106 Nanjing Road, Qingdao 266071, China
[b] Sarite Center for Stem Cell Engineering Translational Medicine, East Hospital, Stem Cell Research Center, Tongji University School of Medicine, Shanghai 200120, China
[c] Key Laboratory of Freshwater Biodiversity Conservation and Utilization of Ministry of Agriculture, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan 430223, China

ARTICLE INFO

Article history:
Received 5 February 2013
Received in revised form
17 July 2013
Accepted 17 July 2013
Available online 6 August 2013

Keywords:
Turbot (*Scophthalmus maximus*)
STAT2
Nuclear export signal (NES)
Vibrio anguillarum
LCDV

ABSTRACT

Signal transducer and activator of transcription 2 (STAT2) is an important molecule involved in the type I interferon signalling pathway. To date, little STAT2 homologue is available in fish except Atlantic salmon and goldfish. In this paper, STAT2 was firstly cloned and characterized from turbot, a marine flatfish with high economic value. Briefly, turbot STAT2 cDNA is 3206 bp in length encoding a predicted protein of 793 amino acids. The phylogenetic tree shows that turbot STAT2 protein shared the closest relationship with Atlantic salmon. Analysis of subcellular distribution indicates that STAT2 is mainly present in the cytoplasm of TK cells. *Stat2* mRNA is constitutively expressed in widespread tissues and induced by several folds in turbot tissues and TK cells after stimulation with *Vibrio anguillarum* and lymphocystis disease virus (LCDV). Unlike the higher vertebrate STAT2, turbot STAT2 nuclear export signal (NES) exists not in the C-terminal 79 amino acids but in N-terminal 137–312 amino acids (STAT_alpha domain). The nuclear translocation of turbot STAT2 after Poly(I:C) treatment proved its transcription activity in TK cells. All these results suggested that STAT2 may be involved in the immune response in turbot as a transcription factor.

1. Introduction

The Janus kinase (JAK) and signal transducers and activators of transcription (STAT) signalling pathway play critical roles in the regulation of cell proliferation, growth, haematopoiesis, and immune response [1,2]. So far, seven STAT family members including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6 and four JAK family members including JAK1–4 have been identified in mammals [3]. The binding of extracellular cytokines and growth factors to their receptors firstly triggered JAKs' activation, followed by tyrosine phosphorylation of STATs. Phosphorylated STATs form dimers which enter the nucleus to activate the transcription of their target genes [3–6].

As an exceptional member of the STAT family, STAT2 does not form homo-dimer like other STATs. Instead, in response to interferon (IFN) α and β, STAT2 forms heterodimer with STAT1 by tyrosine-phosphorylation [7–9]. The STAT2–STAT1 heterodimer subsequently translocates into the nucleus and combines IFN regulatory factor 9 (IRF-9) to form IFN-stimulated gene factor 3 (ISGF3) which activates IFN-stimulated response element driven genes [7–11]. The STAT2 knockout mice exhibit susceptible to viral infection due to their impaired ability to respond to IFN α/β signalling, likely with the phenotype of STAT1 knockout mice [12–14].

Besides the association with STAT1 and IRF-9, STAT2 has also been shown to interact with Interferon-alpha/beta receptor alpha chain (IFNAR1) [15], Interferon-alpha/beta receptor beta chain (IFNAR2) [16], mediator of RNA polymerase II transcription subunit 14 (MED14) [17], and regulator of calcineurin 1 (RCAN1) [18]. Recent studies have shown that STAT2 plays a pivotal role in blood development, immune response, and myogenic differentiation [19,20]. In addition, in response to the evolutionary struggle between host and pathogen, many viruses such as paramyxovirus, measles virus, lymphocytic choriomeningitis virus, and herpes simplex virus have developed a strategy to bypass the IFN antiviral

* Corresponding author. Tel.: +86 532 85844606; fax: +86 532 85811514.
E-mail address: chensl@ysfri.ac.cn (S.-L. Chen).
[1] These authors contributed equally to the paper.

1050-4648/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsi.2013.07.033

system by interacting with STAT2 [21–25]. The understanding of STAT2 inhibition mechanism is helpful for the design of STAT2-directed therapeutics for treatment of diseases.

So far, little study about STAT2 in fish is available except Atlantic salmon [26] and goldfish [27]. In turbot, an important economic marine fish, lymphocystis disease virus (LCDV) and *Vibrio anguillarum* represent two of the major pathogens causing significantly economic losses in China. In order to explore the host–pathogen interaction mechanism, several EST libraries have been constructed in turbot and many immune related EST sequences such as IRF-3, IRF-7, IRF-10 and JAK1 have been identified [28–32].

In this study, a STAT2 homologue was cloned and characterized from turbot. Followed by the phylogenetic tree construction and subcellular location analysis of STAT2, its involvements in immune response of several tissues and TK cell line to *V. anguillarum* and LCDV, were also investigated.

2. Materials and methods

2.1. Fish, cell line and total RNA isolation

Turbots weighing about 100 g were obtained from Haiyang Aquatic Company of Yantai and every six turbots were raised in a 72 l breeding tank with running seawater at 16 °C. The TK cell line, derived from turbot kidney [33], was cultured in Eagle's minimal essential medium (MEM) (Gibco) supplemented with 10% foetal bovine serum (FBS) (Gibco) at 24 °C.

Eleven tissues including brain, gill, skin, muscle, fin, heart, liver, spleen, kidney, head kidney and intestine were collected from three individuals, separately. And total RNAs of tissues were isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions.

2.2. Turbot STAT2 cloning

Based on the 987-bp partial sequence of turbot STAT2 cDNA (DQ848884) identified from turbot spleen cDNA library [30], primers Stat2_51/52, Stat2_31/32 were designed for the 5′ and 3′ RACE followed as the protocol of the BD SMART™ RACE cDNA amplification kit (BD Biosciences Clontech).

In brief, the first strand cDNA synthesis for 5′RACE/3′RACE was performed on liver-derived RNA with primers OligodG, 5′-CDS and 3′-CDS, respectively. To obtain the 5′ fragment, primers Stat2_51/UPM and Stat2_52/NUP were used for the primary PCR and the nested PCR respectively. Similarly, the 3′ fragment of STAT2 was obtained by nested PCR with primers Stat2_31/UPM and Stat2_32/NUP. These two fragments were ligated to pMD-18T vector and sequenced by ABI 3730 DNA Analyzer.

Finally, these three fragments were joined into the full-length STAT2 cDNA, which was further confirmed by sequencing the PCR product amplified with primers Stat2_1 and Stat2_2 within the 5′ and 3′ UTR, respectively.

2.3. Sequence analysis of turbot STAT2

Turbot STAT2 amino acids sequence deduced by Vector NTI 11.5 software was submitted into BLAST program (http://blast.www.ncbi.nlm.nih.gov) in search of its counterpart sequences. Multiple sequences alignment of STATs proteins including STAT1, STAT2, STAT3, STAT4, STAT5 and STAT6 was carried out with ClustalX program. Subsequently, an unrooted phylogenetic tree was constructed with MEGA 5.05, based on proteins alignment. The phylogenetic tree was tested for reliability by 1000 bootstrap replications.

2.4. The subcellular location of STAT2 in TK cell line

The TK cell line was used to analyze the subcellular location of turbot STAT2. Firstly, primers GFPS2-U1, GFPS2-D1, GFPS2-DN1, GFPS2-UN2, GFPS2-U2, GFPS2-D2, GFPS2-U3 and GFPS2-D3 were designed to amplify the corresponding cDNA of the full-length CDS region (1–793 amino acids), N terminal region (1–312 amino acids), N1 terminal region (1–136 amino acids), N2 terminal region (137–312 amino acids), C terminal region (313–793 amino acids), predicted NES (nuclear export signal) region (715–793 amino acids) and C-NES region (313–714 amino acids) of STAT2. Secondly, these seven fragments were ligated into the vector pEGFP-N3 (BD Biosciences Clontech) based on HindIII/EcoRI sites to construct seven GFP vectors pSTAT2-GFP$^{1-793}$, pSTAT2-N-GFP$^{1-312}$, pSTAT2-N1-GFP$^{1-136}$, pSTAT2-N2-GFP$^{137-312}$, pSTAT2-C-GFP$^{313-793}$, pSTAT2-NES-GFP$^{715-793}$ and pSTAT2-C-NES-GFP$^{313-714}$. Finally, seven vectors were transfected into 12-well plates with Lipofetamine 2000 (3 μl:1 μl) follow as the protocol. For the identification of NES in turbot STAT2, Leptomycin B (LMB, the Crm1-dependent NES inhibitor) is introduced at 6 h after transfection with a final concentration of 10 ng/ml as the previous study [34]. At 36 h after transfection, the transfected cells were rinsed with PBS for 5 min, fixed with 4% paraformaldehyde for 15 min, rinsed again with PBS for 5 min, treated with 0.1% Triton X-100 for 5 min, and stained with DAPI (100 ng/ml) for 10 min.

In order to prove the transcription activity of turbot STAT2, Poly(I:C) was introduced with a final concentration of 10 ng/ml at 6 h after transfection with pSTAT2-GFP$^{1-793}$. At 24 h after Poly(I:C) stimulation, the cells were treated as above method for DAPI staining.

All treated samples were observed and captured on a Nikon ECLIPSE TE 2000-U fluorescence microscope.

2.5. Pathogen challenge in turbot tissues and TK cells

The bacterium *V. anguillarum*, which has been shown to be pathogenic in turbot [35], was cultured at 28 °C to mid-logarithmic growth on 2216E medium and a final concentration of 7×10^6 cfu (colony forming units) suspended in 0.9% saline (PS) per fish was intraperitoneally injected in twenty turbots. And the turbots injected with PS were used as the control. At 6 h, 12 h, 24 h, 48 h, 72 h and 96 h post injection, three individuals from each point were anaesthetized and tissues including liver, spleen, kidney and head kidney were collected.

LCDV was used for virus challenge in present study. The isolation, culture and titration determinations were performed as previous described [36]. 1×10^7 $TCID_{50}$ (50% tissue culture infective dose) LCDV per fish was intraperitoneally injected in twenty turbots. And the turbots injected with PS were used as the control. At 6 h, 12 h, 24 h, 48 h, 72 h and 96 h post injection, three individuals from each point were anaesthetized and tissues including liver, spleen, kidney and head kidney were collected.

For TK cells infection, cells in six-well plates were treated for 1 h with 50 CFU *V. anguillarum* or 50 $TCID_{50}$ LCDV suspensions per well. Three wells were collected for RNA extraction at 6 h, 12 h, 24 h post *V. anguillarum* infection and 24 h, 48 h, 96 h post LCDV infection. In addition, the untreated cells were used as the negative control.

Total RNA was extracted from above samples by Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was carried out with ReverTra Ace (Toyobo) in the presence of random 9-mer primer and dNTPs for cDNA synthesis.

2.6. The quantitative RT-PCR analysis

To reveal the expression pattern of *Stat2* under normal and challenge condition, the quantitative RT-PCR (qRT-PCR) was

conducted on an Applied Biosystems 7500 Real-Time PCR System with SYBR® Premix Ex Taq™ (Takara). The cDNA of 11 normal tissues and *V. anguillarum*/LCDV infected tissues or TK cells were chosen for detection of *Stat2* expression pattern with primers Stat2_u and Stat2_d (Table 1). For normalization, the expression of *β-actin* was used as an internal control with primers actin_u and actin_d (Table 1).

All RT-qPCRs were performed in a 20 μl volume containing 1 μl cDNA sample, 10 μl SYBR® Premix Ex Taq™, 0.4 μl ROX Reference Dye II, 0.4 μl PCR forward/reverse primers (10 μM) and 7.8 μl nuclease free water. The PCR conditions were as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 58 °C for 34 s. Finally a dissociation curve analysis was added to verify the amplification of a single PCR product. The reaction was carried out with three duplicates for each sample.

In addition, the efficiency of primers' amplification was detected follow as the dilution method [37]. Briefly, the liver cDNA preparation was diluted over a 20-fold range. For each dilution sample, amplification was carried out with primers of *Stat2* and *β-actin*. A plot of the log cDNA dilution versus ΔCT was made. Slop close to zero means the amplification efficiencies of *Stat2* and *β-actin* are the same. Due to that the absolute value of the slop in present study was 0.087, the $2^{-\Delta\Delta CT}$ method could be used for the analysis of relative quantification.

Finally, the data were submitted to one-way ANOVA (analysis of variance) test followed by an unpaired, two-tailed *t*-test. $p < 0.01$ was considered statistically significant.

3. Results

3.1. Cloning and characterization of turbot STAT2 cDNA

Based on a 987 bp turbot STAT2 fragment, the 5′ and 3′ RACE fragments were acquired according to the BD SMART™ RACE cDNA amplification kit with primers Stat2_51/52, Stat2_31/32 and adaptor primers UPM, NUP. Finally these three fragments were joined into turbot STAT2 cDNA (GenBank number: FJ719015) with length of 3206 bp which consisted of a 5′ UTR of 116 bp, an open reading frame of 2379 bp encoding a polypeptide of 793 amino acids, and a 3′ UTR of 708 bp with a poly (A) tail (Fig. 1).

3.2. Amino acid sequence analysis and phylogenetic relationships

SMART (http://smart.embl-heidelberg.de/) analysis indicated the existence of four domains including STAT_int (STAT protein, protein interaction domain) at 2–122 amino acids, STAT_alpha (STAT protein, all-alpha domain) at 137–312 amino acids, STAT_bind (STAT protein, DNA binding domain) superfamily at 314–560 amino acids and SH2 domain at 568–678 amino acids (Fig. 2) in turbot STAT2 protein. Similar structures were found in zebrafish (*Danio rerio*), goldfish (*Carassius auratus*) and western clawed frog (*Xenopus tropicalis*) STAT2 proteins. However, STAT_alpha domain is missing in Atlantic salmon (*Salmo salar*) STAT2 protein. Besides four conserved domains, human (*Homo sapiens*), bovine (*Bos taurus*), horse (*Equus caballus*), Rhesus monkey (*Macaca mulatta*) and house mouse (*Mus musculus*) STAT2 proteins also has STAT2_C domain which contains a nuclear export signal (NES) allowing export of STAT2 into the cytoplasm [38].

Analyzed with BLASTp of NCBI website, turbot STAT2 predicted protein exhibited 64% identity to Atlantic salmon STAT2, 38% identity to human STAT2 and 33% identity to murine STAT2. In further, the phylogenetic analysis (Fig. 3) showed that turbot STAT2 shared the closest relationship with Atlantic salmon (*S. salar*). All the known fish STAT2 including zebrafish (*D. rerio*), Atlantic salmon (*S. salar*), turbot (*Scophthalmus maximus*) and goldfish (*C. auratus*) form a different group with other species. Two groups are separated with a bootstrap value of 100%, one including STAT1, STAT2, STAT3 and STAT4, the other including STAT5 and STAT6.

3.3. The subcellular location and identification of functional NES of STAT2 in TK cells

To examine the subcellular location of turbot STAT2, pSTAT2-GFP$^{1-793}$ was constructed with a green fluorescent protein fused with wild type STAT2 (Fig. 4A). The transfection of pSTAT2-GFP$^{1-793}$ resulted in uniform distribution of fluorescence in the cytoplasm of TK cells (Fig. 4B).

In order to verify whether turbot STAT2 also contained a NES in the C-terminal similar with human STAT2, NES prediction analysis was conducted according to the NES sequences in NES databases (www.cbs.dtu.dkPdatabasesP NESbase) [39] and the common

Table 1
Primers used in this study.

Primers	Sequences	Primer information
Stat2_31	CGTTCTTGTTGCGCTTCAG	3′RACE
Stat2_32	TGAAAGTGTCATTGGAGGAA	3′RACE
Stat2_51	TCAGCTCAGGAAACTTCACG	5′RACE
Stat2_52	TTAGTGCGGAGAACCAGAGG	5′RACE
Stat2_u	GGAAGCGGCAAGGGAGT	qRT-PCR
Stat2_d	TGCTGCTGGCTGGAGTT	qRT-PCR
actin_u	GCTGTGCTGTCCCTGTA	qRT-PCR
actin_d	GAGTAGCCACGCTCTGTC	qRT-PCR
UPM long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE
UPM short	CTAATACGACTCACTATAGGGC	RACE
NUP	AAGCAGTGGTATCAACGCAGAGT	RACE
OligodG	AAGCAGTGGTAACAACGCAGAGTACGCGGG	5′RACE
5′-CDS	$(T)_{25}$VN	5′RACE
3′-CDS	AAGCAGTGGTATCAACGCAGAGTAC(T)30VN	3′RACE
Stat2_1	ATGGCTCAGTGGGACAGACTG	cDNA confirmation
Stat2_2	TGGGGGCAGGCAATCCAAGTC	cDNA confirmation
GFPS2-U1	ACG***AAGCTT***ATGGCTCAGTGGGACAGACTG	Vector construction
GFPS2-D1	ACG***GAATTC***CGACTTTTGAGTAGGTCTTTCAG	Vector construction
GFPS2-DN1	ACG***GAATTC***CGTTCCATGATTTCCGGCTC	Vector construction
GFPS2-UN2	ACG***AAGCTT***ATGACGAGCAGCAAGCAAGAC	Vector construction
GFPS2-U2	ACG***AAGCTT***ATGTCCTTTGTGGTTGAGACTCAG	Vector construction
GFPS2-D2	ACG***GAATTC***CGTGGGGGCAGGCAATCCAAGTC	Vector construction
GFPS2-U3	ACG***AAGCTT***ATGCTGGAGCCAATGAATGGC	Vector construction
GFPS2-D3	ACG***GAATTC***CGACCTTCACCCTGTGCCACAT	Vector construction

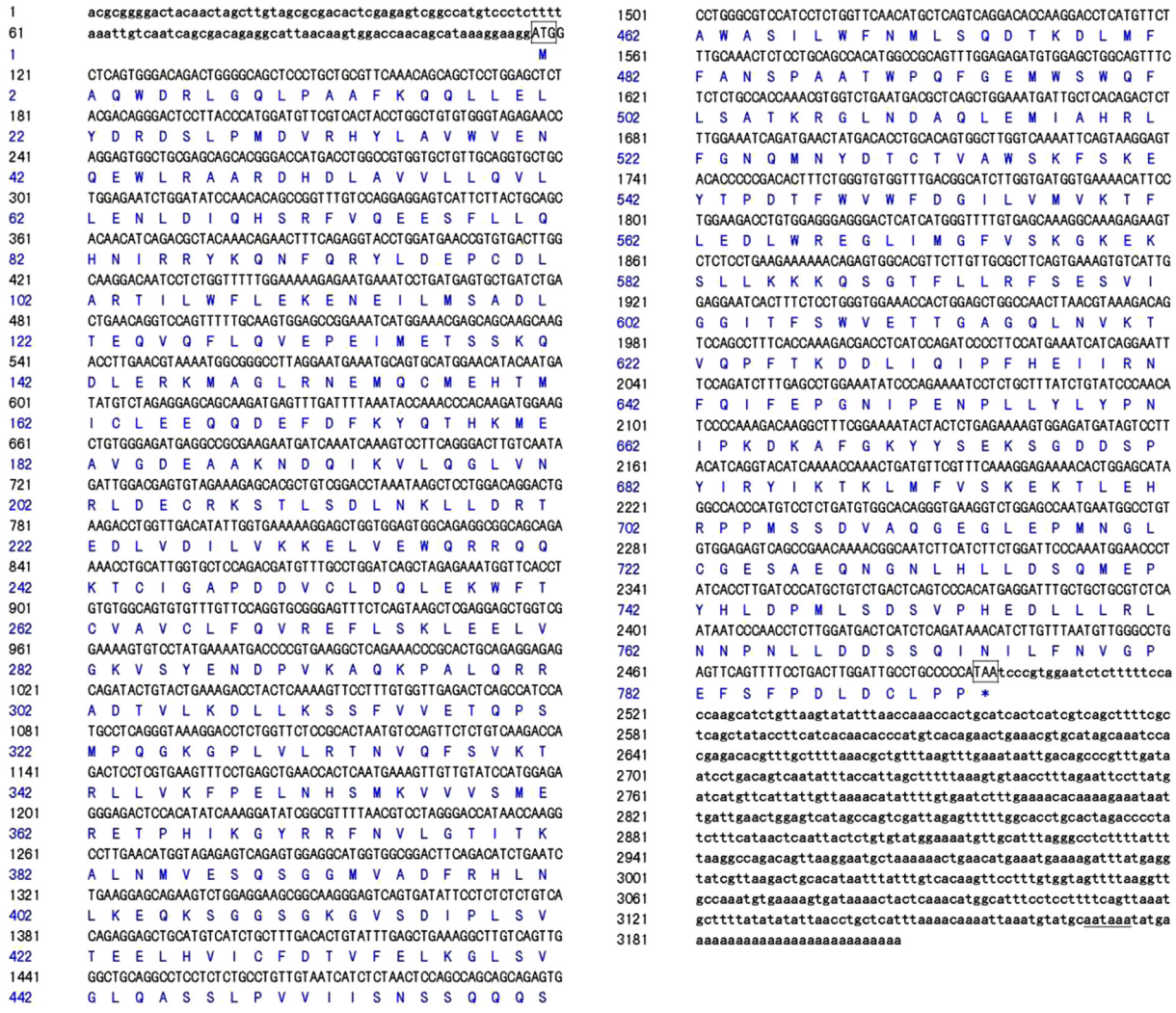

Fig. 1. The cDNA and predicted protein sequence of turbot STAT2. The open reading frame sequences are shown in capital letters. The start and stop codons are marked by box. The poly (A) signal sequence is underlined.

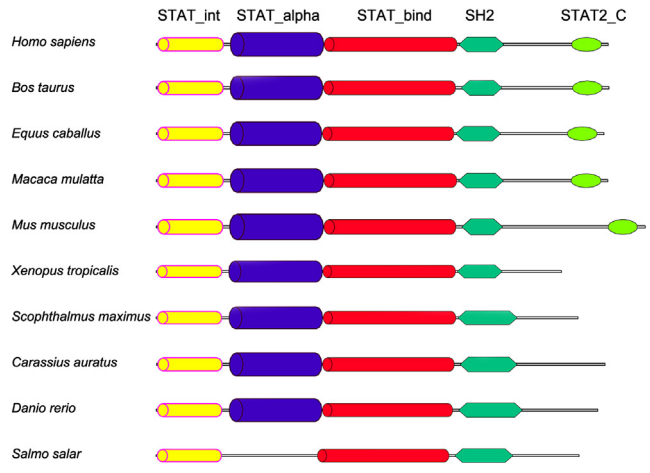

Fig. 2. The schematic structure of turbot STAT2 deduced protein and other STAT2 proteins. Five major structural features are indicated including STAT_int, STAT_alpha, STAT_bind, SH2 and STAT2_C domains. All fish and *Xenopus tropicalis* STAT2 proteins are missing STAT2_C domain. STAT_alpha domain is also lack in *Salmo salar* STAT2.

structures of NES: L-x(2,3)-[LIVFM]-x(2,3)-L-x-[LI]. Firstly, turbot C-terminal 79 amino acids sequence (715–793 amino acids) was considered as the predicted NES region. However, the transfection of the corresponding plasmid pSTAT2-NES-GFP$^{715-793}$ led to a uniform distribution of fluorescence in cytoplasm and nucleus, similar with the pattern of pEGFP-N3 (Fig. 4B).

To identify the functional NES of turbot STAT2, several plasmids were constructed by fusion of GFP with STAT2 truncated motifs. As shown in Fig. 4, transfection of pSTAT2-N-GFP$^{1-312}$, which expressed a GFP fusion protein with domains STAT_int and STAT_alpha, resulted in strong GFP signals in the cytoplasm with punctate distribution; transfection of pSTAT2-C-GFP$^{313-793}$ or pSTAT2-C-NES-GFP$^{313-714}$, which fused GFP protein with STAT_bind, SH2 and other structures, both led to weak GFP signals in the cytoplasm (Fig. 4B). The results showed that the functional NES may be located in the N terminal 1–312 amino acids. So, we constructed two other plasmids pSTAT2-N1-GFP$^{1-136}$ and pSTAT2-N2-GFP$^{137-312}$ which contained GFP fusion protein with STAT_int and STAT_alpha, respectively. The transfection assay revealed that pSTAT2-N1-GFP$^{1-136}$ expressed similar GFP pattern with pEGFP-N3, while pSTAT2-N2-GFP$^{137-312}$ expressed uniform GFP signals exclusively in the cytoplasm (Fig. 4C), which suggested that N terminal 137–312 amino acids in STAT2 may contained the functional

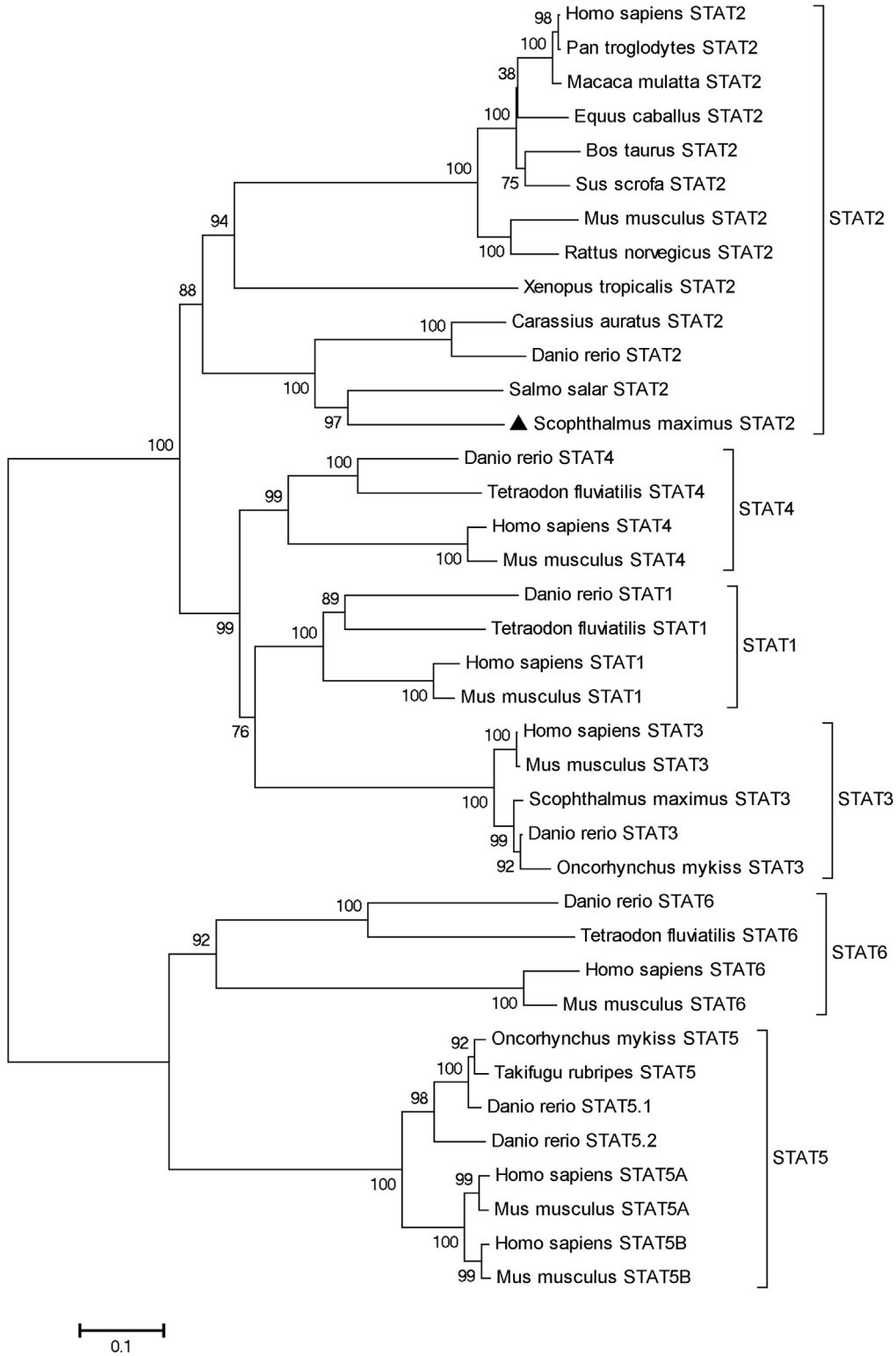

Fig. 3. Phylogenetic analysis of STATs protein sequences. A phylogenetic tree was constructed with the neighbour-joining algorithm in MEGA 5.05. The branches were validated by bootstrap analysis from 1000 replications, which were represented by percentage in branch nodes. STATs proteins sequences used in this analysis: *Homo sapiens* STAT1 (NP_009330), *Mus musculus* STAT1 (NP_033309), *Danio rerio* STAT1 (NP_571555), *Tetraodon fluviatilis* STAT1 (AAL09414); *Homo sapiens* STAT2 (NP_005410), *Bos taurus* STAT2 (XP_588270), *Danio rerio* STAT2 (XP_693577), *Mus musculus* STAT2 (Q9WVL2), *Carassius auratus* STAT2 (JQ804927), *Scophthalmus maximus* STAT2 (ACX69848), *Sus scrofa* STAT2 (BAA20332), *Xenopus tropicalis* STAT2 (AAI67467), *Salmo salar* STAT2 (NP_001138896), *Rattus norvegicus* STAT2 (NP_001011905), *Pan troglodytes* STAT2 (XP_509146), *Macaca mulatta* STAT2 (XP_001115072), *Equus caballus* STAT2 (XP_001504891); *Homo sapiens* STAT3 (NP_644805), *Mus musculus* STAT3 (AAA19452), *Danio rerio* STAT3 (AAH68320), *Oncorhynchus mykiss* STAT3 (NP_001118180), *Scophthalmus maximus* STAT3 (ACX69847); *Homo sapiens* STAT4 (NP_003142), *Mus musculus* STAT4 (NP_035617), *Danio rerio* STAT4 (NP_001004510), *Tetraodon fluviatilis* STAT4 (AAL09416); *Homo sapiens* STAT5A (NP_003143), *Homo sapiens* STAT5B (NP_036580), *Mus musculus* STAT5A (AAH13274), *Mus musculus* STAT5B (NP_035619), *Danio rerio* STAT5.1 (AAI62592), *Danio rerio* STAT5.2 (AAT95392), *Oncorhynchus mykiss* STAT5 (AAG14946), *Takifugu rubripes* STAT5 (AAS80167); *Homo sapiens* STAT6 (NP_003144), *Mus musculus* STAT6 (NP_033310), *Tetraodon fluviatilis* STAT6 (AAO22057), *Danio rerio* STAT6 (AAI62530).

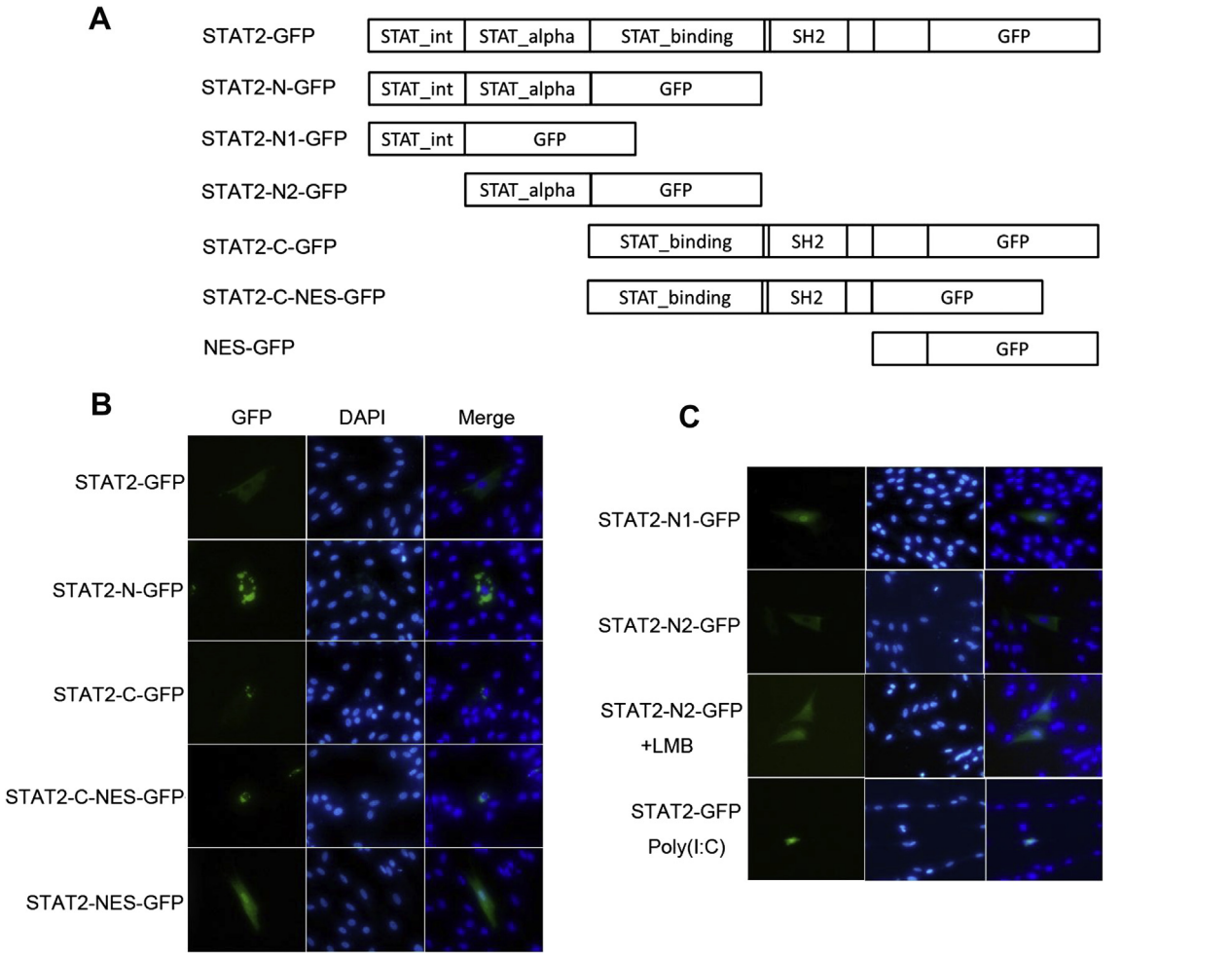

Fig. 4. The subcellular location of turbot STAT2 in TK cells. Part A presents a schematic diagram of wild type STAT2-GFP plasmid and various truncations. Part B shows the subcellular location of STAT2-GFP, STAT2-N-GFP, STAT2-C-GFP, STAT2-C-NES-GFP and STAT2-NES-GFP. The pSTAT2-GFP$^{1-793}$ was uniformly expressed in cytoplasm of TK cell line. The pSTAT2-N-GFP$^{1-312}$ was strongly expressed in cytoplasm of TK cell line with punctate distribution. The pSTAT2-C-GFP$^{313-793}$ and pSTAT2-C-NES-GFP$^{313-714}$ transfected cells showed weak signals in the cytoplasm of TK cells. In pSTAT2-NES-GFP$^{715-793}$ transfected cells, no particular but uniform distribution in cytoplasm and nucleus were observed. Part C showed pSTAT2-N1-GFP$^{1-136}$ expressed similar GFP pattern with pEGFP-N3, while pSTAT2-N2-GFP$^{137-312}$ expressed uniform GFP signals exclusively in the cytoplasm. Also, the introduction of NES inhibitor Leptomycin B (LMB) caused the redistribution of GFP from cytoplasm to nucleus in pSTAT2-N2-GFP$^{137-312}$ transfected cells. In the pSTAT2-GFP$^{1-793}$ transfected cells, Poly(I:C) treatment resulted in the redistribution of GFP from cytoplasm to nucleus.

NES. Also, the introduction of NES inhibitor Leptomycin B (LMB) caused the redistribution of GFP from cytoplasm to nucleus, which further proved that the existence of NES in the N terminal 137—312 amino acids (STAT_alpha domain).

To verify the transcription activity of turbot STAT2, Poly(I:C) was introduced in TK cells transfected with pSTAT2-GFP$^{1-793}$. As a result, the nuclear translocation phenomenon of GFP signal from the cytoplasm to nucleus was observed (Fig. 4C). It indicates that STAT2 acts as a transcriptional activator in TK cells.

3.4. The expression pattern of turbot Stat2

The qRT-PCR was employed to reveal the expression pattern of turbot *Stat2* of tissues or TK cells in normal and pathogen-challenged individuals. The mRNA transcripts of *Stat2* mRNA were detected in all tested 11 tissues with higher expression levels in liver, spleen, brain, gill and lower expression levels in heart and muscle (Fig. 5).

In *V. anguillarum* infected turbot, *Stat2* relative expression levels in detected tissues displayed different degree of increase tendency compared with the saline injected individuals (Fig. 6). In liver and

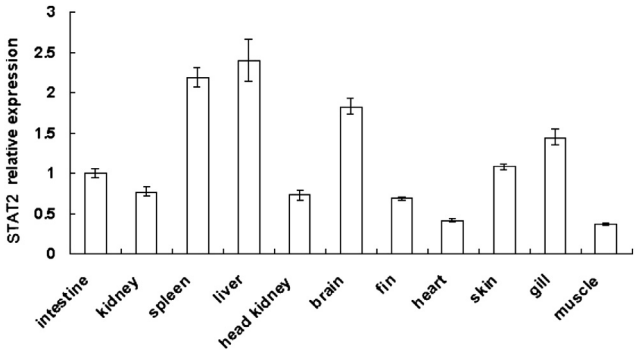

Fig. 5. The expression pattern of *Stat2* in turbot different tissues detected by the quantitative RT-PCR. *Stat2* mRNA was expressed in all tissues detected with higher expression levels in the liver, spleen, brain and gill. The *β-actin* gene was used as an internal control to calibrate the cDNA template for all the samples. Vertical bars represented the mean ± S.D. ($n = 3$).

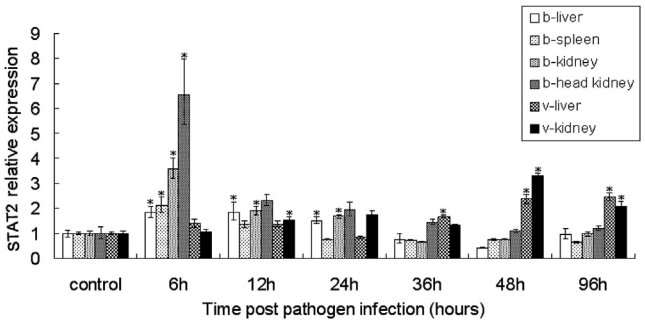

Fig. 6. The *Stat2* expression patterns in *V. anguillarum* and LCDV infected tissues. The b-liver, b-spleen, b-kidney and v-head kidney represent liver, spleen, kidney and head kidney infected with bacteria *V. anguillarum* and v-kidney, v-liver represent kidney and liver infected with virus LCDV, respectively. The *Stat2* expression levels relative to *β-actin* at 6 h, 12 h, 24 h, 48 h, 72 h and 96 h post injection were examined. The sample control means the tissues at 12 h after saline solution injection. All data are expressed as mean ± S.D. ($n = 3$). Asterisk (*) marks the significant difference between experimental group and control group ($p < 0.01$).

kidney, *Stat2* expression levels were significantly increased at 6 h ($p < 0.01$), 12 h ($p < 0.01$) and 24 h ($p < 0.01$) after infection, while the expression levels decreased to the control level from 48 h post infection till 96 h. In spleen and head kidney, *Stat2* expression was significantly up-regulated only at 6 h ($p < 0.01$) after infection.

After LCDV infection, *Stat2* expression level in liver was significantly up-regulated at 36 h ($p < 0.01$), 48 h ($p < 0.01$) and 96 h ($p < 0.01$) respectively. And in kidney, *Stat2* expression level was significantly up-regulated at 12 h ($p < 0.01$), 48 h ($p < 0.01$) and 96 h ($p < 0.01$) respectively.

In TK cells, *Stat2* expression level was significantly up-regulated at 12 h ($p < 0.01$) and 24 h ($p < 0.01$) after *V. anguillarum* infection. *Stat2* expression level was significantly up-regulated at 24 h ($p < 0.01$), 48 h ($p < 0.01$) and 96 h ($p < 0.01$) after LCDV infection (Fig. 7).

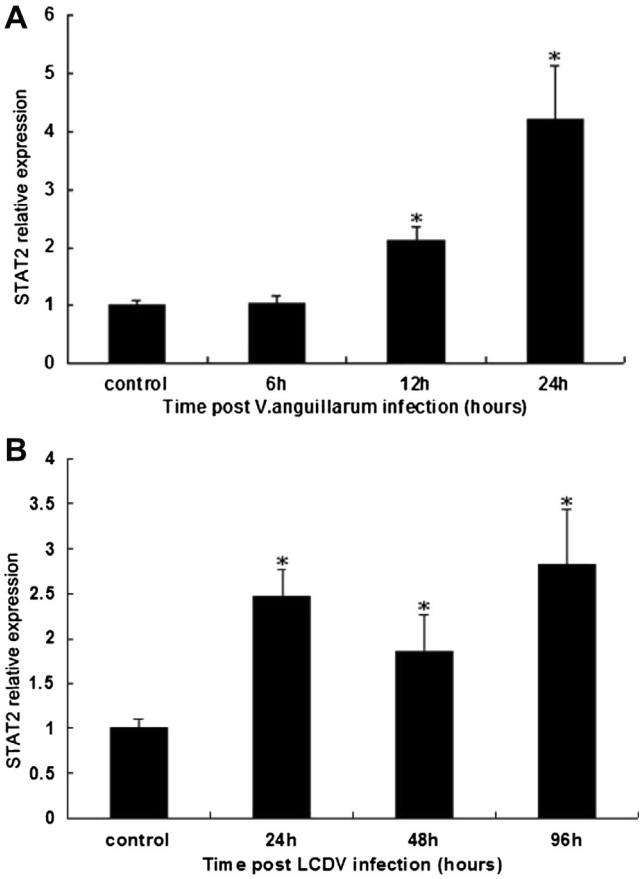

Fig. 7. The *Stat2* expression patterns in *V. anguillarum* and LCDV infected TK cells. The *Stat2* relative expression levels at 6 h, 12 h, and 24 h post *V. anguillarum* infection and at 24 h, 48 h, and 96 h post LCDV infection were examined. Control means untreated TK cells. All data are expressed as mean ± S.D. ($n = 3$). Asterisk (*) marks the significant difference between experiment group and control group ($p < 0.005$).

4. Discussion

STAT2 is an important molecule involved in the type I interferon signalling pathway, which is the key inducer of both innate and adaptive immunity to viruses [40,41]. Although STAT2 gene has been predicted by bio-information analysis from zebrafish genome [42], no STAT2 homologue was cloned from fish [42,43] until the reports in Atlantic salmon [26] and goldfish [27].

In this paper, turbot STAT2 homologue was cloned and identified, which further confirmed the existence of STAT2 in fish. Similar with other species STAT2, the putative 793 amino acids sequences of turbot STAT2 contained four conserved domains: STAT_int$^{2-122}$, STAT_alpha$^{137-312}$, STAT_bind$^{314-560}$ and SH2$^{568-678}$. In further, the phylogenetic tree analysis clusters turbot STAT2 and the other known fish STAT2 genes as one branch. All these results convinced that the turbot STAT2 was indeed a STAT2 homologue.

Similar with Atlantic salmon *Stat2* [26], turbot *Stat2* transcripts were also detected in various tissues without any stimulation. The up-regulation of *Stat2* after pathogen infection in several immune tissues and TK cells suggested that turbot STAT2 plays important roles in immune response. The maximal fold increase of turbot *Stat2* was mainly at 6 h in *V. anguillarum* challenged tissues and at 48 h in LCDV challenged tissues. In pathogen infected TK cell line, the *Stat2* response time in virus infection is also later than one of bacterial infection. This interesting phenomenon is similar with previous findings [44,45] which showed that Poly I: C induced Mx responses were more intense and longer lasting than those induced by the bacterial in Atlantic salmon.

The subcellular location revealed that the green fluorescence of pSTAT2-GFP$^{1-793}$ was uniformly distributed in the cytoplasm of TK cells, which is similar with the immunofluorescence location of human STAT2 [24,25]. The truncation analysis of human STAT2 demonstrated that deletion of the N-terminal 59 amino acids of human STAT2 completely prevented tyrosine phosphorylation and deletion of the C-terminal 50 amino acids killed all induced transcriptional activity [11]. STAT_alpha domain is also important for the phosphorylation of STAT2 and for the STAT2-independent phosphorylation of STAT2 [16]. STAT_binding domain contains several β-sheets and determines DNA sequence specificity of individual STATs. SH2 domain is a well-known common structural motif, which mediates dimerization via SH2-phosphotyrosyl peptide interactions [46]. In present study, turbot STAT_int$^{2-122}$ domain and C terminal 68 (715–793) amino acids were both located in the cytoplasm and nucleus with uniform distribution, and STAT_alpha$^{137-312}$ was exclusively distributed in the cytoplasm, which implied their possible roles in specific regions. Nevertheless, it is worth to investigate whether these domains exhibit similar functions in turbot.

The higher vertebrate STAT2 including human (*H. sapiens*), mouse (*M. musculus*), bovine (*B. taurus*), horse (*E. caballus*) and Rhesus monkey (*M. mulatta*) all contain the domain STAT2_C in the C-terminal which contains a NES allowing export of STAT2 into the cytoplasm (Fig. 2). In order to prove whether turbot STAT2 also contained the NES in the C-terminal, turbot putative NES (LLLR-LNN-PN-L) within 715–793 amino acids was screened out

according to the common structure of NES sequences. And the following subcellular location experiment showed that the fusion protein of pSTAT2-NES-GFP$^{715-793}$ was uniformly distributed in the cytoplasm and nucleus of cells, similar with the pEGFP-N3 fluorescence distribution pattern. It implies that no NES exist in the C-terminal 79 amino acids of turbot STAT2, which is different from that of human STAT2 [11]. The further STAT2-truncated ORF location revealed that pSTAT2-N2-GFP$^{137-312}$ expressed GFP signals only in the cytoplasm, which could be inhibited by NES inhibitor LMB. The region of 127–312 amino acids was rich in hydrophobic amino acid residues with two atypical NES structures: LSD-LNK-LL-D at 211–219 amino acids and LSK-LEE-LV-G at 274–282 amino acids, which is not hydrophobic residue at the last position. The similar atypical NES structures were found in human, bovine, horse, Rhesus monkey and house mouse STAT2 with LPY(C/H)D-LR(M)H-LN-T.

The translocation of turbot STAT2 from the cytoplasm to nucleus indicates the transcription activity of STAT2 in TK cells. As a transcription factor, turbot STAT2 may also contain nuclear localization signal (NLS) as well as NES for its shuttle function like other proteins [47,48]. The identification and detailed location of these two signals will aid in the understanding the interaction mechanism of turbot STAT2 with other genes and pathogen.

In conclusion, the cloning and identification of turbot STAT2 are described in present study for the first time. It not only further proves the existence of STAT2 homologue in teleosts, but also provides a new target for study the fish JAK–STAT pathway and the control of turbot diseases. In future, the exploration of more proteins involved in STAT2 pathway and pathogen–host interaction will help to develop the new strategy for fish diseases treatment.

Acknowledgements

This work was supported by grants from National Natural Science Foundation of China (31001133), National High Technology Research and Development Program of China (2012AA092203, 2012AA10A408), 973 National Basic Research Program of China (2010CB126303), and Taishan Scholar Project Fund.

References

[1] Aaronson DS, Horvath CM. A road map for those who don't know JAK–STAT. Science 2002;296:1653–5.
[2] O'Shea JJ, Pesu M, Borie DC, Changelian PS. A new modality for immunosuppression: targeting the JAK/STAT pathway. Nat Rev Drug Discov 2004;3:555–64.
[3] Schindler C, Plumlee C. Inteferons pen the JAK–STAT pathway. Semin Cell Dev Biol 2008;19:311–8.
[4] Murray PJ. The JAK–STAT signaling pathway: input and output integration. J Immunol 2007;178:2623–9.
[5] Ihle JN. STATs: signal transducers and activators of transcription. Cell 1996;84:331–4.
[6] Darnell Jr JE. STATs and gene regulation. Science 1997;277:1630–5.
[7] Schindler C, Fu XY, Improta T, Aebersold R, Darnell Jr JE. Proteins of transcription factor ISGF-3: one gene encodes the 91-and 84-kDa ISGF-3 proteins that are activated by interferon alpha. Proc Natl Acad Sci U S A 1992;89:7836–9.
[8] Schindler C, Shuai K, Prezioso V, Darnell JE. Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. Science 1992;257:809–13.
[9] Qureshi SA, Salditt-Georgieff M, Darnell Jr JE. Tyrosine-phosphorylated Stat1 and Stat2 plus a 48-kDa protein all contact DNA in forming interferon-stimulated-gene factor 3. Proc Natl Acad Sci U S A 1995;92:3829–33.
[10] Improta T, Schindler C, Horvath CM, Kerr IM, Stark GR, Darnell Jr JE. Transcription factor ISGF-3 formation requires phosphorylated Stat91 protein, but Stat113 protein is phosphorylated independently of Stat91 protein. Proc Natl Acad Sci U S A 1994;91:4776–80.
[11] Qureshi SA, Leung S, Kerr IM, Stark GR, Darnell Jr JE. Function of Stat2 protein in transcriptional activation by alpha interferon. Mol Cell Biol 1996;16:288–93.
[12] Park C, Li S, Cha E, Schindler C. Immune response in Stat2 knockout mice. Immunity 2000;13:795–804.
[13] Durbin JE, Hackenmiller R, Simon MC, Levy DE. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. Cell 1996;84:443–50.
[14] Meraz MA, White JM, Sheehan KC, Bach EA, Rodig SJ, Dighe AS, et al. Targeted disruption of the Stat1 gene in mice reveals unexpected physiological specificity in the JAK–STAT pathway. Cell 1996;84:431–42.
[15] Uddin S, Chamdin A, Platanias LC. Interaction of the transcriptional activator Stat-2 with the type I interferon receptor. J Biol Chem 1995;270:24627–30.
[16] Li X, Leung S, Kerr IM, Stark GR. Functional subdomains of STAT2 required for preassociation with the alpha interferon receptor and for signaling. Mol Cell Biol 1997;17:2048–56.
[17] Lau Joe F, Inna Nusinzon, Darya Burakov, Freedman Leonard P, Horvath Curt M. Role of metazoan mediator proteins in interferon-responsive transcription. Mol Cell Biol 2003;23:620–8.
[18] Lee JW, Kang HS, Lee JY, Lee EJ, Rhim H, Yoon JH, et al. The transcription factor STAT2 enhances proteasomal degradation of RCAN1 through the ubiquitin E3 ligase FBW7. Biochem Biophys Res Commun 2012;420:404–10.
[19] O'Shea JJ, Gadina M, Schreiber RD. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. Cell 2002;109:121–31.
[20] Wang K, Wang C, Xiao F, Wang H, Wu Z. JAK2/STAT2/STAT3 are required for myogenic differentiation. J Biol Chem 2008;283:34029–36.
[21] Horvath CM. Silencing STATs: lessons from paramyxovirus interferon evasion. Cytokine Growth Factor Rev 2004;15:117–27.
[22] Hahm B, Trifilo MJ, Zuniga EI, Oldstone MB. Viruses evade the immune system through type I interferon-mediated STAT2-dependent, but STAT1-independent, signaling. Immunity 2005;22:247–57.
[23] Ramachandran A, Parisien JP, Horvath CM. STAT2 is a primary target for measles virus V protein-mediated alpha/beta interferon signaling inhibition. J Virol 2008;82:8330–8.
[24] Kadeppagari RK, Sanchez RL, Foster TP. HSV-2 inhibits type-I interferon signaling via multiple complementary and compensatory STAT2-associated mechanisms. Virus Res 2012;167:273–84.
[25] Rodriguez JJ, Parisien JP, Horvath CM. Nipah virus V protein evades alpha and gamma interferons by preventing STAT1 and STAT2 activation and nuclear accumulation. J Virol 2002;76:11476–83.
[26] Collet B, Ganne G, Bird S, Collins CM. Isolation and expression profile of a gene encoding for the signal transducer and activator of transcription STAT2 in Atlantic salmon (*Salmo salar*). Dev Comp Immunol 2009;33:821–9.
[27] Shi J, Zhang YB, Liu TK, Sun F, Gui JF. Subcellular localization and functional characterization of a fish IRF9 from crucian carp *Carassius auratus*. Fish Shellfish Immunol 2012;33:258–66.
[28] Pardo BG, Fernández C, Millán A, Bouza C, Vázquez-López A, Vera M, et al. Expressed sequence tags (ESTs) from immune tissues of turbot (*Scophthalmus maximus*) challenged with pathogens. BMC Vet Res 2008;4:37.
[29] Park KC, Osborne JA, Montes A, Dios S, Nerland AH, Novoa B, et al. Immunological responses of turbot (*Psetta maxima*) to nodavirus infection or polyriboinosinic polyribocytidylic acid (pIC) stimulation, using expressed sequence tags (ESTs) analysis and cDNA microarrays. Fish Shellfish Immunol 2009;26:91–108.
[30] Meng L, Chen SL, Liu Y. Construction of a cDNA library from spleen of *Scophthalmus maximus* and identification of immune-related genes by EST sequencing. J Fish China 2009;33:713–8.
[31] Hu GB, Xia J, Lou HM, Chen XL, Li J, Liu QM. An IRF-3 homolog that is up-regulated by DNA virus and poly I: C in turbot, *Scophthalmus maximus*. Fish Shellfish Immunol 2011;31:1224–31.
[32] Hu G, Xia J, Lou H, Liu Q, Lin J, Yin X, et al. Cloning and expression analysis of interferon regulatory factor 7 (IRF-7) in turbot, *Scophthalmus maximus*. Dev Comp Immunol 2011;35:416–20.
[33] Wang N, Wang XL, Sha ZX, Tian YS, Chen SL. Development and characterization of a new marine fish cell line from turbot (*Scophthalmus maximus*). Fish Physiol Biochem 2010;36:1227–34.
[34] Kudo N, Wolff B, Sekimoto T, Schreiner EP, Yoneda Y, Yanagida M, et al. Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. Exp Cell Res 1998;242:540–7.
[35] Chen JX, Li CF, Yan XH, Yun Li, Chi ZH, Xue XW, et al. Studies on biological characteristics of five pathogenic *Vibrio anguillarum* strains isolated from diseased turbot (*Scophthalmus maximus*) in Shandong province of China. High Tech Lett 2005;15:92–6.
[36] Xu HT, Piao CA, Jiang ZL, Wang WX. Study on the causative agent of lymphocystic disease in cultured flounder, *Paralichthys Olivaceus*, in mainland China. Chin J Virol 2000;16:223–6.
[37] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods 2001;25:402–8.
[38] Banninger G, Reich NC. STAT2 nuclear trafficking. J Biol Chem 2004;279:39199–206.
[39] la Cour T, Gupta R, Rapacki K, Skriver K, Poulsen FM, Brunak S. NESbase version 1.0: a database of nuclear export signals. Nucleic Acids Res 2003;31:393–6.
[40] Ellis AE. Innate host defence mechanisms of fish against viruses and bacteria. Dev Comp Immunol 2006;25:827–39.
[41] Robertsen B. The interferon system of teleosts fish. Fish Shellfish Immunol 2006;20:172–91.

[42] Stein C, Caccamo M, Laird G, Leptin M. Conservation and divergence of gene families encoding components of innate immune response systems in zebrafish. Genome Biol 2007;8:251–5.
[43] Guo CJ, Zhang YF, Yang LS, Yang XB, Wu YY, Liu D, et al. The JAK and STAT family members of the mandarin fish *Siniperca chuatsi*: molecular cloning, tissues distribution and immunobiological activity. Fish Shellfish Immunol 2009;27:349–59.
[44] Salinas I, Lockhart K, Bowden TJ, Collet B, Secombes CJ, Ellis AE. An assessment of immunostimulants as Mx inducers in Atlantic salmon (*Salmo salar* L.) parr and the effect of temperature on the kinetics of Mx responses. Fish Shellfish Immunol 2004;17:159–70.
[45] Acosta F, Lockhart K, Gahlawat SK, Real F, Ellis AE. Mx expression in Atlantic salmon (*Salmo salar* L.) parr in response to *Listonella anguillarum* bacterin, lipopolysaccharide and chromosomal DNA. Fish Shellfish Immunol 2004;17:255–63.
[46] Gupta S, Yan H, Wong LH, Ralph S, Krolewski J, Schindler C. The SH2 domains of Stat1 and Stat2 mediate multiple interactions in the transduction of IFN-alpha signals. EMBO J 1996;15:1075–84.
[47] Park SW, Do HJ, Huh SH, Sung B, Uhm SJ, Song H, et al. Identification of a putative nuclear export signal motif in human NANOG homeobox domain. Biochem Biophys Res Commun 2012;421:484–9.
[48] Günther V, Davis AM, Georgiev O, Schaffner W. A conserved cysteine cluster, essential for transcriptional activity, mediates homodimerization of human metal-responsive transcription factor-1 (MTF-1). Biochim Biophys Acta 2012;1823:476–83.