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Identification and functional characterization of IRAK-4 in grass carp (*Ctenopharyngodon idellus*)

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ABSTRACT

IL-1R-associated kinase 4 (IRAK4), a central TIR signaling mediator in innate immunity, can initiate a cascade of signaling events and lead to induction of inflammatory target gene expression eventually. In the present study, we cloned and characterized an IRAK4 orthologue from grass carp (*Ctenopharyngodon idella*). The full length cDNA of *CiIRAK4* was 2057 bp with an ORF of 1422 bp encoding a polypeptide of 472 amino acids. Multiple alignments showed that IRAK4s were highly conserved among different species. Phylogenetic tree analysis revealed that *Ci*IRAK4 shared high homologous with zebra fish IRAK4. Expression analysis indicated that *CiIRAK4* was widely expressed in all tested tissues. It was significantly up-regulated after treatment with poly I:C, especially obvious in liver and spleen. Also, *CiIRAK4* could be induced by poly I:C and LPS in CIK cells. Fluorescence microscopy assays showed that *Ci*IRAK4 localized in the cytoplasm. RNAi-mediated knockdown and overexpression assays indicated that *Ci*IRAK4 might have little effect on NF-kappa B p65 translocation from cytoplasm to nucleus, indicating that *Ci*IRAK4 was dispensable for activation of NF-kappa B p65. In addition, IRAK4 promoted IRF5 nuclear translocation, which has nothing to do with the interaction between IRAK4 and IRF5. It suggested that fish IRAK4 kinase regulated IRF5 activity through indirect ways.

1. Introduction

The innate immune system is the first line of host defense against pathogens, particularly important for lower vertebrates to defense against viral or microbial invasion [1,2]. The recognition of invading pathogens is a pivotal step to trigger intracellular signaling cascades in host cells, and pattern-recognition receptors (PRRs) play a critical role in this process [3]. Different PRRs react with specific pathogen-associated molecular partterns (PAMPs), activate specific signaling pathways, and lead to distinct antipathogen responses [4]. Toll-like receptors (TLRs), the first identified and the most well characterized PRRs, can trigger innate immune responses and prime antigen-specific adaptive immunity [5,6]. To date, 10 and 12 functional TLRs have been identified in human and mouse respectively, and they are evolutionarily conserved from worm to mammals [7]. Containing intracellular Toll–interleukin 1 (IL-1) receptor (TIR) domains, TLRs recruit a specific set of adaptor molecules, such as MyD88 and TRIF, and initiate distinct signaling pathways [8]. In the MyD88-dependent pathway, IL-1 receptor-associated kinases (IRAKs) play a critical role in linking TLR signaling to the nuclear factor κB (NF-kappa B) pathway [8,9].

In mammals, IRAKs is comprised of four family members: IRAK1, IRAK2, IRAK3 (IRAKM), and IRAK4 [10–13]. Although all of them contain an N-terminal death domain (DD), a proST domain, and a conserved central kinase domain, only IRAK1 and IRAK4 have real kinase activity, while IRAK2 and IRAK3 are inactive pseudokinases [14,15]. In the MyD88-dependent pathway, after PAMPs binding with their TLRs, the adaptor protein MyD88 is recruited to the receptors. Then, MyD88 recruits the first protein kinase IRAK4 via interactions between their N-terminal death domains, and IRAK4 undergoes dimerization and trans-autophosphorylation [13,16]. The activated IRAK4 subsequently phosphorylates the downstream kinases IRAK1 and IRAK2, which results in formation of the TRAF6-TAK1-IKK signalosome ultimately leads to the activation of the NF-kappa B and Jun N-terminal kinase pathway [17–19].

So far, some *IRAK4* homologous cDNA were cloned from zebrafish (*Danio rerio*) [20], roughskin sculpin (*Trachidermus fasciatus*) [21], half-smooth tongue sole (*Cynoglossus semilaevis*) [22], rainbow trout (*Oncorhynchus mykiss*) [23], grouper (*Epinephelus coioides*) [24], rock bream (*Oplegnathus fasciatus*) [25], and large yellow croaker (*Larimichthys crocea*) [26]. In zebrafish, *Dr*IRAK4 contains the conserved domains

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that shared with insect and mammalian gene. Also, mRNA expression of *Dr*IRAK4 is significantly upregulated after exposure of zebrafish to *Edwardsiella tarda* [20]. It is suggested that fish posses some conserved TLR-signaling pathways similar to mammals. However, previous studies found that the functions of some fish IRAK4 were quite intriguing. Rainbow trout IRAK4 significantly quenched TLR2-mediated signaling in HEK-293T cells [23]. When co-expressed with MyD88, grouper and large yellow croaker IRAK4 impaired MyD88 induced NF-kappa B activation [24,26]. These results demonstrated that the functions of fish IRAK4 might be diverse.

In the present study, we cloned and identified the full-length cDNA sequence of grass carp (*Ctenopharyngodon idellus*) IRAK4 (*Ci*IRAK4). The expression patterns analysis showed that the mRNA level of *CiIRAK4* was dramatically up-regulated after infection with poly I:C and LPS. Meanwhile, we found that *Ci*IRAK4 was distributed entirely in the cytoplasm in CIK cells. Furthermore, nuclear-cytosol extraction assays found overexpressing *Ci*IRAK4 had very little effect on the nuclear translocation of NF-kappa B p65 in CIK cells. However, *Ci*IRAK4 could promote IRF5 translocation to the nucleus, which has nothing to do with the interaction between IRAK4 and IRF5.

2. Materials and methods

2.1. Reagents, antibodies, and kits

Medium 199 and DMEM were purchased from Corning, and DAPI was commercially acquired from Sangon Biotech. Polyclonal antibody for Histone H3 was purchased from Affinity Biosciences, and mouse monoclonal antibody against GFP was purchased from Abmart, respectively. Goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were purchased from ZSGB-BIO. Anti-Flag and anti-GFP agarose conjugate were purchased from Sigma. Polyclonal antibodies for grass carp p65 (Suppl. Fig. 1) and GAPDH [27] were prepared and preserved in our laboratory. Small interfering RNA (siRNA) for IRAK4 and negative control RNA oligo were purchased from Shanghai GenePharma. Poly I:C and LPS were purchased from Sigma. HiperFect transfection reagent was purchased from QIAGEN. FuGENE®6 Transfection Reagent was purchased from Promega. RNA simple Total RNA Kit was purchased from Tiangen. Super Script III reverse polymerase was purchased from Invitrogen. Script RT reagent kit with gDNA Eraser Perfect Real Time was purchased from TaKaRa. Nuclear and Cytoplasmic Protein Extraction Kit was purchased from Beyotime.

2.2. Fish, cells and vectors

Grass carps (about 20 g body weight) were obtained from Nanchang Shenlong Fisheries Development (Jiangxi, China) and acclimatized to the laboratory conditions for two weeks in a quarantine area. The CIK cell lines were kindly gifted by Professor Pin Nie, Institute of Hydrobiology, Chinese Academy of Sciences. CIK cells were cultured at 28 °C in M199 containing 10% FCS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. pEASY®-T1 and pcDNA3.1 (+) were purchased from TransGen and Invitrogen, respectively. p3 × FLAG-MYC-CMV and pEGFPC1 were purchased from Sigma and Promega, respectively.

2.3. Cloning and sequence analysis of grass carp IRAK4 cDNA

Grass carp IRAK4 full length cDNA sequence (*CiIRAK4*) was obtained by RACE-PCR. Briefly, total RNA was extracted from CIK cells using RNA simple Total RNA Kit. SMART cDNA was prepared using Super Script III reverse polymerase according to manufacturer's protocol. Based on the partial sequence of *IRAK4* from Grass Carp Genome Database (GCGD) (CI_GC_18320), four degenerate primers 5′-IRAK4-Out, 5′-IRAK4-Inner, 3′-IRAK4-Out and 3′-IRAK4-Inner (Table 1) were designed. The 5′-end was amplified by using the primer pair 5′-IRAK4-Out/UPM and 5′-IRAK4-Inner/NUP. The 3′-end was amplified by the primers 3′-IRAK4-Out/UPM, 3′-IRAK4-Inner/NUP. The PCR cycling conditions were: 95 °C/3 min, 35 cycles of 95 °C/30 s, 56 °C/30 s, 72 °C/1.5 min, and 72 °C/10 min. PCR products were ligated into pEASY-T1 vector and transformed to *E. coli* strains DH5α for sequencing by Sangon Biotech.

Polypeptide sequence prediction was determined by online-software ORF Finder (http://www.ncbi.nlm.nih.Gov/gorf/gorf.html). Known IRAK4s from different species were searched with NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast). The domain structures of *Ci*IRAK4 were predicted from SMART program (http://smart.emblheidelberg.de/smart/saveuserpreferences.pl). Multiple alignments were generated using Clustal X 2.0 and the results were subsequently edited by using GeneDoc program. The phylogenetic tree was constructed by the Neighbour-Joining algorithm implemented in MEGA software version 6.0 (http://www.megasoftware.net).

2.4. Plasmids construction

The open reading frame (ORF) of *CiIRAK4* was inserted into pcDNA3.1 (+) vector to construct the expression plasmid pcDNA3.1/*CiIRAK4* using for over-expression experiment. The ORF of *CiIRAK4* was cloned into pEGFP-C1 and p3 × FLAG-MYC-CMV for subcellular localization assay and co-immunoprecipitation assay, respectively. In addition, the ORF of *CiIRF5* was cloned into pEGFP-C1. DNA sequencing confirmed all constructs. The primers used for plasmid construction are shown in Table 1.

2.5. Q-PCR analysis of CiIRAK4 mRNA expression in grass carp tissues and cells

To determine the expression profile of *CiIRAK4*, grass carp were injected 10 mg/g bodyweight Poly I:C. The control group fish were injected with PBS. After challenged with Poly I:C for 0 h, 6 h, 12 h, 24 h, 48 h and 72 h, total RNA were extracted from liver, spleen, kidney, brain, intestine and eye tissues by using RNA simple Total RNA Kit, respectively. CIK cells were seeded in 6-well plates to obtain 80% confluence, then stimulated with poly I:C or LPS. Total RNA were extracted at different time (0 h, 6 h, 12 h, 24 h, 48 h and 72 h) post-stimulation of poly I:C or LPS. cDNA was reverse transcribed using the Prime Script RT reagent kit with gDNA Eraser Perfect Real Time. Quantitative real-time PCR (Q-PCR) was performed to detect the expression of *CiIRAK4* with *β-actin* as an internal reference gene. It was performed as described in our previous study [28]. The primers for *CiIRAK4* amplification were listed in Table 1.

2.6. Knockdown and overexpression of CiIRAK4

In RNAi-mediated gene knockdown assay, the specific siRNA sequence against *CiIRAK4* and negative control RNA oligo were designed and synthesized from Shanghai GenePharma (Table 1). The RNAi-mediated knockdown was performed according to the respective protocol guidelines as described in our previous study [27].

For overexpression of *CiIRAK4*, CIK cells were seeded in 6-well plates overnight. 6 μl FuGENE®6 Transfection Reagent were diluted in 100 μl M199 (free of FCS) and incubated for 5 min. At the same time, 2 μg plasmids of pcDNA3.1/*CiIRAK4* or pcDNA3.1 (+) were diluted in 100 μl M199 (free of FCS). Added the diluted FuGENE®6 Transfection Reagent to the diluted plasmids and mixed by vortexing. The mixture was incubated for 20 min at room temperature and dropped onto the cells.

2.7. Extraction of cytoplasmic and nuclear proteins

CIK cells were seeded in T25 culture flasks to reach ~80% confluence. Cells were harvested at 60 min post LPS treatment or 24 h post transfection and the cytoplasmic and nuclear proteins were extracted

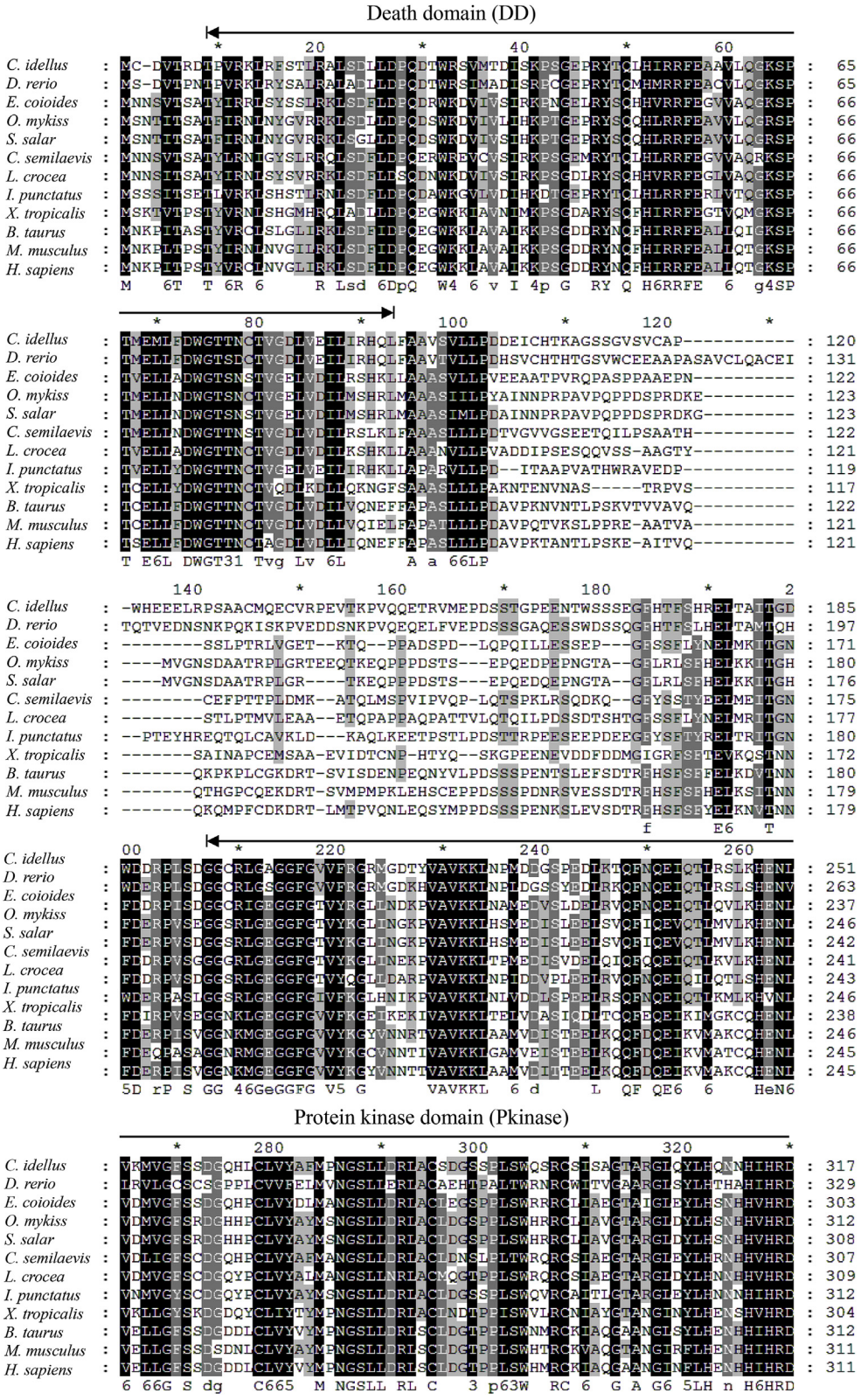

Fig. 1. Multiple alignment of *Ci*IRAK4 with the amino acid sequences of known typical organisms IRAK4s by Clustal X 2.0 program. Identical (shaded in black) and similar (shaded in gray and light gray) residues identified by the ClustalW program are indicated. Both death domain (DD) and protein kinase domain (Pkinase) are indicated with arrows.

from CIK cells using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) according to the manufacturer's instructions as described in our previous study [29]. Nuclear-cytoplasm translocation of *Ci*p65 was detected with the primary antibody against p65 by Western blot as described in Materials and methods 2.9.

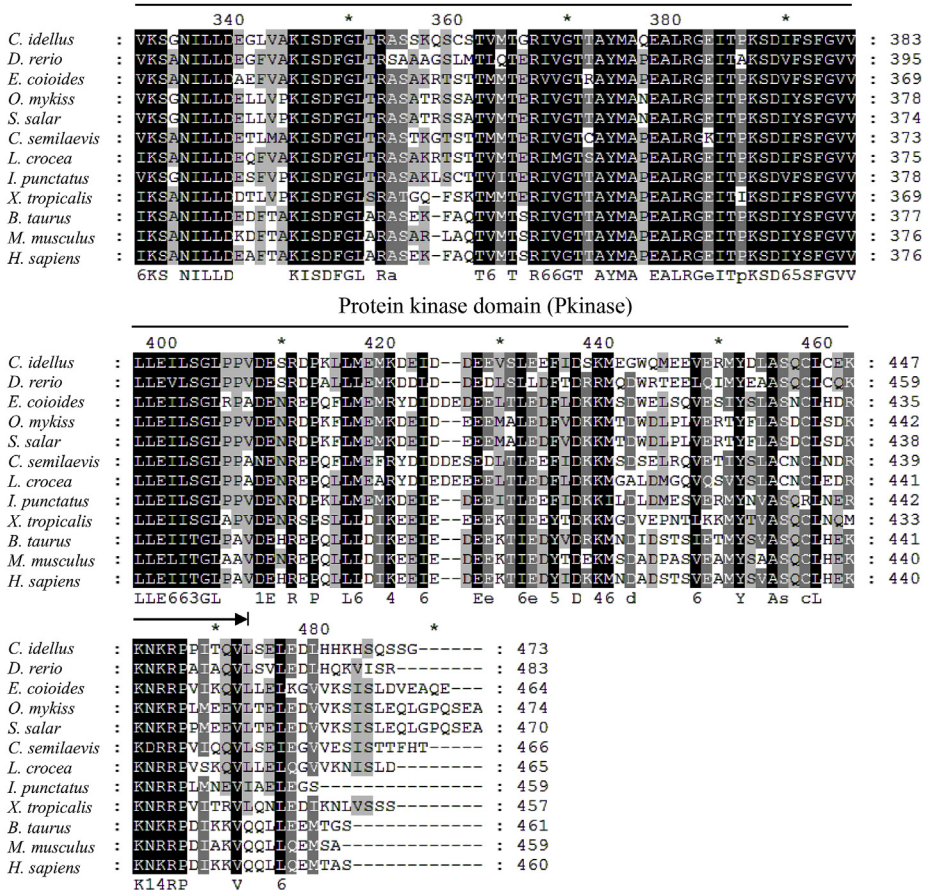

Fig. 1. (*continued*)

2.8. Subcellular localization analysis

CIK cells were plated on microscopic petri dishes to obtain 70–80% confluence, and transfected with pEGFP-C1/*CiIRAK4*. Meanwhile, CIK or HEK-293T cells were co-transfected with pEGFP-C1/*CiIRF5* and pcDNA3.1/*CiIRAK4*. After transfection for 36 h, the cells were washed three times with PBS and fixed with 4% (v/v) paraformaldehyde for 15 min at room temperature. Then, the cells were dyed with DAPI (0.1 μg/ml) and examined under a confocal microscope (Leica).

Meanwhile, the cytoplasmic and nuclear proteins were extracted

Table 1
Primers used for all the experiments.

Name	Sequence (5′ to 3′)	Application
5′-IRAK4-Out	TGCTGAACGGGTTTAGTGAC	Race-PCR
5′-IRAK4-Inner	GATGAGCCCGCTTTAGTGTGG	
3′-IRAK4-Out	GAGATGCTGTTCGACTGGG	
3′-IRAK4-Inner	ATTCTGATCCGACACCAGCTG	
Long	CTAATACGACTCACTATAGGGCAAAGCAGTGGTATCAACGCAGAGT	
Short	CTAATACGACTCACTATA	
NUP	AAGCAGTGGTATCAACGCAGAG	
IRAK4-ORF-F	ATGTGTGACGTCACGCGGG	ORF cloning
IRAK4-ORF-R	TCATCCAGAGCTCTGGGAATG	
IRAK4-RT-F	CTCCACACTGAGAGCTTTATC	Q-PCR
IRAK4-RT-R	ATGTGCAGCTGTGTGTATCT	
β-actin-F	CACTGTGCCCATCTACGAG	
β-actin-R	CCATCTCCTGCTCGAAGTC	
3.1-IRAK4-F	GCGGTACCATGTGTGACGTCACGCGGG	Eukaryotic vector construction
3.1-IRAK4-R	CGGAATTCTCATCCAGAGCTCTGGGAATG	
GFP-IRAK4-F	CGGAATTCTATGTGTGACGTCACGCGGG	
GFP-IRAK4-R	GCGGTACCTCATCCAGAGCTCTGGGAATG	
Flag-IRAK4-F	CGGAATTCTATGTGTGACGTCACGCGGG	
Flag-IRAK4-R	GCGGTACCTCATCCAGAGCTCTGGGAATG	
GFP-IRF5-F	CGGAATTCTATGAGCGGTCAACCACGGAG	
GFP-IRF5-F	GCGGTACCTTAGTGGATGTTTTGAGGCC	
IRAK4 siRNA	GGAGGUGGAGAGGAUGUAUTT	Knockdown
	AUACAUCCUCUCCACCUCCTT	
negative control	UUCUCCGAACGUGUCACGUTT	
	ACGUGACACGUUCGGAGAATT	

from CIK cells as described in Materials and methods 2.7. Nuclear-cytoplasm translocation of *Ci*IRF5 was detected with the primary antibody against mouse monoclonal antibody against GFP by western blot.

2.9. Western blotting

For Western blot analysis, CIK cells were washed twice with PBS and lysed. Proteins were detected by western blotting with different antibody as described in our previous study [27]. The membrane was exposed to a chemiluminescence Imaging System (CLINX, China).

2.10. Co-immunoprecipitation assay

In Co-IP experiments, HEK-293T cells were co-transfected with pEGFP-C1/*CiIRF5* and p3 × FLAG/*CiIRAK4*. At 36 h post-transfection, the cells were lysed and the cellular debris was removed by centrifugation at 12,000 g for 10 min at 4 °C. The supernatant was incubated with anti-Flag or anti-GFP agarose conjugate overnight at 4 °C. Then, the beads were detected by immunoblotting with the indicated antibody. It was performed using the same strategy as described in our previous study [28].

3. Results

3.1. Sequence and phylogenetic analysis of CiIRAK4

In this study, the full-length cDNA of *IRAK4* in grass carp (*CiIRAK4*) had been cloned. *CiIRAK4* was 2057 bp in length with 170 bp of 5′UTR and 465 bp of 3′UTR. The open reading frame was 1422 bp that could code a 472 amino acids peptide (Suppl. Fig. 2). The putative *Ci*IRAK4 protein possessed an N-terminal death domain (DD) and a protein kinase domain (P kinase).

To better understand the sequence similarity of *Ci*IRAK4 and that in other species, amino acid sequence multiple alignments were done. As shown in Fig. 1, IRAK4 proteins showed highly conserved among different species, especially their death domain and protein kinase domain. For instance, *Ci*IRAK4 has a similarity of 66% with IRAK4 of *Danio rerio*, 66% with *Ictalurus punctatus*, 54% with *Gallus gallus*, 55% with *Rattus norvegicus*, 62% with *Salmo salar*, 55% with *Homo sapiens*, which revealed that *Ci*IRAK4 shared homology with other fishes except with *Danio rerio* (65% similarity), and that homology was also found among mammals (Fig. 1).

Meanwhile, a phylogenetic tree was constructed with 18 known IRAK4 members from mammals, amphibian, and fish. The results showed that *Ci*IRAK4 shared high homologous (> 90% bootstrap value) with other fish IRAK4, especially with zebra fish. All of the fish IRAK4 sequences clustered together as well as diverged from their counterparts in other species (Fig. 2).

3.2. Expression analysis of CiIRAK4 in tissues and cells of grass carp

Q-PCR analysis was used to evaluate the expression profile of *CiIRAK4* mRNA in different tissues. As shown in Fig. 3A, *CiIRAK4* was expressed ubiquitously in all detected tissues (eye, liver, spleen, kidney, brain and intestine), with its higher expression in the liver. After injection with poly I:C, the expression levels of *CiIRAK4* peaked at 6 h post-treatment in most tested tissues, especially obvious in liver (74.46-fold) and spleen (10.72-fold) (Fig. 3A). Then, it declined rapidly and reached normal or lower levels at 24 h post-treatment. It reduced continually and came to the lowest level at 72 h post-treatment.

In addition, the expression patterns of *CiIRAK4* in CIK cells were examined by Q-PCR. When CIK cells were challenged with poly I:C, the level of *CiIRAK4* mRNA was increased rapidly (4.6-fold) at 6 h post-treatment, and reached the peak (21.56-fold) at 48 h post-treatment (Fig. 3B). By contrast, *CiIRAK4* was up-regulated and reached the peak (46.73-fold) at 12 h after CIK cells were treated with LPS (Fig. 3B).

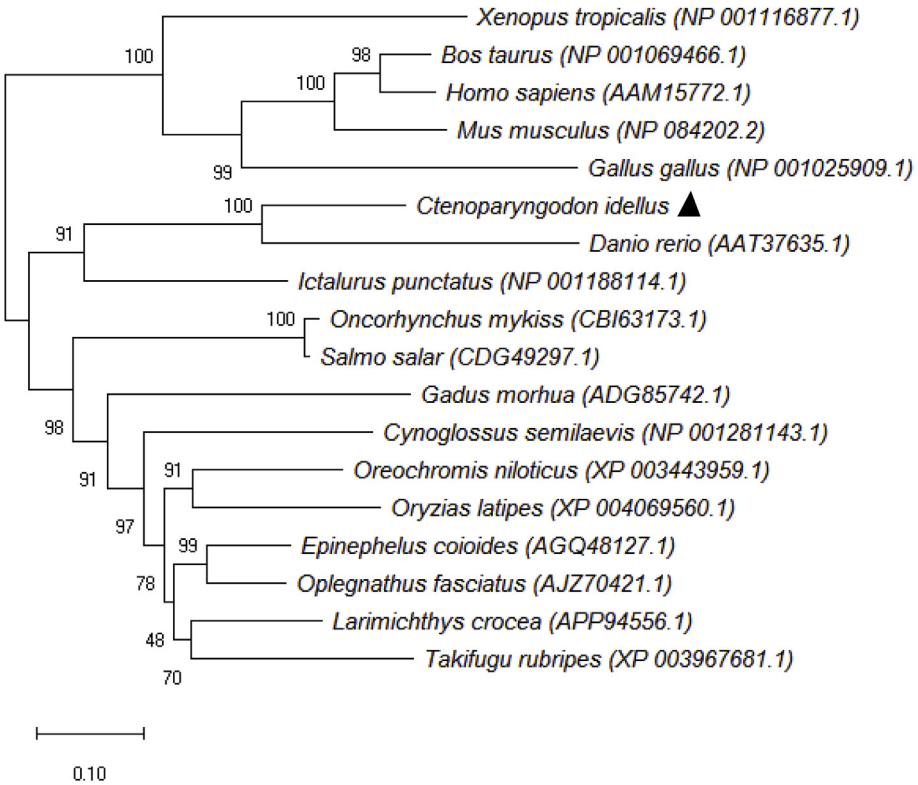

Fig. 2. Phylogenetic analysis of IRAK4s. A neighbour-joining tree was constructed by MEGA 6.0. All sequences used for analysis were derived from GenBank and their accession numbers were shown in parentheses. The bootstrap confidence values shown at the nodes of the tree were based on 1000 bootstrap replications.

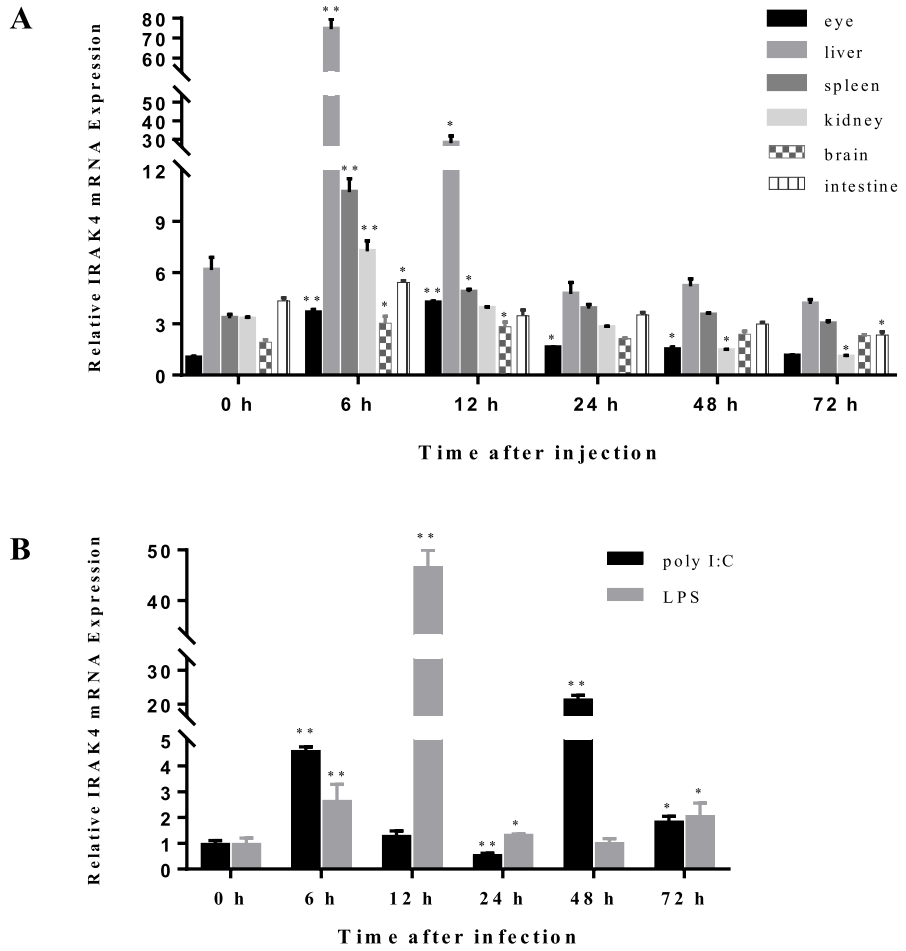

Fig. 3. Expression of *Ci*IRAK4 in response to poly I:C or LPS induction. (A) Expression analysis of *Ci*IRAK4 mRNA in grass carp tissues (eye, liver, spleen, kidney, brain, and intestine) at various time following infected with poly I:C. Eye (treatment for 0 h) was used as the calibrator (B) Expression analysis of *Ci*IRAK4 mRNA in CIK cells at various time following induced with poly I:C or LPS. The data shown were derived from a representative experiment reported as the mean (n = 3) ± S.D. * and ** represented significant ($p < 0.05$) and highly significant ($p < 0.01$), respectively.

3.3. Subcellular localization of *CiIRAK4*

To investigate the subcellular localization of *Ci*IRAK4, pEGFP-C1/*CiIRAK4* was transfected into CIK cells. The results revealed that *Ci*IRAK4 was distributed entirely in the cytoplasm, whereas GFP empty protein was located in both the cytoplasm and the nucleus (Fig. 4A). Also, it was confirmed by Western blotting analysis (Fig. 4B). When CIK cells were transfected with pEGFP-C1/*CiIRAK4*, GFP-IRAK4 fusion protein was detected by GFP antibody at the correct molecular weight.

3.4. Effect of *CiIRAK4* on NF-kappa B p65

To explore the effect of *CiIRAK4* on NF-kappa B p65, knockdown and overexpression of *CiIRAK4* assays were preformed. The level of *CiIRAK4* mRNA was diminished in the cells transfected with siRNA against *CiIRAK4* gene, confirming the siRNA specifically knocked down *IRAK4* in CIK cells. When the cells transfected with pcDNA3.1/*CiIRAK4*, *IRAK4* mRNA level was found to be up-regulated for 3.66-fold compared to the control (Suppl. Fig. 3).

After CIK cells were transfected with pcDNA3.1/*CiIRAK4* or siRNA against *CiIRAK4* gene, the cytoplasmic and nuclear proteins were extracted. As shown in Fig. 5, compared with the mock, CIK cells treated with LPS for 60 min could be observed a part of NF-kappa B p65 translocation into the nucleus. When *CiIRAK4* was overexpressed in CIK cells, it displayed that only a small number of NF-kappa B p65 were translocated into the nucleus. Meanwhile, as *CiIRAK4* knockdown in CIK cells, the proportion of NF-kappa B p65 nuclear translocation was similar to that of negative control RNA oligo and vector (pcDNA3.1) transfection alone. These data suggested that *Ci*IRAK4 might have little effect on NF-kappa B p65 nuclear translocation.

3.5. *IRAK4* promotes *IRF5* nuclear translocation

Recently, IRAK4 was verified to have a minor effect on the activation and nuclear translocation of NF-kappa B p65 [30]. Also, IRAK4 kinase activity was required for IRF5 nuclear translocation but redundant for NF-kappa B [31]. Then, we next investigated whether *Ci*IRAK4 could promote IRF5 translocation to the nucleus. pcDNA3.1/*CiIRAK4* and pEGFP-C1/*CiIRF5* were cotransfected into CIK cells. As shown in Fig. 6A, some green granules were obviously filled in the nuclear regions, suggesting that *Ci*IRF5 was translocated to the nucleus when cells cotransfected with *Ci*IRAK4. Also, this result could be found in HEK-293T cells (Suppl. Fig. 4).

Consistent with the observation above, as shown in Fig. 6B, GFP-IRF5 could be found only in the cytoplasm when CIK cells were transfected with pEGFP-C1/*CiIRF5* alone. Correspondingly, when CIK cells were co-expressed *CiIRF5* with *CiIRAK4*, some of GFP-IRF5 were translocated from the cytoplasm to the nucleus (Fig. 6B).

Subsequently, co-immunoprecipitation was used to detect if IRF5 nuclear translocation controlled by IRAK4 related to the interaction between IRAK4 and IRF5. p3 × FLAG/*CiIRAK4* and pEGFP-C1/*CiIRF5* were co-transfected into HEK-293T cells. As shown in Fig. 7, IRAK4

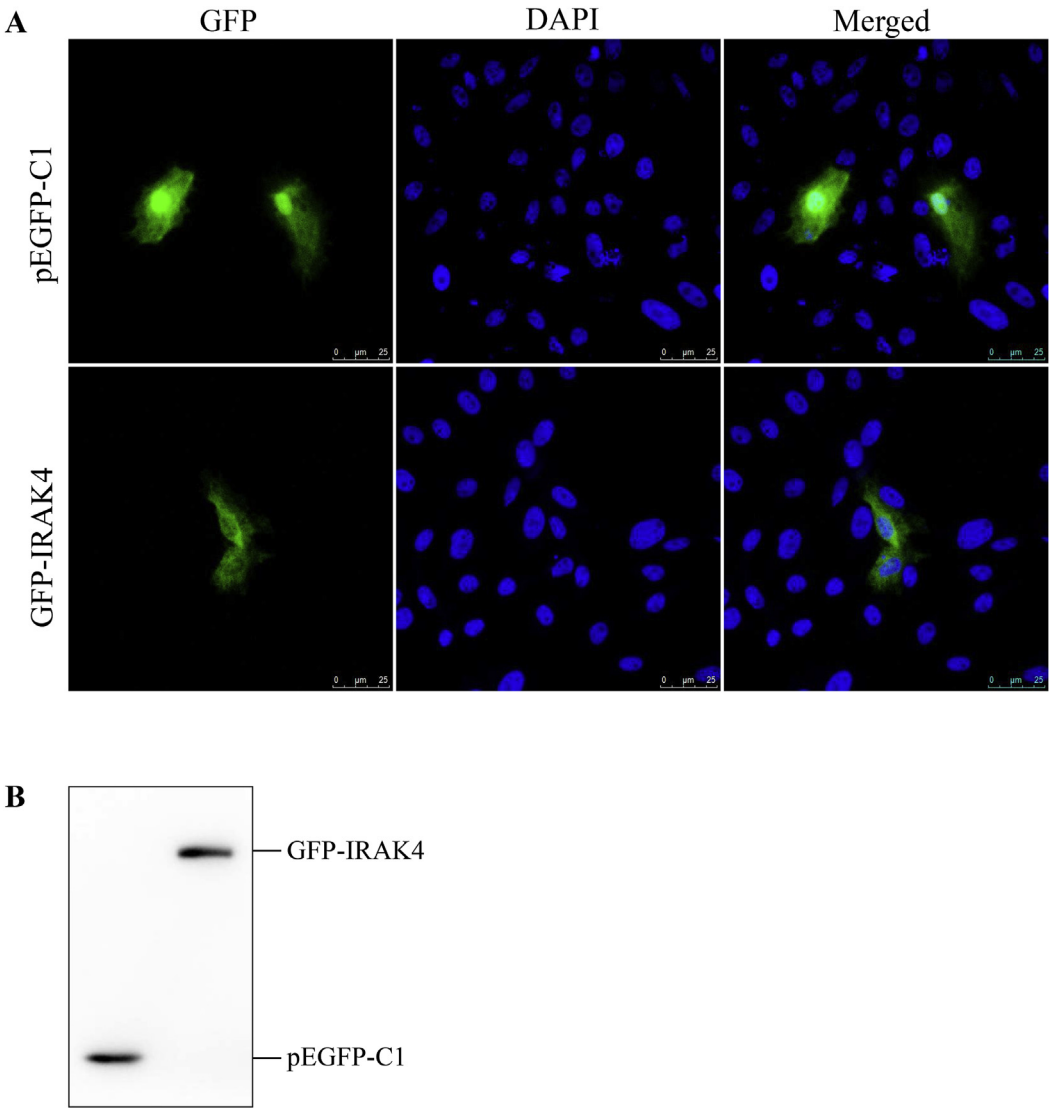

Fig. 4. Subcellular localization of *Ci*IRAK4. (A) CIK cells were seeded on microscopy dishes and transiently transfected with 2 μg of pEGFP-C1/*CiIRAK4* plasmids. After 36 h of transfection, the cells were fixed and examined with confocal microscopy. The CIK cells transfected with pEGFP-C1 were used as control. Green staining represents the *Ci*IRAK4 protein signal, and blue staining indicates the nucleus region. The results are representative of three independent experiments. Scale bars represent 25 μM. (B) The expression of GFP fusion proteins were confirmed by Western blotting analysis using mouse monoclonal antibody against GFP. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

could not directly interact with IRF5. These results suggested that IRAK4 promoted IRF5 nuclear translocation via activating some other signal factors.

4. Discussion

IRAK4 plays a pivotal role in the signaling pathways of TLRs/IL-1Rs. It can initiate a cascade of signaling events and leads to induction of inflammatory target gene expression eventually [32]. To date, some homologs of IRAK4 have been found in some different species of fish except in mammals. In this study, a novel teleost *IRAK4* cDNA was identified from grass carp. Similar to other IRAK4 subfamily member, *Ci*IRAK4 protein contained an N-terminal death domain (DD) and a C-terminal kinase domain. Customarily, MyD88 activates IRAK4 by binding to certain sites in the IRAK4 death domain and transducts signals to the downstream factors of the TLRs pathway [33]. In addition, the alignment and phylogenetic analysis also demonstrated that IRAK4 was highly conserved in vertebrates, and grass carp IRAK4 shared the highest homology with zebra fish IRAK4.

In consistent with the previous reports, *CiIRAK4* was constitutively expressed in different tested tissues, with the higher expression level in the immune-related tissues, such as liver, spleen and kidney. It indicated that *Ci*IRAK4 was essential for immune defense. Meanwhile, the expression of *CiIRAK4* was significantly up-regulated at 6 h after poly I:C stimulation, especially in the liver and spleen. Similarly, The expression of large yellow croaker *IRAK4* was significantly induced by poly I:C treatment in the spleen and liver tissues [26,34]. Additionally, some studies revealed that the expression of fish *IRAK4* is different for various PAMPs. Rock bream *IRAK4* was up-regulated after infection of rock bream iridovirus (RBIV) [25], but large yellow croaker and trout *IRAK4* showed no significant change after infection of *V. parahemolyticus* and *Aeromonas salmonicida*, respectively [23,26]. Zebrafish *IRAK4* could be observably induced by *Edwardsiella tarda*, whereas it was down-regulated after infection with snakehead rhabdovirus (SHRV) [20]. Similarly, as revealed in the present study, *CiIRAK4* mRNA was induced by poly I:C and LPS in CIK cells. The level of *CiIRAK4* mRNA was elevated first at 6 h after poly I:C treatment and declined to the normal levels, then it reached the peak at 48 h. However, *CiIRAK4* mRNA was up-regulated significantly and peaked at 12 h after LPS infection (Fig. 3B). It could be postulated that recognition of

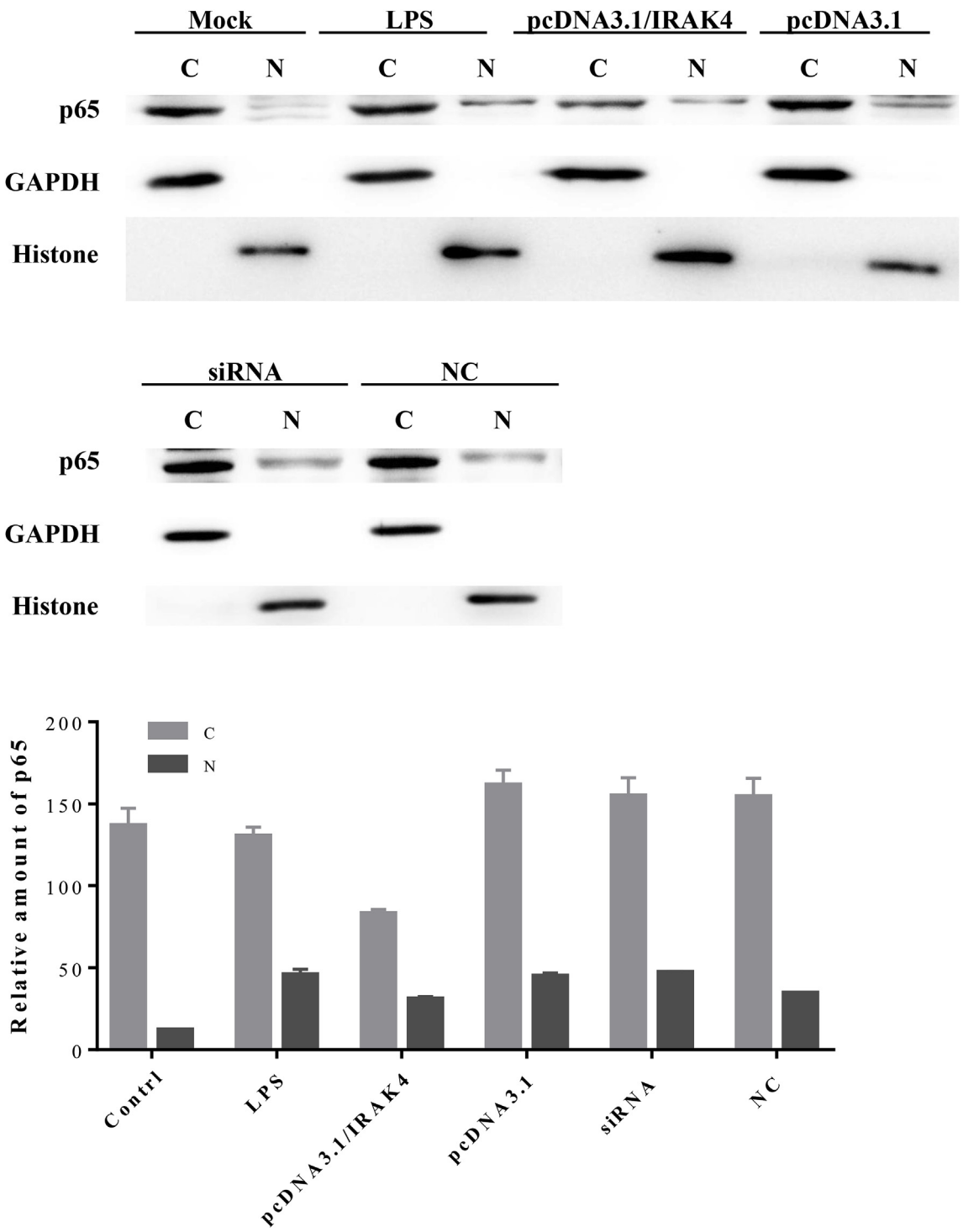

Fig. 5. *Ci*IRAK4 is dispensable for the nuclear translocation of NF-kappa B p65. CIK cells were seeded in T25 culture flasks overnight. LPS treatment, RNAi-mediated gene knockdown and overexpression of *CiIRAK4* assays were preformed, respectively. Cells were harvested at 60 min post LPS treatment or 24 h post transfection. The cytoplasmic and nuclear proteins were extracted from these cells for Western blot. GAPDH and Histone were used as internal references for cytoplasmic and nuclear proteins, respectively. The intensity of bands on a Western blot was measured by Image J (https://imagej.nih.gov/ij/). Results were shown by columns. Values were mean ± SD of three experiments. C, cytoplasmic proteins; N, nuclear proteins; mock, untreated cells; siRNA, RNAi-mediated gene knockdown of *CiIRAK4*; NC, negative control RNA oligo.

pathogenic pattern receptors by different signaling pathways might be responsible for the differential expression of IRAK4 in fish.

In MyD88-dependent pathway, upon binding of ligands, TLRs recruit the adaptor molecule MyD88 through the TIR domain. Then, MyD88 recruits IRAK4 and initiates an intracellular signaling cascade [17]. It's no doubt that IRAK4 is located in the cytoplasm in cells. Similar to other fish species [23,24,26], when GFP-tagged *Ci*IRAK4 was expressed in CIK cells, it could be obviously detected that IRAK4 distributed entirely in the cytoplasm, which was confirmed by Western blot result by using a GFP-specific antibody (Fig. 4).

It is undoubted that IRAK4 plays the critical roles in TLRs signaling. Paradoxically, the function of IRAK4 is quite diverse. In mammals, Yang

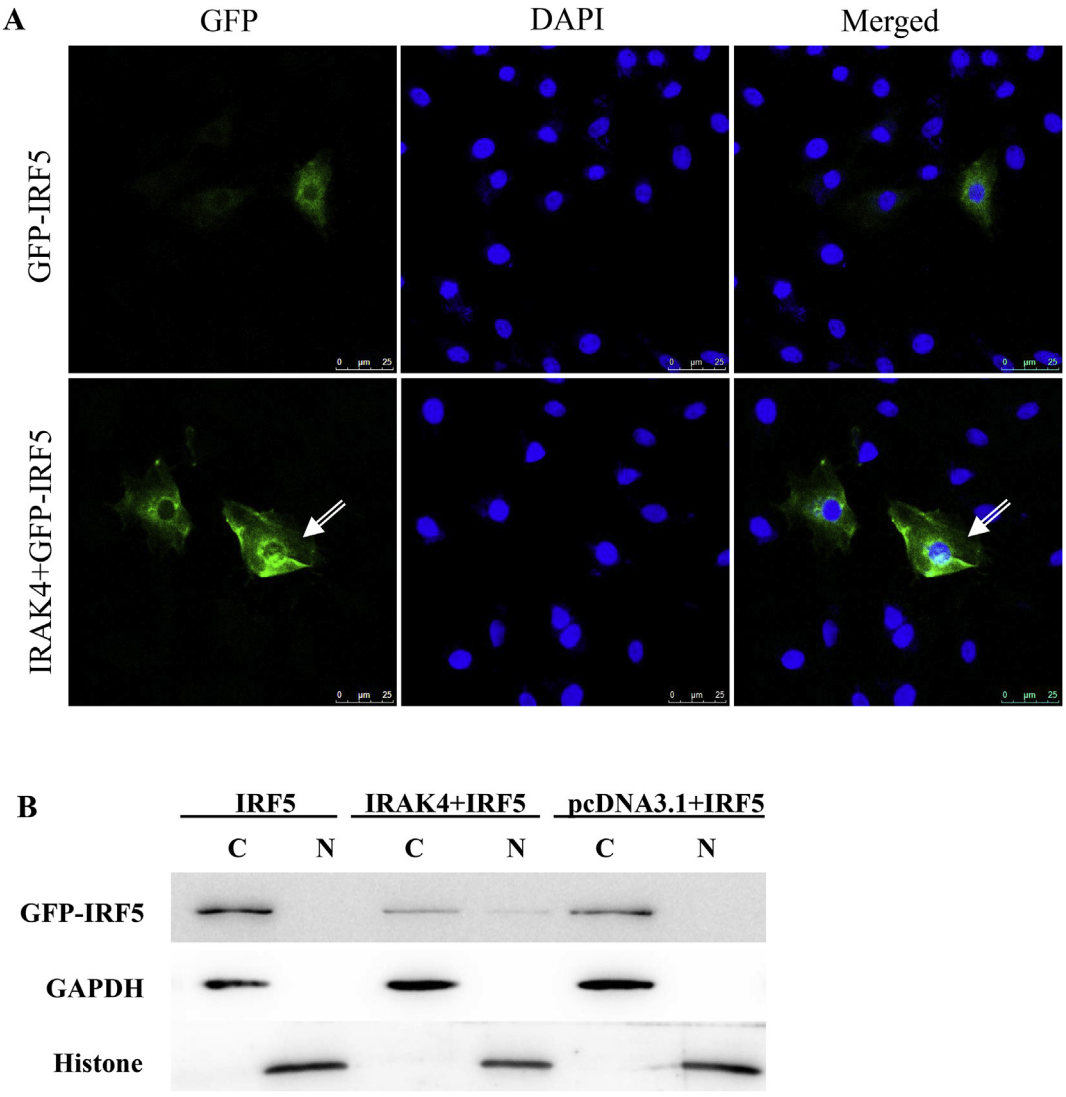

Fig. 6. IRAK4 promotes IRF5 nuclear translocation. (A) CIK cells were seeded on microscopy dishes and co-transfected with pEGFP-C1/*CiIRF5* and pcDNA3.1/*CiIRAK4*. After transfection for 36 h, the cells were fixed and examined with confocal microscopy. The transfection of pEGFP-C1/*CiIRF5* alone into CIK cells acted as a control. Green granules in nucleus represent the nuclear translocation of *Ci*IRF5 protein (indicated with arrow). The results are representative of three independent experiments. Scale bars represent 25 μM. (B) At the same time, the CIK cells were harvested and subjected to separation of subcellular components. The indicated proteins in the cytoplasmic (C) and nuclear (N) fraction were then tested by for Western blot. GAPDH and Histone were used as internal references for cytoplasmic and nuclear proteins, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

et al. found that human IFN-alpha/beta and -lambda could be induced by TLR-3 and TLR-4 agonists in IRAK-4-deficient cells. In contrast, TLR-7, -8, and -9-mediated induction of IFN-alpha/beta and -lambda was strictly IRAK-4 dependent [35]. Recent studies have demonstrated that IRAK4 played a dual role in Myddosome formation and TLR signaling. On the one hand, IRAK4 had a critical scaffold function in Myddosome formation, and the kinase activity of IRAK4 was dispensable for Myddosome assembly and activation of the NF-kappa B and MAPK pathways. On the other, IRAK4 was essential for MyD88-dependent production of inflammatory cytokines [30]. In teleost fishes, IRAK4 seems to perform different functions. In zebra fish, overexpression of IRAK-4 in ZFL cells resulted in NF-kappa B-dependent luciferase expression, indicating that zfIRAK-4 could activate the NF-kappa B signal transduction pathway [20]. In contrast, previous studies found that grouper and large yellow croaker IRAK4 could impair MyD88-mediated NF-kappa B activation in HEK-293T cells [24,26]. In the present study, we identified that *Ci*IRAK4 was unnecessary for nuclear translocation of NF-kappa B p65 (Fig. 5), which might imply that *Ci*IRAK4 has little effects on the activation of NF-kappa B p65.

Recent studies from primary human monocytes revealed that IRAK4 kinase activated TAK1 and IKK, and the activated IKK then phosphorylated IRF5 and induced nuclear translocation of IRF5. During this process, IRAK4 kinase activity was required for IRF5 nuclear translocation but redundant for NF-kappa B [31]. Here, when CIK cells were cotransfected with *CiIRF5* and *CiIRAK4*, it could be noticed that IRAK4 promoted nuclear translocation of some IRF5 (Fig. 6), which has nothing to do with the interaction between IRAK4 and IRF5 (Fig. 7). It suggested that fish IRAK4 kinase regulated IRF5 activity through indirect ways. Therefore, it could be postulated that *Ci*IRAK4 was dispensable for activation of the NF-kappa B pathways, nevertheless, it was critical for TLR7/8 cytokine responses via IRF5 in grass carp. Further work will be needed to test this hypothesis.

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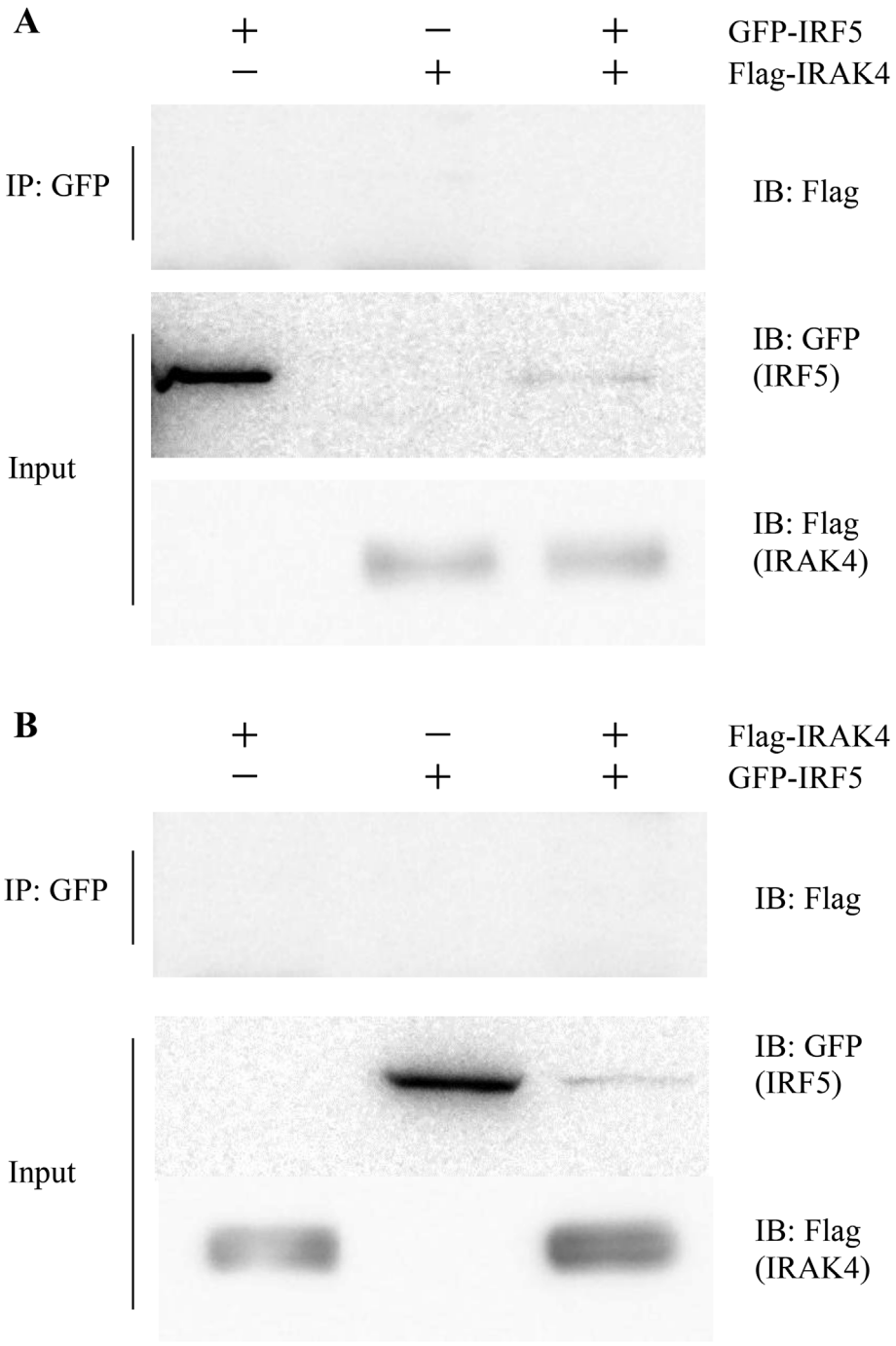

Fig. 7. IRAK4 can not directly interact with IRF5. HEK-293T cells seeded in 10 cm^2 dishes were co-transfected with 5 μg p3 × FLAG/*CiIRAK4* and pEGFP-C1/*CiIRF5* plasmid. At 48 h post-transfection, the whole-cell lysates were immunoprecipitated (IP) with either anti-Flag affinity gel (A) or anti-GFP-agarose beads (B). The immunoprecipitates and cell lysates were then analyzed by immunoblotting (IB) with the anti-Flag and anti-GFP antibody, respectively.

(GJJ161303).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.01.031.

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