Short Communication

Ctenopharyngodon idella IKKβ interacts with PKR and IκBα

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Abstract

Inhibitor of nuclear factor kappa-B kinase β (IKKβ) is a subunit of the IKK complex. It can activate the NF-κB pathway through phosphorylating IκB in response to a wide range of stimuli. In the present study, an IKKβ gene from grass carp (Ctenopharyngodon idella; KT282114) was cloned and identified by homologous cloning and rapid-amplification of cDNA ends (RACE) technique. The complete CiIKKβ cDNA is 3428 bp in length, with the longest open reading frame (ORF) of 2337 bp encoding a polypeptide of 778 amino acids. The deduced amino acid sequence of CiIKKβ has similar domain distribution to those of mammalian. For example, CiIKKβ consists of a serine/threonine kinase domain at the N-terminal, a basic region leucin zipper (BRLZ) domain in the middle, a homeobox associated leucin zipper (HALZ) domain and an IKKβ NEMO (NF-κB essential modulator) binding domain at the C-terminal. Phylogenetic tree analysis also showed that CiIKKβ is highly homologous to zebrafish IKKβ (DrIKKβ) and clearly distinct from the mammalian and amphibian counterparts. The expression of CiIKKβ was ubiquitously found in the liver, intestine, kidney, gill, spleen, heart, and brain tissues of grass carp and significantly up-regulated in CIK cells under the stimulation with Poly I:C and UV-inactivated grass carp hemorrhagic virus. To investigate the activation mechanism of NF-κB pathway in fish and the role of CiIKKβ in the pathway, we explored the protein interactions of protein kinase R (PKR) with IKKβ and IKKβ with IκBα by co-immunoprecipitation and GST-pull down assays. The interaction between each pair was confirmed. The results suggest that CiIKKβ may be a primary member in the activation of NF-κB pathway in fish.

Key words: IKKβ, PKR, IκBα, protein interaction, fish

Introduction

Nuclear factor κB (NF-κB), as a member of significant transcription factor, plays an important role in immunoreaction, inflammation, cell survival, and cell proliferation [1,2]. In mammals, NF-κB comprises five subunits, i.e. Rel A (p65), Rel B, C-Rel, NFκB1 (p50), and NFκB2 (p52) [3]. In un-stimulated cells, NF-κB is located in the cytoplasm as homodimer or heterodimer by binding with its inhibitor (IκB) which can inhibit the nuclear translocation and DNA binding activity of NF-κB dimmers [4]. When cells are exposed to various stimuli, such as bacterial or viral components, cytokines, UV irradiation, and oxidant, IκB becomes phosphorylated and then destructed by proteasome and released from the NF-κB/IκB complex [5]. Thus, the activated NF-κB is allowed to enter the nucleus and carries out its function.
Fish IKKβ interacts with PKR and IκBα

IκBα, the well-known member of the IκB family, can be phosphorylated by IKK under different kinds of stimulations. The activated IKK can bind with the serine rich region located at the N-terminus of IκBα and induces its phosphorylation [4]. So, the binding of IKKβ with IκBα and the subsequent phosphorylation are crucial to the activation of NF-κB.

IKK complex is composed of two catalytic subunits, IKKα (IKK1) and IKKβ (IKK2) [6,7], and one regulatory subunit called IKKγ or NF-κB essential modulator (NEMO) [8]. IKKα is involved in the non-canonical NF-κB activation pathway, whereas IKKβ participates in the canonical pathway when cells are subject to various types of stimulation [9]. The non-canonical pathway is activated by B-cell activating factor of the TNF family (BAFF), CD40, lymphotxin-B receptors, LPS, and some viral proteins [10,11]. In the non-canonical pathway, the response is slow but lasts longer to stimuli. However, in the canonical pathway, the response is rapid, generally brief and triggered mainly by TNF-α, IL-1, LPS, and viruses [12–13]. IKKβ is not only a key element in canonical NF-κB activation, but also involved in tumorigenesis and anti-apoptosis effect independent of NF-κB activation [14–15].

Double-stranded RNA (dsRNA)-dependent protein kinase R (PKR) is well known for its roles in immune and inflammatory reactions in response to various stimuli, such as bacterial infection, viral infection, and inflammatory cytokines [16]. Once PKR is activated by dsRNA, it can phosphorylate the alpha subunit of the eukaryotic initiation factor 2 (eIF-2α) to inhibit protein translation initiation [17]. Apart from eIF-2α, PKR is known to mediate the phosphorylation of IκBα [18]. In fact, the interaction of PKR with IKKβ is a prerequisite for the activation of NF-κB pathway [19].

Over the past few years, tremendous progress has been made in NF-κB signaling pathway in fish. Many NF-κB pathway-related genes and PKR gene have been cloned and identified in fish [20–28], however little is known about the activation mechanism of NF-κB pathway [29]. In this study, to explore the molecular mechanism of PKR-IKKβ-IκB involved in NF-κB activation in fish, we cloned and identified the full-length cDNA of Ctenopharyngodon idellus IKKβ (CiIKKβ). Phylogenetic tree analysis showed that it has a higher homologous to zebrafish IKKβ (DrIKKβ) than to the other teleost counterparts. CiIKKβ was ubiquitously expressed and significantly up-regulated after treatment with Poly I:C and UV-irradiated grass carp hemorrhagic virus (GCHV). The direct protein interactions between CiIKKβ and CiPKR, CIKKβ and CIKβα were demonstrated by co-immunoprecipitation and GST-pulldown assays. The results showed that the activation mechanism of NF-κB pathway in fish is similar to that in mammals.

Materials and Methods

Fish

Grass carp (average weight 50 g), were purchased from Nanchang Shenlong Fisheries Development Co., Ltd (Nanchang, China). They were kept in an aerated freshwater aquarium at room temperature for 2 weeks. Grass carp which had no physical malformation, swimming behavior abnormalities, or disease signs were identified to be healthy and were used in this study.

Plasmid construction

pEASY-T1, p3xFLAG-Myc-CMV™-24, pCMV-HA, pcDNA3.1, pGEX-4 T-1, and pET-32a were purchased from Transgen (Beijing, China), Sigma (St Louis, USA), Clontech (Mountain View, USA), Invitrogen (Carlsbad, USA), GE Healthcare (Bethesda, USA), and Novagen (Gibbstown, USA), respectively. pcS2-HA was kindly provided by Prof. Yiping Zhang, Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China).

The ORF of CiKKβ was generated by PCR and inserted into the HindIII/Sal I sites of p3 × FLAG-Myc-CMV™-24 to obtain the plasmid pCMV-CiIKKβ–FLAG. The PCR product of CiPKR–ORF was digested with BamHI and Xhol and then subcloned into the pcDNA3.1 vector digested at the same sites to obtain the plasmid pcDNA3.1-CiPKR.

According to a known Cikbα sequence (KF581195.1), the primers IκBα-ORF-HA-EcoRI/IκBα-ORF-HA-XhoI-R were used to clone the ORF of Cikbα. Then the sequence was inserted into the EcoRI/Xhol sites of pcS2-HA to get the plasmid pcS2-Cikbα-HA.

CiIKKβ–ORF was identified and double digested with EcoRI/ Xhol, respectively, and then subcloned into the same site of pGEX-4 T-1 expression vector. Cikbα–ORF was amplified and double digested with BamHI/Xhol, respectively, and then subcloned into the same site of pGEX-4 T-1 expression vector.

Cell lines and treatment

HEK 293 T cells were obtained from ATCC (Manassas, USA) and were grown in Dulbecco’s modified Eagle’s medium (DMEM; GE Healthcare, Bethesda, USA) supplemented with 10% fetal bovine serum at 37°C with 5% CO2. CIK (C. idellus kidney) cells were kept in our lab and cultured at 28°C in M199 (Thermo Fisher Scientific, Waltham, USA) with 10% fetal bovine serum (Invitrogen) and 1% (v/v) penicillin-streptomycin.

CIK cells were cultured overnight in 24-well plates at the density of 5 × 10⁵ cells/well at 28°C to reach a confluence of 80%–95%. The experiment group was incubated with 50 μl of Poly I:C (1 mg/ml) or UV-inactivated GCHV (~10⁷ TCID5₀ ml⁻¹) well per different time points (3, 6, 12, 24, 48, 72, and 96 h), while the control group was added with an equal volume of PBS (pH 7.4). Each group with the same time interval was repeated in triplicate. UV-inactivated GCHV was kindly provided by Prof. Pin Nie, Institute of Hydrobiology, Chinese Academy of Sciences.

Cloning of the full-length cDNA sequence of C. idellus IKKβ

RNA simple Total RNA Kit (Tiangen, Beijing, China) was used for extracting total RNA from CIK cells. SMART cDNA was prepared by SuperScript III reverse polymerase (Invitrogen). PCR was carried out in a volume of 50 μl mixture containing 5 μl 10 × LA Buffer (plus Mg²⁺), 2 μl SMART cDNA, 1 μl each primer (10 μM), 2 μl dNTP (10 μM), 0.5 μl LA Taq (5 U/ml; TaKaRa, Dalian, China), and 38.5 μl ddH₂O. All primers used in the study were listed in Table 1. Based on the DriKKβ cDNA sequence (NP_001167,377), a pair of homologous primer IΚKβ-homo-F and IΚKβ-homo-R was designed to obtain partial cDNA of grass carp IκKβ. PCR cycling parameters were: 94°C/5 min, followed by 35 cycles of 94°C/30 s, 58°C/30 s, and 72°C/1 min 30 s and then a final elongation step at 72°C/10 min. PCR products were cloned into pEASY-T1 vector (Transgen, Beijing, China) and sequenced (Shanghai Sangon Ltd, Shanghai, China). Full length of grass carp IκKβ cDNA sequence (CiIKKβ) was obtained by RACE-PCR. Primers IκKβ-5RACE-out and UPM (4 μl long + 20 μl short + 76 μl ddH₂O) were used for the first step of 5’-RACE. Conditions of the PCR cycling were: 1 cycle of 94°C/5 min; 25 cycles of 94°C/30 s, 52°C/30 s, 72°C/1 min; and 1 cycle of 72°C/10 min. Then the PCR product which was diluted by a 100 times
was used as the template for the second step of 5ʹ-RACE with primers IKKβ-3RACE-inner and NUP. The thermal cycling parameter was: 1 cycle of 94°C/5 min; 35 cycles of 94°C/30 s, 60°C/30 s, 72°C/1 min; and 1 cycle of 72°C/10 min. Primers IKKβ-3RACE-out and UPM were used for the first round of 3ʹ-RACE. PCR cycling conditions were as follows: 1 cycle of 94°C/5 min; 25 cycles of 94°C/30 s, 55°C/1 min 30 s, 72°C/1 min; and 1 cycle of 72°C/10 min. IKKβ-3RACE-inner and NUP were used to amplify 3ʹ-RACE of CiIKKβ with the product of the first round of 3ʹ-RACE which was diluted by a hundred folds. The PCR program was: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 1 min, followed by 72°C for 10 min.

**Sequence analysis and phylogenetic tree construction**

The online-software ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to predict the open reading frame (ORF) of CiIKKβ. The domain feature of the protein was analyzed by SMART program (http://smart.embl-heidelberg.de/smart/save_user_preferences.pl). The sequences of IKKβ from different species were searched by the NCBI blast server (http://www.ncbi.nlm.nih.gov/blast). The phylogenetic tree of IKKβ was constructed using Neighbor-Joining algorithm from MEGA6.0 program. Bootstrap values were calculated with 1000 replications to estimate the robustness of internal branches.

**Quantitative real-time PCR**

To eliminate the individual differences, nine healthy fishes were sacrificed for RNA extraction. Total RNA were extracted from liver, intestine, kidney, gill, spleen, heart, and brain tissues by using RNA simple Total RNA Kit (Tiangen). The mRNA was reverse transcribed into cDNA by using the PrimeScript RT reagent kit with gDNA Eraser Perfect Real Time (TaKaRa). Quantitative real-time PCR (qRT-PCR) was performed to detect the expression of CiIKKβ with β-actin as an internal reference gene on CFX ConnectTM Real-Time System (Bio-Rad, Hercules, USA). Amplification reactions were performed in triplicate in 20 μl containing 2.0 μl cDNA sample, 10 μl SYBR premix Ex Taq (TaKaRa), 0.4 μl of each primer, and 7.2 μl ddH₂O. PCR was performed under following conditions: 1 cycle of 94°C/5 min; 40 cycles of 94°C/30 s, 55°C/30 s, and 72°C/30 s. The result was analyzed by using the 2−ΔΔCt method. All experiments were repeated three times. The primers for qRT-PCR were listed in Table 1. All group data were given in terms of relative mRNA expressed as the mean (n = 3) ± SD, and then subject to Student’s t-test. Differences were considered as significant at P < 0.05, and highly significant at P < 0.01.

**Co-immunoprecipitation assay**

HEK 293 T cells were grown in complete medium in 10-cm culture plates for 24 h, and co-transfected with the plasmid pCMV-CiIKKβ-FLAG and pcDNA3.1-CiPKR. After 36 h, cells were washed twice with PBS and lysed directly on the plate with 1 ml RIPA lysis buffer containing the phosphatase/protease inhibitor cocktail (Beyotime, Shanghai, China). Cell lysates were centrifuged at 12,000 g for 10 min at 4°C to remove cell debris. For immunoprecipitation of the FLAG-tagged CiIKKβ, 50 μl of cell lysates were stored at −20°C as an input and 500 μg of the other cell extractions were incubated with 5 μg anti-FLAG antibody (Sigma) at 4°C for 3 h. As a negative control, 5 μg normal mouse IgG (Beyotime) was incubated with 500 μg of whole cell lysates. After incubation, cell lysates were added to 20 μl of protein A/G agarose (Santa Cruz) and incubated...
Figure 1. Nucleotide sequence and deduced amino acid sequence of CIKKβ

The nucleotide and amino acid sequences were numbered on the left of each line. The start codon (ATG) is boxed and the stop codon (TGA) is marked with an asterisk. The UBQ domain (339–596 aa), BRLZ domain (537–753 aa), and IKKβ NEMO binding domain (733–800 aa) are marked with a double line. The Ser/Thr kinase domain (154–408 aa), BRLZ domain (537–753 aa), and IKKβ NEMO binding domain (733–800 aa) are marked with a wave line, respectively. The start codon (ATG) is boxed and the stop codon (TGA) is marked with an asterisk. The UBQ domain (339–596 aa), BRLZ domain (537–753 aa), and IKKβ NEMO binding domain (733–800 aa) are marked with a wave line, respectively. The Ser/Thr kinase domain (154–408 aa), BRLZ domain (537–753 aa), and IKKβ NEMO binding domain (733–800 aa) are marked with a wave line, respectively. The Ser/Thr kinase domain (154–408 aa), BRLZ domain (537–753 aa), and IKKβ NEMO binding domain (733–800 aa) are marked with a wave line, respectively. The Ser/Thr kinase domain (154–408 aa), BRLZ domain (537–753 aa), and IKKβ NEMO binding domain (733–800 aa) are marked with a wave line, respectively. The Ser/Thr kinase domain (154–408 aa), BRLZ domain (537–753 aa), and IKKβ NEMO binding domain (733–800 aa) are marked with a wave line, respectively. The Ser/Thr kinase domain (154–408 aa), BRLZ domain (537–753 aa), and IKKβ NEMO binding domain (733–800 aa) are marked with a wave line, respectively. The Ser/Thr kinase domain (154–408 aa), BRLZ domain (537–753 aa), and IKKβ NEMO binding domain (733–800 aa) are marked with a wave line, respectively. The Ser/Thr kinase domain (154–408 aa), BRLZ domain (537–753 aa), and IKKβ NEMO binding domain (733–800 aa) are marked with a wave line, respectively. The Ser/Thr kinase domain (154–408 aa), BRLZ domain (537–753 aa), and IKKβ NEMO binding domain (733–800 aa) are marked with a wave line, respectively. The Ser/Thr kinase domain (154–408 aa), BRLZ domain (537–753 aa), and IKKβ NEMO binding domain (733–800 aa) are marked with a wave line, respectively. The Ser/Thr kinase domain (154–408 aa), BRLZ domain (537–753 aa), and IKKβ NEMO binding domain (733–800 aa) are marked with a wave line, respectively. The Ser/Thr kinase domain (154–408 aa), BRLZ domain (537–753 aa), and IKKβ NEMO binding domain (733–800 aa) are marked with a wave line, respectively. The Ser/Thr kinase domain (154–408 aa), BRLZ domain (537–753 aa), and IKKβ NEMO binding domain (733–800 aa) are marked with a wave line, respectively. The Ser/Thr kinase domain (154–408 aa), BRLZ domain (537–753 aa), and IKKβ NEMO binding domain (733–800 aa) are marked with a wave line, respectively.

overnight at 4°C. Agarose beads were collected by centrifugation at 1,000 g for 5 min at 4°C and washed five times with RIPA buffer, then suspended in 50 μl of 1x SDS-loading buffer and boiled for 5 min. The equal loading of sample was analyzed by western blot analysis with anti-PKR antibody which was used in our previous study [30]. For immunoprecipitation of CIKKβ, 3.5 μg anti-PKR antibody was added to the protein extracts while an equal dose of normal rabbit IgG was incubated with the cell extractions as a negative control, other steps were the same with immunoprecipitation of CIKKβ. Western blot analysis was used to detect the equivalent loading of sample with anti-FLAG antibody.

HEK 293 T cells were co-transfected with the plasmid pCMV-CiKKβ-FLAG and the plasmid pCS2-GlkBα-HA, while another two groups of HEK 293 T cells were transfected with the plasmid pCMV-CiKKβ-FLAG or the plasmid pCS2-GlkBα-HA, respectively. After 24 h, cell lysates were obtained as above. For immunoprecipitation of the FLAG-tagged CIKKβ, 50 μl of cell lysates were stored at −20°C as an input and 500 μg of the other cell extractions were incubated 40 μl anti-FLAG M2 affinity gel (sigma) at 4°C for 12 h. For immunoprecipitation of HA-tagged GlkBα, anti-HA affinity gel (sigma) was added to the cell lysates. After incubation, the affinity gels were washed five times with RIPA buffer and boiled with 2 × SDS-loading buffer. Western blot analysis was performed to test the same number of samples using anti-FLAG and anti-HA antibodies (Abmart, Shanghai, China).

Expression and purification of the recombinant CIKKβ and GlkBα

Recombinant vector pGEX-4 T-1/CiKKβ-ORF, pGEX-4 T-1/CiKβBα-ORF and empty vector pGEX-4 T-1 were transformed into BL21 (DE3; Promega, Madison, USA), respectively. Bacteria were cultured in LB medium containing 200 μg/ml ampicillin at 37°C to reach an OD600 of 0.6–0.8, then induced with 1 mM IPTG. The GST-CiKKβ, GST-GlkBα fusion protein and GST protein were induced for 12 h at 16°C. After induction, cells containing the recombinant plasmid pGEX-4 T-1/CiKKβ-ORF, pGEX-4 T-1/CiKβBα-ORF or pGEX-4 T-1 were suspended in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.3) containing 0.2 mM PMSF and 1 mM DTT. Cells were broken by using ultrasonication and the lysates were centrifuged at 12,000 g for 10 min at 4°C. Each supernatant containing GST-CiKKβ, GST-GlkBα fusion protein and GST protein was purified using glutathione Sepharose 4B resins (GE Healthcare) according to the manufacturer’s protocol. All proteins were electrophoresed in SDS-PAGE gel. Protein concentration was measured by Bradford assay and stored at −80°C for pull-down assay.

GST-pull down assay

The purified GST-CiKKβ, GST-GlkBα fusion protein and GST protein were incubated with glutathione Sepharose 4B beads (GE Healthcare), respectively, and rotated gently at 4°C for 6 h. The resins were then washed three times with washing buffer (PBS, pH 7.3, 1 mM DTT, 0.1% Triton X-100, 0.2 mM PMSF). CIPKR-ORF was generated by PCR and inserted into the EcoRI/XhoI sites of pCMV-HA. The HEK 293 T cells were transfected with the plasmid...
pCMV-GPKR-HA or pCMV-CiKKβ-FLAG. After 24 h, cell lysates were obtained and 50 μl of cell lysates were stored at −20°C as an input separately. The cell lysates which expressed GPKR-HA were mixed with the GST-CiKKβ beads, while the cell extractions which contained CiKKβ-FLAG were incubated with GST-CiBα beads. GST beads were used as a control to incubate with the above cell lysates. After overnight incubation at 4°C, the resins were washed five times with washing buffer. HA-tagged or FLAG-tagged protein was released from the beads separately by boiling in SDS-loading buffer. The same number of samples were separated on SDS-PAGE and subsequently transferred to nitrocellulose membrane (Millipore, Billerica, USA). The presence of GPKR was detected by western blot analysis with anti-HA antibody, while CiKKβ was detected with anti-FLAG antibody.

Results

Sequence characteristics of CiKKβ
The complete CiKKβ cDNA is 3428 bp in length consisting of 185 bp 5’ UTR, 906 bp 3’ UTR and an ORF of 2337 bp. No polyadenylation signal (AATAAA) or AU sequences (ATTTA) are found (Fig. 1). The ORF encodes a polypeptide of 778 amino acids containing a serine/threonine (Ser/Thr) kinase domain of the N-terminal (15–349 aa) in which there is a Ser/Thr kinase active site ‘RIHRDLK PENILVQ’ (139–154 aa) and a conserved MAPKK activation loop motif ‘SLCIT’ (177–181 aa). The ORF of CiKKβ also contains a ubiquitin homologues (UBQ) domain (339–408 aa), a basic region leucine zipper (BRLZ) domain (537–596 aa), a homeobox associated leucine zipper (HALZ) domain (547–589 aa) and an IkKβ NEMO binding domain (733–771 aa). This cDNA sequence and deduced protein sequence have been submitted to GenBank (KT282114). Phylogenetic tree analysis showed that CiKKβ is more homologous to DrIkKβ than to other teleost IkK proteins, and it is clearly distinct from its mammalian and amphibian counterparts (Fig. 2).

Tissue specific expression of CiKKβ mRNA
To quantify tissue expression profile of CiKKβ mRNA in healthy fish, qRT-PCR was performed. As shown in Fig. 3A, CiKKβ was constitutively expressed in all tested tissues including the liver, intestine, kidney, gill, spleen, heart, and brain. The CiKKβ mRNA level in liver was the highest (4.69-fold) when normalized to that in brain (1-fold).

Expression of CiKKβ in response to Poly I:C and UV-inactivated GCHV induction in CiK cells
qRT-PCR analysis showed that CiKKβ mRNA was significantly up-regulated after stimulation with Poly I:C and UV-inactivated GCHV in CiK cells (Fig. 3B,C). The trend of up-regulation of CiKKβ was the same under the treatment of Poly I:C and UV-inactivated GCHV. The expression was significantly up-regulated at 3 h post-stimulation and reached a peak at 6 h post-induction, then declined at 12 h. Later, the expression of CiKKβ was increased to a second peak at 24 h and maintained the increasing status until 96 h post-induction.

Interaction of GPKR with CiKKβ
The interaction of CiKKβ with GPKR was confirmed by co-immunoprecipitation assay. After the cell lysates were incubated with the anti-FLAG antibody pre-immobilized on protein A/G beads, GPKR was detected by western blot analysis in the anti-FLAG IP samples, but was not detected in the control (Fig. 4A). Conversely, IkKβ-FLAG was detected in IP samples which were obtained by using the anti-PKR antibody (Fig. 4B). The results confirmed the physical interaction of CiKKβ with GPKR.

GST pull-down assay was performed to further confirm the interaction between CiKKβ and GPKR in vitro. CiKKβ was expressed as a GST-fusion protein (Fig. 4C), while a GST protein of 26 kD was obtained as a negative control (Fig. 4D). GST pull-down assay was performed by incubation of immobilized GST-CiKKβ with the cell lysates containing GPKR-HA. The result demonstrated that GPKR can directly interact with GST-CiKKβ (Fig. 4E).

The interaction between CiKKβ and CiBα
The FLAG-tagged CiKKβ and HA-tagged CiBα were co-expressed in HEK 293T cells to perform co-immunoprecipitation assay. When the cell lysates were incubated with anti-HA affinity gel, HA-tagged CiBα can specifically co-immunoprecipitate with FLAG-CiKKβ (Fig. 5A). On the other hand, when the cell lysates were incubated with anti-FLAG M2 affinity gel, FLAG-CiKKβ also combined with HA-CiBα. These results confirmed a direct binding of CiKKβ with CiBα.

GST pull-down was conducted by using the beads with GST-CiBα and cell extractions expressing FLAG-tagged CiKKβ. These results also showed that GST-CiBα directly binds to FLAG-CiKKβ (Fig. 5B).
Figure 3. Expression of CiIKKβ mRNA in grass carp tissues and CIK cells

(A) Expression analysis of CiIKKβ mRNA in various tissues of healthy grass carp. The expression level of CiIKKβ mRNA in the brain was used as the calibrator (1-fold). (B,C) qRT-PCR analysis of CiIKKβ mRNA in CIK cells was carried out under Poly I:C and UV-inactivated GCHV treatment. The sample untreated with Poly I:C (0 h) and UV-inactivated GCHV (0 h) were used as the calibrator (1-fold). Each sample was run in triplicate. *P < 0.05 and **P < 0.01.

Figure 4. Binding of CiIKKβ to CPKR

(A) Co-immunoprecipitation were performed with agarose-conjugated FLAG antibody or normal mouse IgG. Western blot analysis was performed to detect PKR and IKKβ-FLAG. (B) Cell extractions were used in immunoprecipitation assays with either anti-PKR antibody or normal rabbit IgG, then IKKβ-FLAG and PKR were detected by western blot analysis. (C,D) The purified CiIKKβ and GST proteins were analyzed by SDS-PAGE respectively. Lane 1: total protein non-induced; lane 2: total protein induced by IPTG; lane 3: purified CiIKKβ and GST protein. M: PageRuler™ Prestained Protein Ladder (Pierce). (E) The purified GST-CiIKKβ or GST bound to glutathione-Sepharose beads were incubated with cell extracts which expressed CPKR-HA. After incubation, CPKR-HA was detected by western blot analysis to verify the protein binding. Sample composition is indicated above each lane.
Discussion

IKKβ is considered as the essential subunit responsible for the response of NF-κB to viral stimulation [31]. So, it is involved in the immune response to viral infection. HSV-1 can induce persistent activation of transcription factor NF-κB by activating IKK in the early phase of infection [32]. Our study showed that CiIKKβ was ubiquitously expressed in different tissues of grass carp and up-regulated by Poly I:C and UV-inactivated GCHV infection in CIK cells (Fig. 3). Similar to SclKKβ [33], CiIKKβ expression was also mainly in the liver. Furthermore, there were more than one peak in their expression patterns. So, the expression patterns are consistent with those of IKKβ in other types of organisms [34,35]. The complexity of IKKβ expression patterns may be related to the NF-κB activation. Cip65 of grass carp, one of the subunits of NF-κB, is significantly up-regulated after stimulation with Poly I:C from 3 to 96 h [24], and the expression of Cip65 is very similar to IKKβ expression. Guo et al. [36] found that viruses and viral products not only stimulate the expression level of IKKβ but also influence the activity of NF-κB. However, the biological significance is unclear and needs further investigation. Just like the expression pattern of CiIKKβ, CiPKR was also expressed ubiquitously [37] and was up-regulated in response to Poly I:C stimulation in CIK cells [38]. CiIkBa can be significantly induced by classical immune activator LPS [22]. All these results above reinforced the point that CiPKR, CiIKKβ, and CiIkBa are involved in immune response.

In mammals, PKR was reported to activate IKK through protein-protein interaction with the IKKβ subunit. Further investigations have shown that the N-terminus rather than the C-terminus of PKR participates in the binding and activation of IKK complex [19,39,40]. The two dsRBMs (1–180 aa) participate in interacting with IKKβ. In this study, the physical interaction of CiIKKβ with CiPKR was demonstrated by co-immunoprecipitation (Fig. 4A,B); while in vitro binding between CiIKKβ and CiPKR was confirmed by GST pull-down assay (Fig. 4E). The specific interaction of CiIKKβ with CiPKR is crucial for the activation of IKKβ.

IKKβ, the immediately direct kinase of IkBa, is essential for the phosphorylation of IkBa [41]. As a distinct feature of the NF-κB pathway, IkBa is phosphorylated by IKKβ through the direct combination [42]. CiIKKβ protein has the same domain distribution as mammalian’s. For example, a conserved MAPK activation loop motif ‘SLCTS’ (177–181 aa) which is essential to IKKβ activation [43] is found in CiIKKβ (Fig. 1). CiIkBa also contains highly conserved domains like those mammalian orthologues [22]. Two serine sites ‘DSGVDS’ related to its phosphorylation [44] located at the N-terminus of CiIkBa; six ankyrin repeats involved in the interaction of the nuclear localization signals (NLS) and dimerization domains of NF-κB [45] are also discovered in CiIkBa. The structural resemblance between CiIKKβ, CiIkBa and their mammalian counterparts gives a hint that CiIKKβ and CiIkBa may play the same role in the conserved NF-κB pathway. In this study, the interaction between CiIKKβ and CiIkBa was demonstrated by co-immunoprecipitation and GST pull-down (Fig. 5). However, the phosphorylation of CiIKKβ and CiIkBa induced by PKR under stimulation needs to be further investigated.

In conclusion, our results suggest that there are the same or similar molecular mechanisms as mammalian’s for NF-κB activation in fish and IKKβ may act as a primary member in the NF-κB pathway.

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References