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Identification and functional characterization of the transcription factor NF-κB subunit p65 in common carp (*Cyprinus carpio* L.)

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ABSTRACT

p65 is an important subunit of the transcription factor NF-κB in the regulation of immune response. In the present study, the p65 cDNA was identified from common carp (*Cyprinus carpio* L.) (named *Cc*p65). Phylogenetic analysis revealed that *Cc*p65 located in the same clade as piscine p65 and exhibited closest relationship to that of *Ctenopharyngodon idella*. *Cc*p65 was constitutively expressed in all the examined tissues. *Aeromonas hydrophila* and poly(I:C) can induce the expression of *Cc*p65 in the designated tissues and the *Cc*p65 expression was up-regulated in HKLs following LPS and poly(I:C) stimulation. In addition, the nuclear localization signal (NLS) and C-terminal domain are the important elements of *Cc*p65. Immunofluorescence assay revealed that the nuclear localization signal deletion mutation of *Cc*p65 (*Cc*p65ΔNLS) failed to translocate to the nucleus even though stimulation with poly(I:C) or LPS, and the C-terminal domain deletion mutation of *Cc*p65 (*Cc*p65ΔC) did not up-regulate the luciferase activity. Furthermore, *Cc*p65 can induce the expression of *il-1β* and *tnf-α*. And LPS and poly(I:C) inducing the expression of *il-1β* and *tnf-α*, is dependent on the *Cc*p65. Taken altogether, these findings lay the foundations for future research to investigate the mechanisms underlying fish p65.

1. Introduction

The nuclear factor-κB (NF-κB) pathway is an evolutionarily conserved pathway. The NF-κB consists a family of transcription factors involved in many biological processes such as immune response, inflammation, oncogenesis, cell growth [1–3], including NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), c-Rel and RelB [4,5]. NF-κB functions in the homodimer or heterodimer. Among them, the most abundant NF-κB dimer is the p65/p50 complex [6]. p65 can regulate the expression of a wide variety of genes, including TNF-α, IL-1β, IL-6, IL-8, β-defensin, iNOS, IFN-β [7,8].

Like other members of the NF-κB family, the mammalian p65 contains the conserved domain and regions, containing the N-terminal Rel-homology domain (RHD) and the transcription activation domain (TAD). Among these, the RHD with a length of 300 amino acids consists of a nuclear localization sequence (NLS), a DNA binding domain, a NF-κB inhibitor protein (IκB) family binding region and a dimerization domain [9]. And the C-terminal TAD can regulate the transcription activity of target genes [10,11]. A wide range of soluble and membrane-bound extracellular ligands activate the NF-κB pathway, most notably through the members of TNFR, TLR, IL-1R, and antigen receptor superfamilies [12]. When the signaling pathway triggered, IκB is phosphorylated and degraded by proteasome [13], and then the NF-κB is released from IκB proteins and translocates into the nucleus [14]. In the nucleus, NF-κB can up-regulate the transcription of specific genes. Does fish p65 possess the same function as the mammalian counterparts?

The p65 has been reported in freshwater and marine organisms. For instance, the *Carcinoscorpius rotundicauda* NF-κB plays an archaic but fundamental role in regulating the expression of critical immune defense molecules [15]. *Carcinoscorpius rotundicauda* p65 is evolutionarily and functionally conserved and functions in the expression of immune-related genes [16]. NF-κB/IκBα pathway in *Siniperca chuatsi* plays a role in the immune response against ISKNV [17]. *Ctenopharyngodon idella* p65 can regulate the expression of IκBα and work as a negative feedback loop in NF-κB pathway [18]. Common carp (*Cyprinus carpio* L.) is a freshwater fish that is widespread in Europe and Asia and accounts for

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up to 10% (over 3 million metric tons) of global annual freshwater aquaculture production [19]. Moreover, it is an auspicious symbol in traditional Chinese culture. There is currently increased interest in understanding the molecular mechanisms underlying the immune defenses of common carp (*Cyprinus carpio* L.). NF-κB pathway plays an important role in the TLRs signaling and other receptors-dependent signaling in mammals. Recent years, several TLRs are found in common carp [20–23], however, the underlying mechanism between TLRs and NF-κB pathway is still uncertain.

The aim of the present study was to characterize the p65 subunit of NF-κB from common carp and provide new sights into the molecular mechanism of the TLRs-dependent signaling. In this study, we identified the common carp p65 (*Cc*p65) and showed the expression of *Cc*p65 under normal condition and different stimuli. Then its activation mechanism and transactivation properties are evolutionarily entrenched. Furthermore, the expression of the NF-κB target genes such as il-1β and tnf-α was analyzed.

2. Materials and methods

2.1. Cloning of Ccp65

Partial cDNA sequence of *Cc*p65 was obtained from the conserved region of reported p65 sequences. First strand cDNA was synthesized from head kidney derived RNA. To obtain a full-length cDNA sequence of *Ccp65*, rapid amplification of cDNA ends (RACE) was used. The 3′ and 5′-RACE was performed using 3′-full RACE core set (TaKaRa) and SMARTer® RACE 5′ Kit (Clontech) following the manufacture's instructions. The primers used for gene cloning are shown in Table 1.

2.2. Sequence and phylogenetic analysis

The protein structures of the target genes were predicted using Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). A phylogenetic tree was constructed by neighbor-joining method in MEGA 6.0 software. The GenBank accession numbers for these sequences were listed in Supplementary Table 1.

2.3. Fish rearing

The common carp (*C. carpio* L.) were obtained from a local fish farm. These fish, with body weight of approximately 180 g, were reared in tanks at 25 °C and were fed daily on a commercial carp diet. After one week of acclimatization, the fish were subjected for further experiments. All surgery was performed under anesthesia, and all efforts were made to minimize the suffering of fishes.

2.4. Cell culture

293T cells and HeLa cells were cultured in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin (Gibco) and 100 mg/ml streptomycin (Gibco). Both cells were maintained in cell culture flasks and incubated at 37 °C in a humidified atmosphere of 5% CO_2. Epithelioma papulosum cyprinid (EPC) cells, obtained from Fresh Water Fishery Research Institute of Shandong Province, were maintained in M199 medium (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco), 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco).

2.5. Immune challenge and sampling

The immune challenges devided into *Aeromonas hydrophila* and poly (I:C) challenge groups. The *A. hydrophila* challenge was performed according to the previous protocols [21,24]. Briefly, *A. hydrophila* was incubated in Luriae Bertani (LB) medium at 30 °C overnight with shaking. Fish were injected intraperitoneally with formalin (overnight at 4 °C in 0.5% formalin) inactivated *A. hydrophila* (5×10^7 CFU per fish).

Immunostimulation of poly(I:C) was performed according to the previously described protocols [25,26]. Briefly, poly(I:C) (polyinosinic-polycytidilic acid, SIGMA) was dissolved in sterile phosphate buffered saline (PBS). All experimental fishes were injected intraperitoneally with poly(I:C) at a dose of 0.8 mg/ml per fish, while control carps were injected with the same volume of sterile PBS.

Six tissues (i.e. liver, spleen, head kidney, foregut, hindgut and skin) of three infected fishes were collected at the indicated time points.

Table 1
Primer sequences used in this study.

Primer	Sequence (5′-3′)	Application
p65-F	ATGGACGGACTGTTTCACCAGTGG	cloning for p65
p65-R	ACGCTTCTCCATCAGCCTGTGCTC	cloning for p65
p65-5'outer	GCACTCTCAGCGGCCCGCTGTAGT	3′-RACE
p65-5'inner	GGAAGCGCATCCCTCGGGCTTTCG	5′-RACE
p65-3'outer	GGTGGCTATCGTCTTCCGCACG	5′-RACE
p65-3'inner	GGTGCGTGTGAAGATGCAGCTG	3′-RACE
5′Outer	TAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	5′-RACE
5′Inner	CTAATACGACTCACTATAGGGC	5′-RACE
3′ Outer	TACCGTCGTTCCACTAGTGATTT	3′-RACE
3′ Inner	CGCGGATCCTCCACTAGTGATTTCACTATAGG	3′-RACE
S11-F	CCGTGGGTGACATCGTTACA	Real-time PCR
S11-R	TCAGGACATTGAACCTCACTGTCT	Real-time PCR
rtp65-F	AACCAAGAACCAGCCGTACAAGC	Real-time PCR
rtp65-R	ACTGTGTATCCTCCGCTCCTGTAG	Real-time PCR
p65-*Hin*dIII-F	CCCAAGCTTATGGACGGACTGTTTCACCAGT	Recombinant plasmid
p65-*Kpn*I-R	CGGGGTACCGTGGTGGGGTGTCCGGACAGA	Recombinant plasmid
P65-ΔNLS-F	ATGAGCACCGGCTGATGGAGACAGAAGGGATGCTGCAGAA	NLS deletion
P65-ΔNLS-R	TTCTGCAGCATCCCTTCTGTCTCCATCAGCCGGTGCTCAT	NLS deletion
P65-ΔT-F	GTATAGCTAAACGAACACTGACGGTACCGCGGGCCCGGGA	C-terminal domain deletion
P65-ΔT-R	TCCCGGGCCCGCGGTACCGTCAGTGTTCGTTTAGCTATAC	C-terminal domain deletion
EPCTNF-α-F	GTGATGGTGTCGAGGAGGAAG	Real-time PCR
EPCTNF-α-R	TCTGAGACTTGTTGAGCGTGAA	Real-time PCR
EPCIL-1β-F	CCCAGACCAATCTCTACCTCGCT	Real-time PCR
EPCIL-1β-R	GAGGAGGTTGTCATTCTGGTCACC	Real-time PCR
EPCβ-actin-F	GCCGTGACCTGACTGACTACCT	Real-time PCR
EPCβ-actin-R	GCCACATAGCAGAGCTTCTCCTTG	Real-time PCR

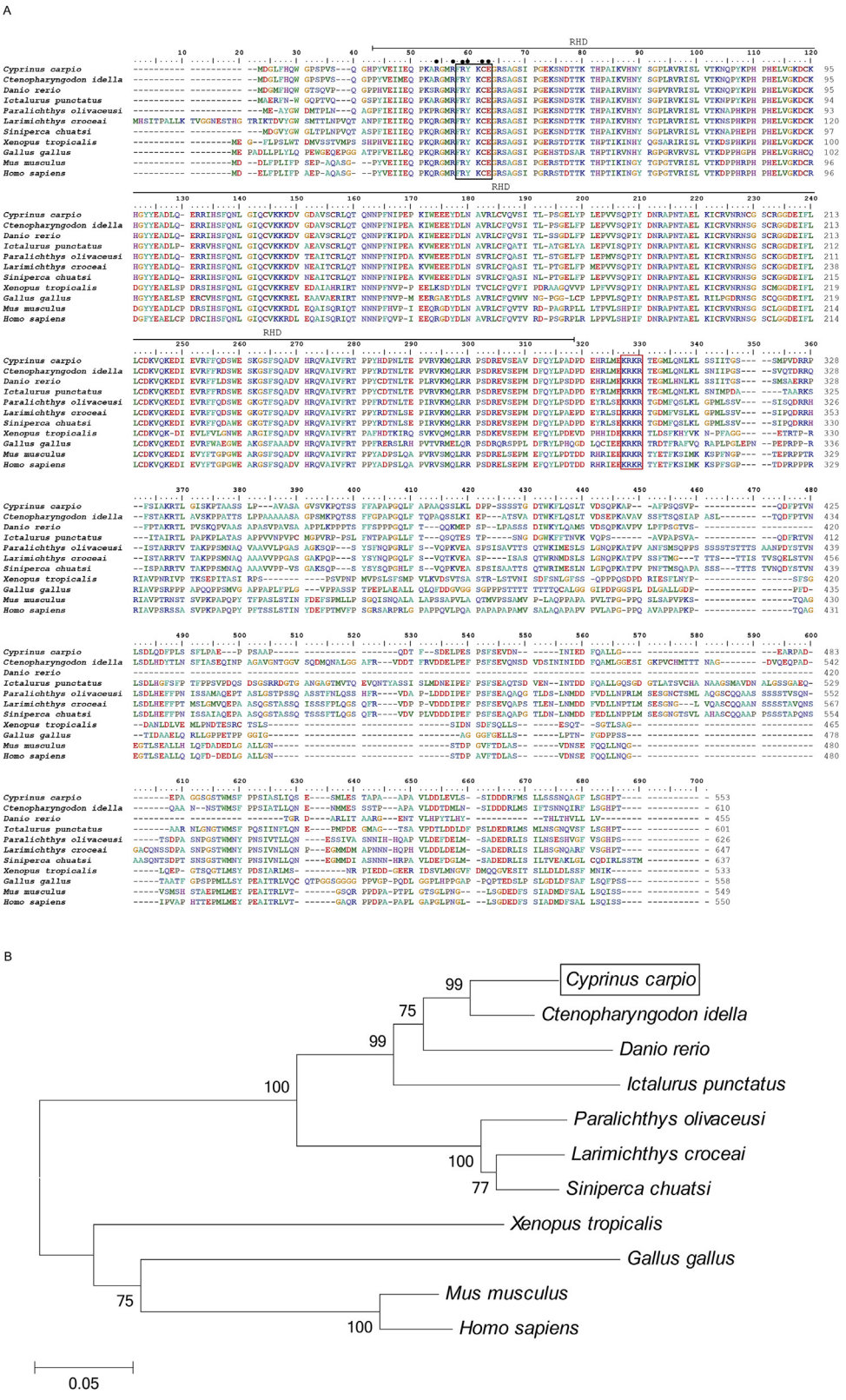

Fig. 1. Multiple alignment and Phylogenetic analysis of p65. (A) The sequences were aligned using the Clustal W method. The identical, conservative and highly conservative substituted amino acid residues are indicated in (*), (.) and (), respectively. The Rel-homology domain was indicated in the picture. The black filled circles denote the binding motifs, the black box denotes the Rel protein signature, and the red box indicates the nuclear localization signal. The GenBank accession numbers are shown in Supplementary Table 1 (B) Phylogenetic analysis between *Ccp*65 and other p65 proteins. Phylogenetic tree was constructed by the neighbor-joining method in MEGA 6.0. The numbers at tree nodes indicate the boot-strap percentage of 1000 bootstrap samples. The frame represents the *Cyprinus carpio* p65. The GenBank accession numbers for these sequences are listed in Supplementary Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Tissue samples were stored in liquid nitrogen until subsequent analyses.

2.6. Isolation of carp leukocytes from head kidney

Carp leukocytes were prepared by percoll density gradient centrifugation as described previously [27,28]. For the isolation of head kidney leukocytes (HKLs), head kidneys were collected from freshly killed carp under sterile conditions by gently pressing through a 100-m stainless steel mesh with the aid of a plunger. Subsequently, head kidney leukocytes (HKLs) were isolated using a 51/34% non-continuous percoll gradient. The cells were resuspended in complete L-15 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco). After recovering overnight at 25 °C, drug treatment was performed using poly

Table 2
Percent identity of p65 between common carp and other species.

Species	Identity (%)	
	Full length	RHD
Homo sapiens	50.6	71.3
Mus musculus	49.5	73.0
Gallus gallus	45.6	64.5
Xenopus tropicalis	49.9	70.6
Danio rerio	76.3	91.0
Ictalurus punctatus	71.0	88.2
Ctenopharyngodon idella	84.3	96.2
Siniperca chuatsi	63.6	85.8
Paralichthys olivaceus	63.3	84.4
Larimichthys crocea	64.5	84.4

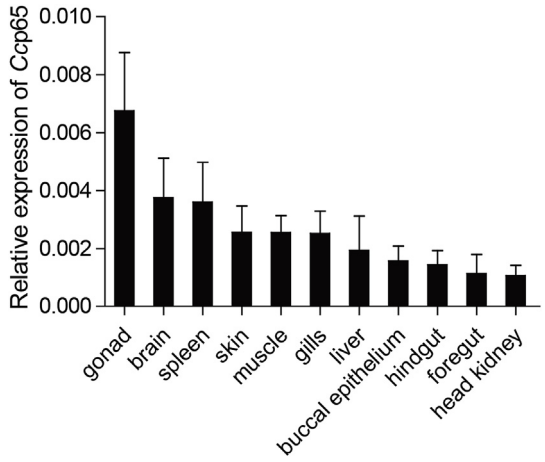

Fig. 2. Tissue expression of *Cc*p65 in normal common carp. The mRNA expression of *Cc*p65 in the liver, spleen, head kidney, foregut, hindgut, skin, gills, gonad, muscle, buccal epithelium and brain, which detected by real-time PCR. The 40S ribosomal protein *s11* was amplified in each tissue sample as internal control and the results were calculated relative to the expression of the 40S ribosomal protein *s11*, n = 3.

(I:C) (5 μg/ml, SIGMA), LPS (10 μg/ml, SIGMA), peptidoglycan (PGN) (10 μg/ml, SIGMA), flagellin (10 ng/ml, SIGMA), and Pam3CSK4 (10 ng/ml, InvivoGen).

2.7. RNA extraction and qRT-PCR assay

Total RNA was extracted from various tissues by using the RNA simple Total RNA kit (TIANGEN) according to manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) analysis was used to detect the expression of studied genes. The sequences of the gene-specific primers used for the qRT-PCR are listed in Table 1. qRT-PCR was performed on a LightCycler® 96 using TransStart Tip Green qPCR SuperMix (Transgen). Reaction was initiated at 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s and 60 °C for 30 s. The 40S ribosomal protein S11 gene was amplified in parallel as a housekeeping control for normalization [29,30]. All samples were analyzed in triplicate. The real-time PCR data were analyzed using the $2^{-\triangle\triangle CT}$ method.

2.8. Construction of recombinant plasmid

The pEGFP-N1 plasmids were used in the construction of expression vectors. Full-length open reading frames of *Ccp65* were amplified with the specific primers (Table 1) and then digested with the corresponding restriction enzymes (*Hin*dIII/*Kpn*I for *Cc*p65). Next, digested *Cc*p65 sequence was ligated into pEGFP-N1 to construct *Cc*p65-pEGFP-N1 recombinant plasmid. The p65 C-terminal domain deletion mutation (*Cc*p65ΔC-pEGFP-N1) and the nuclear localization sequence (NLS) deletion mutation of p65 (*Cc*p65ΔNLS-pEGFP-N1) was amplified with the specific primers (Table 1).

2.9. Transfection and expression profiles of immune-related genes

EPC cells were transfected in 24-well plates using JetPEI transfection reagent (PolyPlus Transfection) with *Cc*p65-pEGFP-N1 or empty vector according to the manufacturer's instructions. Briefly, EPC cells were seeded in 24-well plates at a concentration of 2×10^5 cells and transfected with plasmids using *Cc*p65-pEGFP-N1 or empty vector according to the manufacturer's instructions. After transfection with *Cc*p65-pEGFP-N1 or empty vector for 36 h, the cells were incubated for 12 h in the presence of PBS or 10 μg/ml of LPS or 5 μg/ml of poly(I:C). Then EPC cells were collected for quantification of associated immune molecules expressions (*il-1β* and *tnf-α*). The primers are shown in Table 1.

2.10. Confocal fluorescence microscopy

HeLa cells were plated onto coverslips in a 24-well plate. After reaching 50% confluency, HeLa cells were transfected with the indicated plasmids using Lipofectamine 2000 reagent (Invitrogen). At 36 h post-transfection, the cells were incubated for 1 h in the absence or presence of 5 μg/ml of poly(I:C) or 10 μg/ml of LPS, then fixed with 4% paraformaldehyde in PBS, mounted using DAPI-mounting medium. Finally, the stained cells were viewed under a Leica TCS SP8 MP multiphoton microscope and subsequently analyzed with ImageJ software.

2.11. Luciferase activity assays

293T cells were transfected in 96-well plates using Lipofectamine 2000 reagent (Invitrogen) with *Cc*p65-pEGFP-N1, or *Cc*p65 ΔC-pEGFP-N1 or empty vector together with κB element-driven reporter plasmid. The rhRL-TK vector was used as an internal control. After transfection for 24 h, the cells were washed with PBS and lysed with Dual-Glo® Luciferase Reagent (Promega). Afterwards, *Firefly* and *Renilla* luciferase activities were measured. The *Firefly* luciferase activity was normalized to that of *Renilla*. Data from three independent replicates were calculated.

2.12. Statistical analysis

Statistical analysis was carried out using the GraphPad Prism 5.0 software. The results of three independent experiments are expressed as means ± SD. The two-way ANOVA was used to assess the significant difference of gene expression levels in various tissues at different time points post-induction. *P* values of less than 0.05 were considered statistically significant ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

3. Results

3.1. Sequence characteristics of Ccp65

The full-length of *Cc*p65 cDNA was 2352 bp along with 5′UTR of 116 bp, 3′UTR of 574 bp and a 1662 bp largest open reading frame (GenBank accession No. MN167531). The open reading frame encoded a polypeptide of 553 amino acids with a molecular weight of 60.947 kDa. The protein structure of *Cc*p65 was predicted by SMART analysis, containing a well conserved Rel-homology domain (RHD) at N-terminal and a putative transcription activation domain (TAD) at C-terminal. RHD contains DNA binding motif "RGMRFRYKC" (29–37 aa), Rel protein signature "FRYKCE" (33–38 aa) and a nuclear localization signal "KRKR" (300–303 aa) (Fig. 1A).

Sequence alignment of p65 showed that identities of 50.6, 49.5, 45.6, 49.9, 76.3, 71.0, 84.3, 63.6, 63.3, 64.5% between the *Cc*p65 and

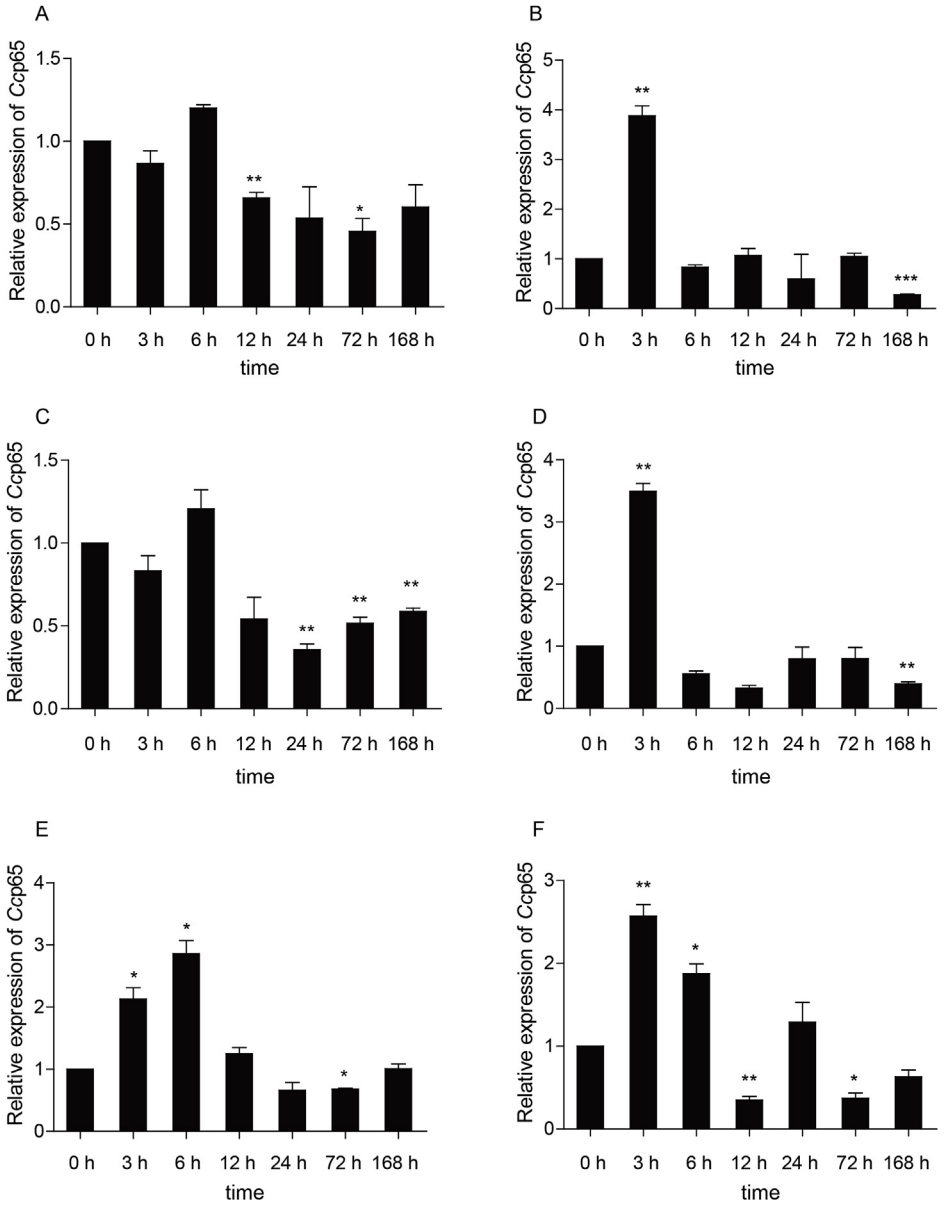

Fig. 3. The relative expression of *Cc*p65 in various tissues of common carp after i.p. injection with *A. hydrophila*. The expression of *Cc*p65 in the liver (A), spleen (B), head kidney (C), foregut (D), hindgut (E) and skin (F) at different time points is shown. The results were calculated relative to the expression of the 40S ribosomal protein *s11*. Data are presented as a fold increase to the unstimulated control group (denoted by 0 h). Mean ± SD (n = 3), $^{*}P < 0.05$, $^{**}P < 0.01$.

that of *Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Xenopus tropicalis*, *Danio rerio*, *Ictalurus punctatus*, *Ctenopharyngodon idella*, *Siniperca chuatsi*, *Paralichthys olivaceus*, *Larimichthys crocea* (Table 2). Particularly, the RHD of *Cc*p65 showed high identity with other species, with 64.5–96.2% identity with its counterparts (Table 2).

To examine the phylogenetic relationship between common carp p65 and p65 from other species, an unrooted phylogenetic tree was built with the neighbor-joining method based on deduced amino acid sequence using the MEGA6 program. Phylogenetic tree analysis showed that fish p65 clustered into one clade. Furthermore, *Cc*p65 had the closest relationship with *C. idella* p65, followed by *D. rerio* p65 (Fig. 1B).

3.2. Tissue expression profile of *Ccp65*

To investigate the expression profile of *Cc*p65, real-time PCR analysis was performed. The results showed that *Cc*p65 was constitutively expressed in different tissues or organs of healthy common carp, including the liver, spleen, head kidney, foregut, hindgut, gills, skin, muscle, brain, gonad and buccal epithelium. The most predominant expression of *Cc*p65 was observed in the gonad, whereas the lowest expression was observed in the head kidney (Fig. 2).

3.3. Expression profiles of *Ccp65* after *A. hydrophila* injection

To determine if *Cc*p65 was involved in response to different stimulants, real-time PCR analysis was conducted in six tissues, i.e., the liver, spleen, head kidney, foregut, hindgut and skin following stimulation with inactivated *A. hydrophila* (Fig. 3) and poly(I:C) (Fig. 4). The expression profile of *Cc*p65 after killed *A. hydrophila* injection was shown in Fig. 3. Significant up-regulation of *Cc*p65 was observed in the spleen, foregut, hindgut and skin, whereas down-regulation was observed in the liver and head kidney. In the spleen, foregut and skin, the *Cc*p65 expression was induced and peaked at 3 hpi (3.9-fold, $P < 0.01$; 3.5-fold, $P < 0.01$; 2.6-fold, $P < 0.01$; respectively) and then subsequently down-regulated (Fig. 3B, D and F). *Cc*p65 expression in the

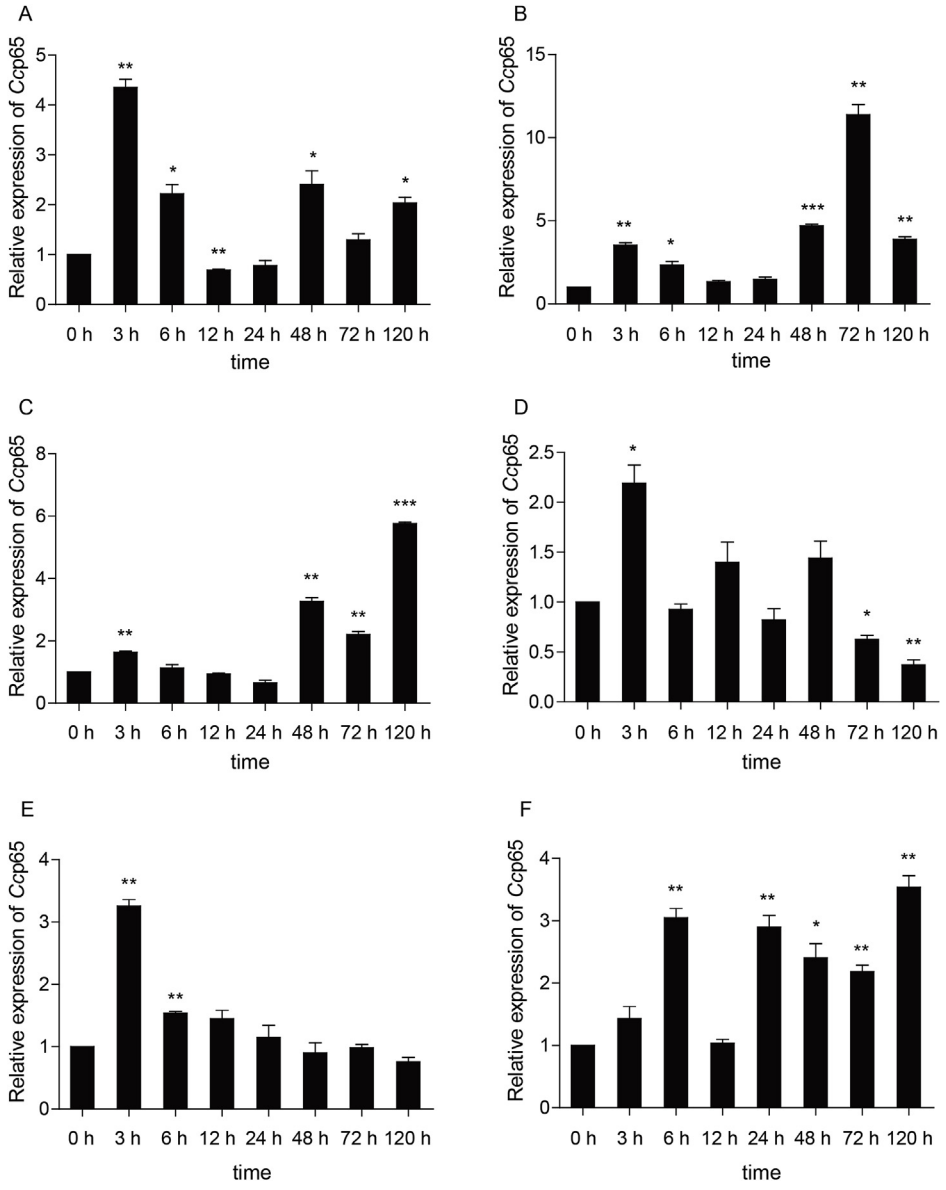

Fig. 4. The relative expression of *Cc*p65 in various tissues of common carp after intraperitoneal injection with poly(I:C). The mRNA expressions of *Cc*p65 in the liver (A), spleen (B), head kidney (C), foregut (D), hindgut (E) and skin (F) at different time points are shown. Gene expression results were calculated relative to the expression of 40S ribosomal protein *s11*. Data are presented as the fold changes based on unstimulated control group (denoted by 0 h). Mean ± SD (n = 3), $^{*}P < 0.05$, $^{**}P < 0.01$.

hindgut was induced at 3 h and reached a peak value at 6 h (2.9-fold, $P < 0.05$) after *A. hydrophila* stimulation (Fig. 3E). However, in the liver and head kidney, the expression of *Cc*p65 was down-regulated to a low level at 72 hpi (0.46-fold, $P < 0.05$) and 24 hpi (0.36-fold, $P < 0.01$) respectively (Fig. 3A and C).

3.4. Expression profiles of Ccp65 after poly(I:C) injection

The above results showed that expression of *Cc*p65 was up-regulated in the spleen, foregut, hindgut and skin after challenge with *A. hydrophila*, indicating that *Cc*p65 might be involved in the antibacterial immune response. Whether *Cc*p65 participates in antiviral immunity was also investigated. The common carp was injected intraperitoneally with poly(I:C), and the mRNA expression level of *Cc*p65 was detected at 3 h, 6 h, 12 h, 24 h, 48 h, 72 h and 120 h post injection. After injection with poly(I:C), significant up-regulation of *Cc*p65 was observed in all the tested tissues, including the liver, spleen, head kidney, foregut, hindgut and skin. In the liver, foregut, and hindgut, the expression of *Cc*p65 was induced and reached a peak level at 3 h (4.4-fold, $P < 0.01$; 2.2-fold, $P < 0.05$ and 3.3-fold, $P < 0.01$, respectively) and subsequently down-regulated following poly(I:C) stimulation (Fig. 4A, D, E). The expression level of *Cc*p65 were fluctuated in the spleen, head kidney and skin. The expression level of *Cc*p65 in the spleen was increased at 3 h, peaked at 72 h (11.4-fold, $P < 0.01$) and down-regulated at 120 h after stimulation with poly(I:C) (Fig. 4B). Similarly, the expression of *Cc*p65 reached a peak value at 120 h (5.8-fold, $P < 0.001$ and 3.5-fold, $P < 0.01$, respectively) in the head kidney and skin (Fig. 4C and F).

3.5. Inductive expression of Ccp65 in isolated head kidney leukocytes (HKLs) after stimulation with TLR ligands

Furthermore, the expression levels of *Cc*p65 in isolated HKLs were determined after stimulation with poly(I:C), LPS, PGN, flagellin and Pam3CSK4. As shown in Fig. 5, the expression of *Cc*p65 was significantly up-regulated after stimulation with poly(I:C) and LPS,

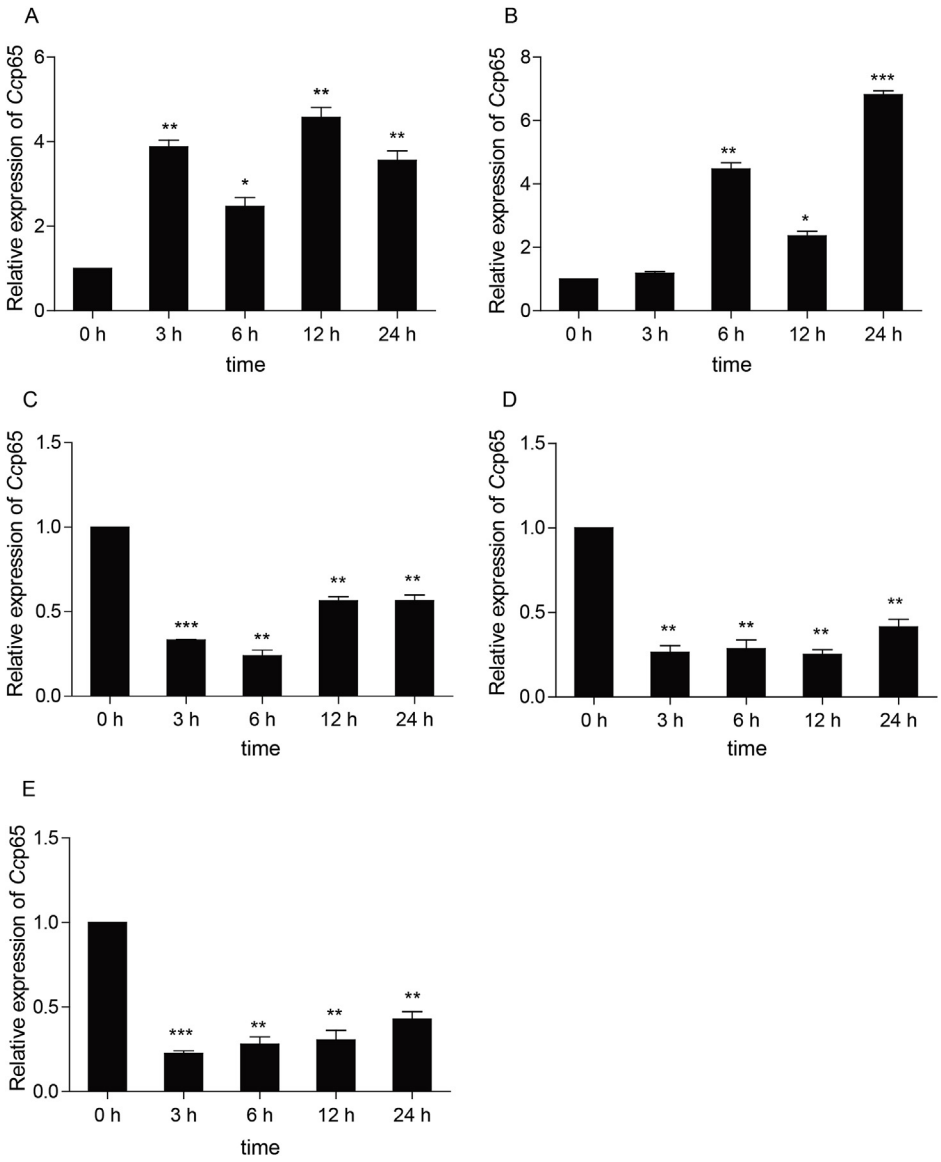

Fig. 5. The relative expression of *Cc*p65 in the HKLs of common carp after treatment with LPS (A), PGN (B), flagellin (C) and Pam3CSK4 (D) at different time points. The results were calculated relative to the expression of the 40S ribosomal protein *s11*. Data are presented as a fold increase to the unstimulated control group (denoted by 0 h). Mean ± SD (n = 3), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

however, down-regulation was observed after PGN, flagellin and Pam3CSK4 treatment. After stimulation with poly(I:C), the expression of *Cc*p65 was induced at 3 h, peaked at 12 h (4.6-fold, $P < 0.01$) (Fig. 5A). *Cc*p65 expression was increased at 6 h and reached a peak value at 24 h (6.7-fold, $P < 0.001$) after LPS stimulation (Fig. 5B). After challenge with PGN, the expression of *Cc*p65 was down-regulated and reached a minimal value at 6 h (0.57-fold, $P < 0.01$) (Fig. 5C). Similarly, *Cc*p65 expression was decreased at a minimal level at 12 h (0.25-fold, $P < 0.01$) when challenged with flagellin (Fig. 5D). After Pam3CSK4 treatment, the expression of *Cc*p65 was down-regulated and reached a minimal value at 3 h (0.23-fold, $P < 0.001$) (Fig. 5E).

3.6. Subcellular localization of *Ccp65*

To gain a better understand of *Cc*p65 functions, the subcellular localization of *Cc*p65 was investigated. Firstly, we transfected HeLa cells with EGFP-tagged *Cc*p65, and followed by stained with DAPI to label the nucleus. As illustrated in Fig. 6B, *Cc*p65 was uniformly distributed in the nucleus and cytoplasm. Furthermore, when treatment with LPS or poly(I:C), the *Cc*p65 translocated into the nucleus. In contrast, EGFP alone was localized in both the cytoplasm and nucleus regardless of LPS or poly(I:C) stimulation (Fig. 6A). The results suggest that the nuclear translocation of *Cc*p65 is depend on the TLRs-mediating signaling cascades.

3.7. Subcellular localization of *Ccp65ΔNLS*

The nuclear localization signal KRKR is an important sequence that determine whether p65 can localize to the nucleus. In the present study, we deleted the nuclear localization signal KRKR from the *Cc*p65 (named *Cc*p65ΔNLS) and analyzed its localization. We found that the *Cc*p65ΔNLS only localized to the cytoplasm in the control group (Fig. 7). Furthermore, the Ccp65ΔNLS failed to localize to the nucleus when stimulated with LPS or poly(I:C) (Fig. 7). The results suggest that the function of *Cc*p65NLS is the same or similar with its mammalian counterparts.

3.8. Activation of a κB element-driven reporter by *Ccp65*

The multiple sequence alignment of p65 between common carp and

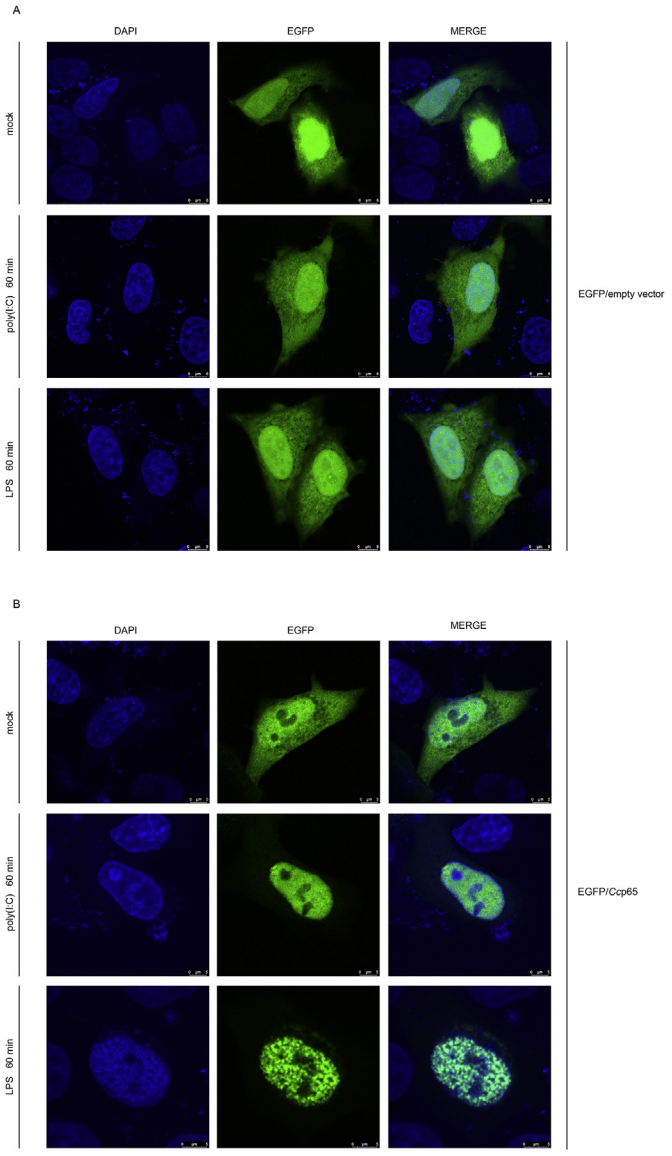

Fig. 6. Localization of *Cc*p65. The empty vector (A), or *Cc*p65 with C-terminal EGFP (B), is expressed in HeLa cells. At 36 h post-transfection, the cells were incubated for 1 h in the absence or presence of 5 μg/ml of poly(I:C) or 10 μg/ml of LPS, then fixed with 4% paraformaldehyde in PBS, mounted using DAPI-mounting medium. Subsequently, the cells were viewed under a Leica TCS SP8 MP multiphoton microscope and analyzed using ImageJ software.

other species showed that the homology of fish with that of mammalian is lower. To test the ability of *Cc*p65 or p65 C-terminal domain in regulating the activation of NF-κB-dependent reporter gene, we performed a transient luciferase reporter assay in 293T cells. A mammalian expression vector carrying full-length wild-type *Cc*p65 (residues 1–553) or a *Cc*p65 C-terminal domain deletion mutation (*Cc*p65ΔC, residues 1–336) or empty vector was transfected into 293T cells along with a κB element-driven reporter plasmid (κB-luc). Luciferase activities were measured in cell lysates at 24 h post transfection. As illustrated in Fig. 8, it was shown that the full-length wild-type *Cc*p65 significantly increased the luciferase activity compared with the control group. However, *Cc*p65ΔC did not up-regulate the luciferase activity. The results suggest that the C-terminal of *Cc*p65 is an important element that activate the κB element-driven reporter (kB-luc).

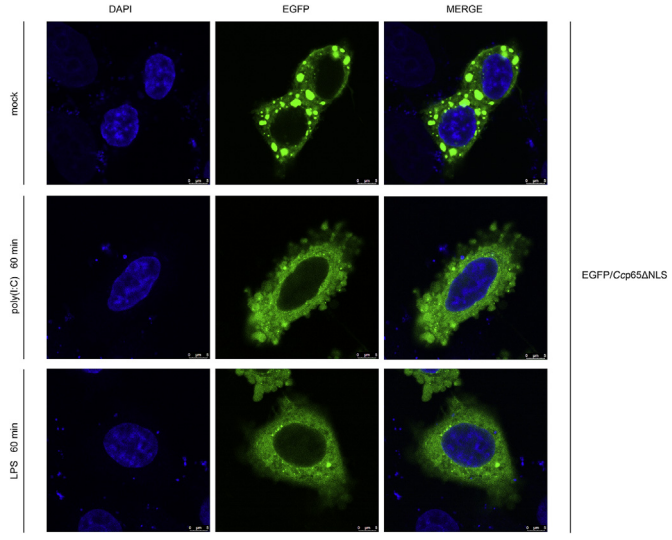

Fig. 7. Localization of *Cc*p65ΔNLS. *Cc*p65ΔNLS with C-terminal EGFP is expressed in HeLa cells. At 36 h post-transfection, the cells were incubated for 1 h in the absence or presence of 5 μg/mL of poly(I:C) or 10 μg/ml of LPS, then fixed with 4% paraformaldehyde in PBS, mounted using DAPI-mounting medium. Subsequently, the cells were viewed under a Leica TCS SP8 MP multiphoton microscope and analyzed using ImageJ software.

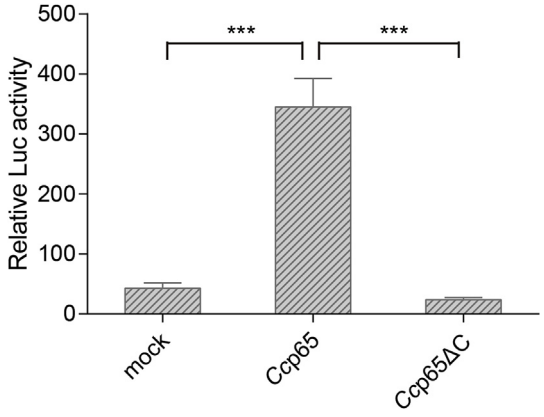

Fig. 8. Activation of a κB element-driven reporter by *Cc*p65. 293T cells were transfected with the constructed *Cc*p65 expression vectors. After transfection for 24 h, *Firefly* and *Renilla* luciferase activities were measured. Relative luciferase activity was represented by the ratio of firefly fluorescence to *Renilla* fluorescence. Mean ± SD (n = 3), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.9. Regulation of the expression of *Ccp65* target genes in EPC cells

NF-κB regulates the inducible expression of many cytokines, chemokines, adhesion molecules, acute phase proteins, and antimicrobial peptides. The proinflammatory cytokines such as IL-1β and TNF-α, which are targets of NF-κB, are involved in systemic inflammation and regulates immune cells. To investigate the involvement of *Cc*p65 subunit of NF-κB in inducing cytokines, we analyzed the gene expression levels of *il-1β* and *tnf-α*. As shown in Fig. 9, the expression of *il-1β* and *tnf-α* was significantly increased in *Cc*p65 overexpressed EPC cells, but not in the control group. Furthermore, we used the TLRs ligands-LPS or poly(I:C) to stimulate the transfected or non-transfected EPC cells, and measured the expression of target genes. When stimulated with LPS, the mRNA expression of *il-1β* and *tnf-α* was up-regulated in *Cc*p65 overexpressed EPC cells. Similarly, poly(I:C) could induce the expression of *il-1β* and *tnf-α* in *Cc*p65 overexpressed cells. The results suggest that *Cc*p65 functions in the expression of *il-1β* and *tnf-α*, and LPS and poly (I:C) inducing the immune-related genes, *il-1β* and *tnf-α*, is dependent

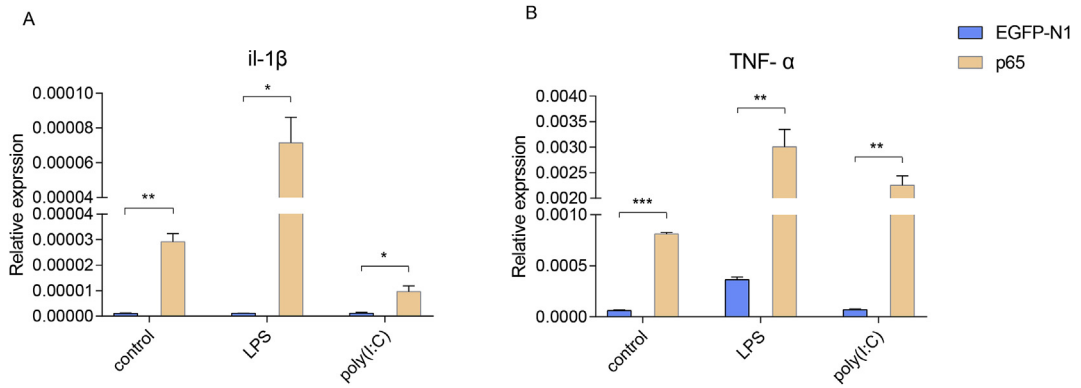

Fig. 9. The relative expression of *il-1β* (A) *and tnf-α* (B) in *Cc*p65-transfected EPC cells. The empty vector, or *Cc*p65 with C-terminal EGFP, is expressed in EPC cells. At 36 h post-transfection, the cells were incubated for 12 h in the presence of PBS or 10 μg/ml of LPS or 5 μg/mL of poly(I:C). The expression of *il-1β* and *tnf-α* was determined. The results were calculated relative to the expression of the β-actin using real-time PCR. Mean ± SD (n = 3), $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

on the *Cc*p65.

4. Discussion

Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor that, by regulating the expression of multiple inflammatory and immune genes, plays a critical role in host defence. As the major subunit of NF-κB, p65 has drawn great attention with respect to the nature of its post-translational modifications. In the present study, we identified the cDNA of *Cc*p65 and showed its activation mechanism and transactivation properties.

The gene expression of p65 has been investigated in many fish species and was found to be constitutively expressed in various tissues of teleost fish. However, the expression profiles of p65 vary among different species. In this study, *Cc*p65 was constitutively expressed in all tissues examined with the highest expression in the gonad and the lowest expression in the head kidney. However, the tissue distribution of *Cc*p65 was somewhat different *P. olivaceus* p65 [18]. The highest expression of *P. olivaceus* p65 was observed in the intestine and the lowest expression was observed in the skin. The observed discrepancies may result from species variations, individual status of the immune system, developmental stage and genetic background.

p65 is reported to be involved in the innate immunity when infected with microbial pathogen or viruses. Many human viruses, including several human pathogens such as human immunodeficiency virus (HIV-1), the human T-cell leukemia virus HTLV-1, influenza virus, hepatitis B and C viruses as well as herpes virus can activate NF-κB [31]. In addition, many pathogenic microorganisms are involved in mechanisms to interfere with the function of NF-κB or modulate NF-κB signal transduction [32]. NF-κB pathway is reported in several fishes and fish p65 is demonstrated to be associated with viruses [16–18]. In the present study, the expression of *Cc*p65 can be induced by *A. hydrophila* and the viral mimic poly(I:C). Leukocytes modulate the expression of their TLR inventory following encounters with pathogens or exposure to specific TLR ligands [33,34]. HKLs consist of heterogeneous cells and are widely used as an experimental system to study immune responses [35,36]. The expression of *Cc*p65 was greatly enhanced in these cells, which further confirmed the *in vivo* results. Moreover, *Cc*p65 translocated into the nucleus when stimulated with LPS or poly(I:C) 1 h. Furthermore, the expression of inflammatory cytokines-il-1β and tnf-α was significantly up-regulated after challenge with LPS or poly(I:C). These results suggest that *Cc*p65 participates in the bacterial and viral infection.

Basal gene expression is often recognized as an indicative measure of gene function in fish immunology. However, additional efforts are needed to investigate the detailed mechanisms of fish p65. Mammalian cells (i.e. HeLa cells) are commonly used *in vitro* models for studying the subcellular localization of fish genes [37,38]. Therefore, we conducted immuofluorescence assay to explore the localization of *Cc*p65 in HeLa cells. In this study, immuofluorescence clearly showed that *Cc*p65 was uniformly distributed in the nucleus and cytoplasm in the resting state. Moreover, the *Cc*p65 translocated into the nucleus when challenged with LPS or poly(I:C) for 1 h (Fig. 6). The results was similar with that of *P. olivaceus* and its mammalian counterparts [2,16]. The reporter gene assays are commonly used to assess the capacity of certain receptors and signaling molecules in triggering immune responses. Of interest, 293T cells have been used for the study of reporter assay in several fish [23,37]. Thus, in this study, the κB element-driven reporter assays were conducted. When the full-length wild-type *Cc*p65 overexpressed, the luciferase activity was significantly increased (Fig. 6), which was consistent with that of *P. olivaceus* and its counterparts [9,16]. Interleukin (IL)-1β can be produced by many cell types, including monocytes, activated macrophages and endothelial cells, which can induce proinflammatory gene expression to further enhance the inflammatory responses [39]. Tumor necrosis factor-alpha (TNF-α) is an important meditator of inflammatory and immune defense mechanisms [40]. To date, identification of il-1β and tnf-α genes of EPC cells has been reported [41] and relevant studies have shown that NF-κB induce the expression of proinflammatory cytokines [9]. In the current study, *Cc*p65 induced the expression of *il-1β* and *tnf-α* in EPC cells (Fig. 9), which was similar with that of *P. olivaceus*. The expression of *tnf-α* was up-regulated in *P. olivaceus* p65 overexpressed HINAE cells [16].

RHD domain is reported to be associated with DNA binding, dimerization, nuclear localization [9]. In the current study, SMART and multiple sequence analysis showed that *Cc*p65 had a conserved N-terminal RHD domain (Fig. 1). And DNA binding motif “RGMRFRYKC” (29–37 aa) and Rel protein signature “FRYKCE” (33–38 aa) were observed in the RHD domain of *Cc*p65 (Fig. 1). In addition, a conserved nuclear localization signal “KRKR” (300–303 aa) was observed in the *Cc*p65 (Fig. 1). The results suggest that function of fish p65 RHD maybe same or similar with those of mammals. Then we deleted the nuclear localization signal from *Cc*p65 and investigated its role. It was shown that *Cc*p65ΔNLS failed to localize to the nucleus when stimulated with poly(I:C) and LPS (Fig. 7), which was similar with that of mammals. While the C-terminal domain (residues 337–553), which, by analogy to other p65 proteins, was likely to be the putative TAD of *Cc*p65, showed lower homology with that of its counterparts. To test the function of C-terminal domain, we deleted the C-terminal domain and investigate its transcriptional activity. However, the luciferase reporter assay showed that the *Cc*p65ΔC did not up-regulate the luciferase activity compared with *Cc*p65 (Fig. 8). It is possible that although the lower homology in

TAD between carp p65 and other species, it may have some important functions because many putative phosphorylation sites have been found [18].

5. Conclusions

Taken altogether, *Cc*p65 was identified and characterized from common carp. *Cc*p65 was evolutionarily and functionally conserved in common carp and mammals. The mRNA expression profile showed that *Cc*p65 was expressed in all the eleven tissues. The subcellular localization suggested that *Cc*p65 was uniformly distributed in the nucleus and cytoplasm in un-challenge statement. In addition, *Cc*p65 could activate the κB element-driven reporter and induce the expression of proinflammatory cytokines-il-1β and tnf-α. Furthermore, *Cc*p65 expression could be induced by *A. hydrophila* and the viral mimic poly (I:C). LPS or poly(I:C) could activate *Cc*p65 to translocate into the nucleus. And LPS and poly(I:C) inducing the immune-related genes, *il-1β* and *tnf-α*, was dependent on the *Cc*p65. This study will provide basic information for studying the molecular mechanisms between fish TLRs and NF-κB signaling.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.10.014.

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