Fish & Shellfish Immunology 54 (2016) 564–572

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Ctenopharyngodon idella NF-κB subunit p65 modulates the transcription of *IκBα* in CIK cells

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ARTICLE INFO

Article history:
Received 15 February 2016
Received in revised form
20 April 2016
Accepted 29 April 2016
Available online 30 April 2016

Keywords:
p65
NF-κB pathway
IκB
Transcriptional regulation
Teleost fish

ABSTRACT

NF-κB is an important transcription factor for regulating the multiple inflammatory and immune related gene transcription. It can bind with the nuclear factor κB site within the promoter of target genes to regulate their transcriptions. p65, the all-important subunit of NF-κB, is ubiquitously expressed in cells. In the present study, we cloned and identified the *p65* subunit from grass carp (*Ctenopharyngodon idella*) (named *Cip65*) by homologous cloning and RACE technique. The full length of *Cip65* cDNA is 2481 bp along with 9 bp 5′ UTR, 639 bp 3′ UTR and the largest open reading frame (1833 bp) encoding a polypeptide of 610 amino acids with a well conserved Rel-homology domain (RHD) in N-terminal and a putative transcription activation domain (TAD) in C-terminal. *Ci*p65 gathers with other teleost p65 proteins to form a fish-specific clade clearly distinct from those of mammalian and amphibian counterparts on the phylogenetic tree. In CIK (*C. idellus* kidney) cells, the expression of *Cip65* was significantly up-regulated under the stimulation with Poly I:C. As one member of the NF-κB inhibitor protein (IκB) family, IκBα can dominate the activity of NF-κB by interacting with it. To study the molecular mechanisms of negative feedback loop of NF-κB signaling in fish, we cloned grass carp *IκBα* (*CiIκBα*) promoter sequence. *CiIκBα* promoter is 414 bp in length containing two RelA binding sites and a putative atypical TATA-box. Meanwhile, *Ci*p65 and its mutant proteins including C-terminus deletion mutant of *Ci*p65 (*Ci*p65-ΔC) and N-terminus deletion mutant of *Ci*p65 (*Ci*p65-ΔN) were expressed in *Escherichia coli* BL21 and purified by affinity chromatography with the Ni-NTA His-Bind resin. *In vitro*, *Ci*p65 rather than *Ci*p65-ΔC and *Ci*p65-ΔN showed high affinity with *CiIκBα* promoter sequence by gel mobility shift assays. *In vivo*, the cotransfection of pcDNA3.1-*Ci*p65 (or pcDNA3.1-*Ci*p65-ΔC, pcDNA3.1-*Ci*p65-ΔN respectively) with pGL3-*Ci*IκBα and pRL-TK renilla luciferase plasmid into CIK cells showed that pcDNA3.1-*Ci*p65 rather than pcDNA3.1-*Ci*p65-ΔC and pcDNA3.1-*Ci*p65-ΔN, can increase the luciferase activity. Taken together, these results suggested that *Ci*p65 can regulate the expression of *CiIκBα* and works as a negative feedback loop in NF-κB pathway.

1. Introduction

As one member of the important transcription factor family, nuclear factor κB (NF-κB) can specifically combine the nuclear factor κB site [1] within multiple gene promoters to activate the transcription of cell surface receptor genes [2,3], cytokine genes [4], growth factor genes [5], acute-phase protein genes [6] and cell adhesion molecule genes [7], etc. Thus, NF-κB plays a significant role in the regulation of inflammatory, immune, cell survival and cell proliferation responses [8,9]. In mammals, NF-κB comprises the five sub-units, i.e. Rel A (p65), Rel B, C-Rel, NFκB1 (p50) and NFκB2 (p52) [10]. The N-terminal of all the sub-units contain a conserved region called Rel homology domain (RHD, about 300 amino acids) including nuclear localization sequence (NLS), DNA binding domain, NF-κB inhibitor protein (IκB) family binding region and dimerization domain [11]. A transactivation domain (TAD) which can activate the transcription activity of target genes only locates in the C-terminal sequences of p65, RelB and C-Rel.

NFκB executes function in the form of homodimer or

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http://dx.doi.org/10.1016/j.fsi.2016.04.132
1050-4648/

heterodimer. Among them, p65/p50, p50/c-Rel, p65/c-Rel and p65/p65 can regulate and control the transcriptional activity of target genes, however, p50/p50 and p52/p52 are inactive or repressive [12]. The most common and abundant NFκB dimer is the p65/p50 complex in most cells [13]. p65 can regulate the expression of genes such as *TNF-α* [14], *IL-1β* [15], *IL6* [16], *IL8* [17], *β-defensin* [18], *iNOS* [19], *IFNβ* [20]. However, as the product of precursor protein p105 [21], p50 don't have the capacity to regulate the target genes owing to the lack of the TAD domain.

IκB belongs to a huge family and consists of eight members including IkBα, IkBβ, IkBγ, IkBδ, IkBε, IkBζ, IkBε and Bcl-3 [22]. Of which, IkBα is the well-known one. In unstimulated cell, p65/p50 is in an inactive form by interaction with IκBα [23]. When cells are stimulated with cytokines, LPS, viruses, UV irradiation and oxidant, IκBα is phosphorylated and degraded by proteosome 26S [24]. Subsequently IκBα is separated from the p65/p50 complex and then p65/p50 is activated; finally the activated NFκB translocates into the nucleus and induces gene transcription [25].

In recent years, an increasing number of fish p65 genes, including *Danio rerio p65* [26], *Ictalurus punctatus p65*, *Siniperca chuatsi p65* [27], *Paralichthys olivaceus p65* [28] and *Larimichthys crocea p65* [29], have been cloned and identified. Fish p65 shares some similar functions with the mammalian counterparts. For instance, *Siniperca chuatsi* p65 (*Sc*p65) was proved to have an affinity with *IκBα* [27]. *Paralichthys olivaceus* p65 (*Po*p65) regulates the expression of *IκBα* [28], suggesting that IκBα-p65 pathway in fish may be similar to that in mammal. Despite some progresses have been achieved in fish p65, there is sill lack of direct evidence of the effect of p65 on *IκBα*. To further explore the role of fish p65 in negative feedback loop of NF-κB pathway, in this study, we cloned and identified the full-length cDNA sequence of grass carp (*Ctenopharyngodon idellus*) *p65* (*Cip65*) and *IκBα* (*CiIκBα*) promoter. Phylogenetic tree showed that *Ci*p65 shared high homology with other teleost counterparts, especially with zebra fish (*Danio rerio*) p65. *Cip65* was significantly up-regulated after stimulated by Poly I:C. *In vitro*, *Ci*p65 bound to *CiIκBα* promoter with high affinity by gel mobility shift assay. *In vivo*, *Ci*p65 up-regulated the transcription activity of *CiIκBα* gene in CIK cells by transient co-transfection assay.

2. Materials and methods

2.1. Fish, vectors, strains and cell lines

Grass carp, about 50 g in body weight, were bought from Nanchang Shenlong Fisheries Development Co., Ltd., Jiangxi, China and Institute of Aquatic Science of Jiujiang city, Jiujiang, China. The fish were kept in an aerated freshwater tank at room temperature for 2 weeks prior to experiments. pEASY-T1, pcDNA3.1, pGL3-basic and pET-32a were purchased from Transgen (China), Invitrogen (USA), Promega (USA) and Novagen (USA) respectively. DH5α and BL21 (DE3) pLysS were purchased from Promega (USA). CIK (*C. idellus* kidney) cells were kindly provided by Professor Pin Nie, Institute of Hydrobiology, Chinese Academy of Sciences and kept in our lab. CIK cells were maintained in M199 (Thermo Fisher Scientific, USA) with 10% fetal bovine serum (Invitrogen, USA) and 1% (v/v) penicillin-streptomycin at 28 °C.

2.2. Cloning and sequence analysis of Ctenopharyngodon idellus p65

Total RNA was isolated from CIK cells by RNA simple Total RNA Kit (Tiangen, China). SMART cDNA was prepared using SuperScript III reverse polymerase (Invitrogen). PCR was performed in a volume of 50 μl mixture consisting of 5 μl 10 × LA Buffer (plus Mg^{2+}), 2 μl SMART cDNA, 1 μl each primer (10 mmol/L), 2 μl dNTP (10 mmol/L), 0.5 μl LA Taq (TAKARA) (5 U/ml) and 38.5 μl ddH_2O. All primers used in the study were listed in Table 1. Partial cDNA of grass carp *p65* was cloned with a pair of homologous primers p65—homo-F and p65—homo-R (designed based on the *Drp65* cDNA sequence

Table 1
Primer sequences and their applications in this study.

Primer name	Primer sequence (5′—3′)	Application
p65 —homo-F	CCCTGGAGAGAAGAGCAACGA	Homology-based cloning
p65 —homo-R	TCAGTTCTCTTGCGCTTCTCCATC	
Smart	AAGCAGTGGTAACAACGCAGAGTACGCGGG	Reverse transcription
CDS	AAGCAGTGGTAACAACGCAGAGTA(T)30	
p65-5RACE-Outer	ATCTCCCACATCCTTCTTCTTTAC	5′RACE
p65-5RACE-Inner	GTGGCTTGTATGGCTGGTTCTTGG	
p65-3RACE-Outer	ATCTACGACAACAGAGCTCCAA	3′RACE
p65-3RACE-Inner	TCGTGGGAAAGTAAGGGCTCGT	
Long	CTAATACGACTCACTATAGGGCAAAGCAGTGGTATCAACGCAGAGT	RACE-PCR
Short	CTAATACGACTCACTATAGGGC	
NUP	AAGCAGTGGTATCAACGCAGAGT	
p65-F-F	CGGGACATGGACGGACTGTTT	Full-length sequence validation
p65-F-R	AGCAAAGCTGCACAACAGAGG	
p65-RT-F	AACCAAGAACCAGCCATACAAG	Real-time PCR
p65-RT-R	CGCTTCAGGAATATTAAAGGGG	
β-actin-F	CACTGTGCCCATCTACGA	
β-actin-R	CCATCTCCTGCTCGAAGTC	
p65-ORF-F	CGGAATTCATGGACGGACTGTTTCAC	Prokaryotic/eukaryotic expression vector construction
p65-ORF-R	CGCTCGAGTTAGGTCGGGTGTCCAGACAG	
p65-ΔC -R	CGCTCGAGTCACCCTTCTGTCCTCTT	
p65-ΔN -F	CGGAATTCATGCTGCAGAATCTCAA	
IκBα-SP1	TTGGATTTGCACTTGGGTTTGG	Promoter cloning
IκBα-SP2	GTCAAACTCTCCATTTCCTCCACA	
IκBα-SP3	CTCATCCTCCTTTAGCGAATCCAC	
AAP	CGGGATCCCGGAATTCCCCCICCCCICCCCIC	
IκBα-Pro-KpnI	CGGGGTACCTCGCGATTAATCACATCTAAC	
IκBα-Pro-MluI	CGACGCGTGCCCTTTTATAGCTGCGTTTC	
IκBα-mut-R	CAAGATGACTACGCGAA	

Nucleotides shown in underline indicate restriction sites.