Fish & Shellfish Immunology 30 (2011) 406–411

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Cloning and functional characterization of the p65 subunit of NF-κB from olive flounder (*Paralichthys olivaceus*)

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ARTICLE INFO

Article history:
Received 3 September 2010
Received in revised form
23 November 2010
Accepted 28 November 2010
Available online 3 December 2010

Keywords:
p65
NF-κB
Paralichthys olivaceus
Transactivation
Poly I:C

ABSTRACT

NF-κB is a master transcription factor found in almost all cell types that responds to diverse cellular stimuli by activating the expression of stress response genes, including immune-related genes. cDNA encoding the p65 subunit of olive flounder (*Paralichthys olivaceus*) NF-κB (Po-p65) was isolated through an EST analysis of an olive flounder cDNA library, a screen of BAC library, and rapid amplification of cDNA ends (RACE). The cDNA for Po-p65 encodes a polypeptide 626 amino acids in length containing a well-conserved Rel-homology domain (RHD). The primary sequence of Po-p65 showed strong homology with p65 from perch and zebrafish (82.7 and 64.4%, respectively), and shared 43.4–42.1% homology with p65 from other species, including mammals, while the N-terminal RHD of Po-p65 showed strong identity (95.6–67.8%) with that of other species. Po-p65 mRNA expression was detected in all flounder tissues examined. The over-expression of full-length Po-p65 (Po-p65f), but not of a Po-p65 C-terminus deletion mutant (Po-p65ΔC), stimulated κB element-driven reporter (κB-luc) activity in a dose-dependent manner and regulated the expression of p65 target genes, including TNF-α and IκB-α, in HINAE olive flounder cells. Po-p65f translocated to the nucleus following stimulation with poly I:C in HINAE cells. Together, these results suggest that Po-p65 is evolutionarily and functionally conserved in flounder and mammals and may provide clues to the detailed molecular mechanism(s) underlying immune response regulation in flounder.

1. Introduction

Innate immunity is the first line of defense against infectious agents. The basic mechanisms underlying pathogen recognition and activation are conserved throughout much of the animal kingdom [1]. Recent studies have revealed striking similarities in the signaling pathways used by humans and flies to activate their innate immune responses [2]. In both, infection leads to the activation of Toll-like receptor (TLR)-mediated signaling cascades that promote the activation of NF-κB family transcription factors [3]. NF-κB plays a key role in triggering and coordinating innate and adaptive immune responses [4–7].

NF-κB is a homodimeric or heterodimeric complex formed by the Rel homology domain (RHD)-containing proteins RELA/p65, RELB, NF-κB1/p105, NF-κB1/p50, REL, and NF-κB2/p52; the heterodimeric p65–p50 complex appears to be the most abundant form [8–10]. The N-terminal RHD is a conserved region 300 amino acids in length that is responsible for DNA binding, dimerization, and interaction with I-κB family members [4]. The C-terminal transcription activation domain (TAD) is responsible for the induction of target genes following the activation of NF-κB; consequently, NF-κB functions as an activator of transcription [11]. Under unstimulated conditions, NF-κB dimers are held in an inactive cytoplasmic complex with I-κB family inhibitory proteins. More than 150 extracellular signals, including bacterial lipopolysaccharide, proinflammatory cytokines (e.g., tumor necrosis factor-alpha [TNF-α] and interleukin [IL]-1), viral infection or the expression of certain viral gene products, hormones, and mitogenic agents, have been linked to NF-κB activation [4]. These signals lead to the activation of TLRs, which, in turn, initiate intracellular signaling cascades that culminate in phosphorylation, ubiquitination, and ultimately the degradation of IκB [12,13]. IκB degradation makes possible the nuclear translocation of NF-κB components and the initiation of target gene transcription following the binding of NF-κB to gene promoters [4,14].

NF-κB regulates the transcription of a wide variety of genes, including those encoding cytokines (e.g., IFN-β, IL-1, IL-2, IL-6, IL-12, TNF-α, LTα, LTβ, and GM-CSF), chemokines (e.g., IL-8, MIP-1α,

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doi:10.1016/j.fsi.2010.11.024

MCP1, RANTES, and eotaxin), adhesion molecules (e.g., ICAM, VCAM, and E-selectin), acute phase proteins (e.g., SAA), inducible effector enzymes (e.g., iNOS and COX-2), antimicrobial peptides (e.g., β-defensins), genes encoding molecules important for the adaptive immune response (e.g., MHCs), and costimulatory molecules (e.g., B7.1) [4,15,16]. Additionally, NF-κB has been implicated in the overall immune response through its ability to activate genes encoding regulators of apoptosis and cellular proliferation, such as c-IAP-1, c-IAP-2, A1 (Bfl1), Bcl-X_L, Fas ligand, c-*myc*, and cyclin D1 [17].

There is some evidence for the presence of the NF-κB/I-κB pathway in freshwater and marine organisms [18–20]. The horseshoe crab, *Carcinoscorpius rotundicauda*, possesses a primitive NF-κB/I-κB pathway, which plays a fundamental role in regulating the expression of key immune defense molecules. The NF-κB/I-κB pathway in the mandarin fish, *Siniperca chuatsi*, may play a role in the immune response against viral infection. Cg-Rel was the first Rel/NF-κB homolog characterized in a mollusk, the Pacific oyster, *Crassostrea gigas*.

There is currently increased interest in understanding the molecular mechanisms underlying the immune defenses of olive flounder, *Paralichthys olivaceus*, an economically important species in eastern Asia. Another key molecule in the NF-κB/I-κB pathway, I-κBα, and NF-κB target genes in mammals such as TNF-α, IL-6, IL-8, β-defensins, and MHC have also been characterized in flounder [21–25].

The aim of the present study was to clone and characterize the p65 subunit of NF-κB from *P. olivaceus*. We then examined its function in the regulation of immune responses through the transcriptional activation of immune-related genes in flounder.

2. Materials and methods

2.1. Cloning of the p65 subunit of NF-κB from olive flounder, P. olivaceus

A flounder BAC library was screened by PCR with a primer set (p65-BF, 5′-CTC ATT TCC GTG TGG ACG CAC-3′ and p65-BR, 5′-ACT GGG GTG TCC TGA GAC AAA-3′) based on the sequence of a contig, CL1058, containing the partial cDNA sequence of the p65 subunit of NF-κB identified from an EST analysis of an olive flounder cDNA library. One BAC clone, 116-L-12, was isolated and sequenced using primer p65-RT-R (5′-GGG TTC AGA AGG TCC ACA AA-3′). 5′-Rapid amplification of cDNA ends (RACE) was performed using a SMART™ RACE cDNA amplification kit (Clontech) to obtain the full-length olive flounder p65 cDNA sequence. Based on the BAC sequence, we designed internal primers (R3, 5′-CTC AGG CTC TCC ATG ATC TTC CAT-3′) and used them in combination with the universal primer supplied with the kit to amplify the 5′-end of the p65 transcript. DNA sequencing was performed with the T7 and T3 universal primers (Promega) and internal primers using an autosequencer (ABI3730xl; Applied Biosystems). The full-length p65 cDNA sequence was obtained by combining the partial DNA sequences and 5′-RACE PCR products. The cDNA sequence was searched in GenBank using BLASTX (http://www.ncbi.nlm.nih.gov/BLAST/).

2.2. Construction of expression plasmids

Amplification of the ORF of p65 was carried out using Vent DNA polymerase (New England Biolabs) and primers specific to the 5′ (beginning at the start codon) and 3′ ends of the p65 cDNA, based on the nucleotide sequence. To create expression constructs, primers were designed so that the amplified DNA would contain *Sma*I and *Xho*I restriction sites at its 5′ and 3′ ends, respectively. The primer sequences were: p65 ORF-FS (for full-length p65), 5′-ATA CCC GGG ATG GAG GCG TAT GGA TGG GAC-3′; p65 ORF-RX, 5′-CAT CTC GAG TTA ACT GGG GTG TCC TGA GAT AT-3'; p65 ORF-FS (for p65 delta-C), 5′-ATA CCC GGG ATG GAG GCG TAT GGA TGG GAC-3′; and p65 337R, 5′-CAG CTC GAG TTA CTT GGA TGT GAC TGT TCT-3′. The amplified cDNA fragment was cloned into the *Stu*I and *Xho*I restriction sites at the 5′ and 3′ ends of pCS2 + MT [26]. To create a GFP-fused construct, primers were designed so that the amplified DNA would contain *Bam*HI and *Xho*I restriction sites at its 5′ and 3′ ends, respectively. The primer sequences were as follows: p65 ORF-FB, 5′-TAT GGA TCC ATG GAG GCG TAT GGA TGG GAC-3′, and p65 ORF-RX, 5′-CAT CTC GAG TTA ACT GGG GTG TCC TGA GAT AT-3′. The amplified cDNA fragment was cloned into the *Bgl*II and *Xho*I sites at the 5′ and 3′ ends of pEGFP-C2 (Clontech). The sequences of the constructs were confirmed by sequencing.

2.3. Fish maintenance and tissue sampling

Olive flounder were maintained at the Genetics and Breeding Research Center of the National Fisheries Research and Development Institute (NFRDI; Geoje, Republic of Korea). The fish were fed a commercial diet (Suhyup Feed; crude protein: 52%, crude fat: 11%) three times per day. The temperature of the rearing tanks was maintained at 18 °C. The tissues were removed, immediately frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

2.4. Quantitative real-time RT-PCR

Total RNA was prepared from tissues using TRIzol® reagent (Invitrogen) according to the manufacturer's protocol. The total RNA concentration was quantitatively determined, and 1 μg of total RNA was used for reverse transcription. First-strand cDNA was synthesized using an Advantage® RT-for-PCR kit (BD Biosciences). Quantitative real-time PCR was performed using a LightCycler® FastStart DNA Master SYBR Green I (Roche) and the following forward and reverse primers: for Po-p65, p65-RT-F (5′-GCT TCT CTG GGT AGC ACA CC-3′) and p65-RT-R (5′-GGG TTC AGA AGG TCC ACA AA-3′); for IκBα [21], IκBα-RT-F (5′-ATG GAC CTG CAC CGG ACC AG-3′) and IκBα-RT-R (5′-ATG TCC ATT CCA GTG AAT GT-3′); for TNF-α [22], TNFα-RT-F (5′-ATG GTG AAA TAC ACA AGT GCA-3′) and TNFα-RT-R (5′-TCA AAG TGC AAA GAC ACC GAA-3′); and for 18S rRNA, 18S rRNA-F (5′-ATG GCC GTT CTT AGT TGG TG-3′) and 18S rRNA-R (5′-CAC ACG CTG ATC CAG TCA GT-3′). Following an initial 10-min *Taq* activation step at 95 °C, LightCycler PCR was performed for 40 cycles using the following cycling conditions: 95 °C for 10 s, 57 °C for 5 s, 72 °C for 40 s, and fluorescence reading. Statistical analyses were carried out using unpaired or paired *t*-tests as appropriate. All data are reported as means ± SD. P values <0.05 with respect to the mock transfectants were deemed to indicate statistical significance.

2.5. Cell culture

Olive flounder embryonic HINAE cells (a gift from Dr. T. Aoki) were maintained in Leibovitz L-15 medium (GIBCO BRL) with 10% heat-inactivated fetal bovine serum (FBS; GIBCO BRL) and 1% (v/v) penicillin–streptomycin (PS; GIBCO BRL) in a 20 °C incubator.

2.6. Transient transfection

For luciferase assay, HINAE cells were seeded in 24-well culture plates and transfected with a reporter vector and β-galactosidase expression plasmid, along with each indicated expression plasmid using JetPEI transfection reagent (PolyPlus Transfection). Mammalian expression vectors carrying Po-p65 or Po-p65ΔC were transfected into HINAE cells along with a κB element-driven