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The archaic roles of the lamprey NF-κB (lj-NF-κB) in innate immune responses

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ABSTRACT

The nuclear factor-kappa B (NF-κB) is a pleiotropic transcription factor regulating the expression of genes involved in various biological processes including the immune response and inflammation. Lamprey is regarded as a key species to provide meaningful clues for understanding the evolution of immune system; nevertheless, no information about lamprey NF-κB is reported. Thus, we have characterized a NF-κB homolog in lamprey (lj-NF-κB) for the deeper understanding of the role it played in lamprey immune system. The sequence and 3D structure analyses demonstrate that lj-NF-κB contained a Rel homology domain (RHD) and seven ankyrin repeats domains (ANKs), which would exhibit functional similarities to NF-κB superfamily proteins. This hypothesis was further proved by experiments. We found that the RHD of lj-NF-κB could interact with a mammalian κB response element, translocate to the nucleus to modulate gene (*IL-6*, *IL-1β* and *TNF-α*) expression, and the nuclear localization signals (NLS) was essential for the nuclear translocation. Furthermore, the ANKs of lj-NF-κB are the inhibition signal for the RHD of lj-NF-κB. The present results allow us to surmise that the lj-NF-κB should play a key role in immune response of lamprey, and the function of NF-κB has been maintained during evolution.

1. Introduction

The nuclear factor-kappa B (NF-κB) is a pleiotropic transcription factor regulating the expression of genes involved in various biological processes (Zhang and Sun, 2015). In mammals, the NF-κB family includes five members, RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52), all of which contain a Rel homology domain (RHD) that is responsible for formation of homo- and hetero-dimers as well as for binding of κB sequence elements (Bhatt and Ghosh, 2014). The RelA, RelB, and c-Rel share a conserved C-terminal transactivation domain (TAD) that can directly promote gene transcription (Hayden and Ghosh, 2008). The C-terminal portions of p105 and p100 harbor multiple ankyrin repeat domains (ANKs) that allow them to bind NF-κB dimers and retain them an inactive form in the cytoplasm (Perkins, 2007). In resting cells, NF-κB dimers are sequestered in the cytoplasm by the inhibitor of NF-κB (IκB) proteins. In response to numerous stimuli NF-κB is rapidly activated by degradation of the IκB proteins through proteasome to be released as active NF-κB dimers which migrate to the nucleus to induce the genes expression (Hou et al., 2003).

Until now, NF-κB homologs have been reported in many species ranging from single-cell organisms to mammals (Gilmore and Wolenski, 2012). In *Drosophila*, an IκB homolog (Cactus) and three NF-κB homologs (Dorsal, Dif, and Relish) are involved in multiple biological and immunological processes (Hetru and Hoffmann, 2009; Valanne et al., 2011). Relish is an NF-κB-like protein with an RHD and ANKs, which need be removed for activation. The *Drosophila* immune deficiency (IMD) pathways (Kleino and Silverman, 2014) regulates the activity of the Relish, leads to the production of antimicrobial peptides (AMPs) (Myllymäki et al., 2014), which is different from activation of mammalian NF-κB. The Gram-negative bacterium triggers the IMD pathway that leads to cleavage of Relish by the signal-dependent endoproteolysis Dredd. In contrast to mammalian counterparts, cleaved Relish can serve as a transcription activator by itself (Wiklund et al., 2009; Ganesan et al., 2011), whereas cleaved p105/p100 requires RelA/RelB for their

Abbreviations: NF-κB, nuclear factor-kappa B; RHD, Rel homology domain; ANKs, ankyrin repeats; NLS, nuclear localization signals; TAD, transactivation domain; GRR, glycine-rich region; IMD, immune deficiency; AMPs, antimicrobial peptides; IPT, immunoglobulin-plexin-transcription; ORF, open reading frame; IPTG, isopropyl-1-thio-β-D-galactopyranoside; BCA, bicinchoninic acid; EMSA, electrophoretic mobility shift assay; HRP, hypothalamic regulatory peptides; ECL, electrochemical-luminescence; FBS, fetal bovine serum; UTR, untranslated region; HMM, hidden Markov models; NJ, neighbor-joining; NTD, N-terminal domain; DimD, dimerisation domain

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transactivation function. Moreover, after the endoproteolytic cleavage (Stoven et al., 2003), the ANKs of Relish remains stable in the cytoplasm, but the C-terminal domain of p105/p100 is completely degraded by the proteasome. In *Branchiostoma*, bbtp105 (NF-κB-like protein) can inhibit the transcriptional activity of bbtRel (Rel-like protein). Similar to the Relish, bbtp105 contains an N-terminal RHD, a conserved GRR (glycine-rich region) motif and C-terminal ANKs. In addition, the N-terminal RHD with an immunoglobulin-plexin-transcription (IPT) domain can promote gene transcription, which is independent of forming dimmers (Yuan et al., 2013; Tiwari et al., 2013).

Lamprey, one of the most primitive vertebrates, is the living representative of agnathans whose lineage can be dated back to over 550 million years (Osório and Rétaux, 2008). Owing to its unique phylogenetic position that intermediate between urochordates and jawed vertebrates, it can be regarded as a key species to provide meaningful clues for understanding the evolution of immune system (Amemiya et al., 2007; Forey and Janvier, 1993; Cooper and Alder, 2006). There are both the innate and adaptive immune response systems in lamprey. On the one hand, lampreys use variable lymphocyte receptors (VLR) as counterparts of the immunoglobulin-based receptors that jawed vertebrates use for antigen recognition (Pancer et al., 2004; Han et al., 2008; Rogozin et al., 2007; Guo et al., 2009; Kasamatsu et al., 2010a, 2010b). On the other hand, it also possess the TLR family members with similar functional profile of those in mammals, and the lamprey immune-related TLR orthologs are independent of the VLR system (Ishii et al., 2007; Kasamatsu et al., 2010a, 2010b). Up to now, the information of intracellular NF-κB signal pathways of lamprey has not been clarified. Our previous research identified a lamprey *IκBε-like* gene, which participates in lamprey immune response (Su et al., 2013). Here, the molecular cloning and characterization of a NF-κB homolog (lj-NF-κB) from lamprey are first reported, which will help us to understand how lj-NF-κB functioned in response to the immune stimulation.

2. Materials and methods

2.1. Animals, cells culture and separation of lamprey leukocytes

The handling of lampreys (*Lampetra japonica*) and all experimental procedures were approved by the Animal Welfare and Research Ethics Committee of the Institute of Dalian Medical University (Permit No. SYXK2004–0029). Adult lampreys (length: 36.4–58.4 cm, weight: 112–273.5 g) were captured from the Tongjiang Valley of Heilongjiang province, China, in December. HEK293T cells (human embryonic kidney) were cultured in DMEM supplemented with 10% FCS. Lamprey leukocytes were separated using the method of FicollePaque gradient centrifugation with the FicollePaque medium (concentration, 1.092 g/ml) (Wu et al., 2012).

2.2. Cloning of lj-NF-κB cDNA from lamprey

A NF-κB homolog was found in lamprey leukocytes cDNA library, which was constructed in our laboratory before. Total RNA was extracted from lamprey leukocytes using the Catrimox-14™ RNA Isolation Kit (TaKaRa Biotechnology, Dalian, China). Then the reverse transcription was performed with 1 μg of total RNA and reverse transcriptase (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions. A Full-length *lj-NF-κB* cDNA was amplified using the 3′- and 5′-RACE (rapid amplification of cDNA ends) Core Set Kit (TaKaRa Biotechnology, Dalian, China) based on the 3′- and 5′-RACE primer listed in Table 1.

2.3. Amino acid sequence conservation analysis, 3D structure modeling and phylogenetic analysis

According to (Livingstone and Barton, 1993), the amino acids conservation score was calculated and plotted as a moving average with a sliding window. Protein sequence features retrieved through online tool ScanProsite (http://www.expasy.org/tools/scanprosite). Sequence logo of the 12 sequences alignment was generated using LOGOMAT-M (http://www.sanger.ac.uk/science/tools/logomat-m). The 3D structure of lj-NF-κB N-terminal sequence with RHD (residues 37–341) and C-terminal sequence with ANK repeats (residues 604–921) were predicted respectively with swissmodel (http://swissmodel.expasy.org/interactive). 72 amino acid sequences of NF-κB family including lj-NF-κB were aligned with ClustalX 1.81 based on default settings. The result of multiple sequence alignments was converted into a mega format and imported into MEGA 4.1 for constructing a phylogenetic tree based on the NJ method, 1000 bootstrapped replicates and the phylogenetic tree was visualized using iTOL.

2.4. Recombinant proteins expression and purification

Two truncated fragments of *lj-NF-κB* open reading frame (ORF) which contain RHD (1068-bp) and ANK (999-bp), respectively, were prokaryoticly expressed. Two fragments flanked by a *BamH* I and a *Not* I restriction site were respectively amplified and inserted into the pET-32a expression plasmid, and named rlj-RHD and rlj-ANK, respectively. The recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) with the isopropyl-1-thio-β-D-galactopyranoside (IPTG, 0.1 mM) induction for 3.5 h. Subsequently, the cells were collected by centrifugation at 7500 rpm for 10 min at 4 °C, and re-suspended with binding buffer containing 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 20 mM imidazole. Then the sonicated supernatant was collected, and the fusion protein was purified with His-Bind affinity chromatography (GE Healthcare, New York, NY, USA). The purity of the recombinant proteins was analyzed using 12% SDS-PAGE and the concentration of the sample was measured by the bicinchoninic acid (BCA) protein assay kit (Sangon Biotech, Shanghai, China).

2.5. Electrophoretic mobility shift assay (EMSA)

EMSA experiments were performed as the instructions of the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, Waltham, PA, USA). Two 22-bp DNA sequences were synthesized by TaKaRa Biotechnology (Dalian, China) [NF-κB sense, 5′-AGCTTCAGAGGGGACTTTCCGAGAGG-3′ (biotin-labeled probes) and NF-κB antisense, 5′-TCGACCTCTCGGAAAGTCCCCTCTGA-3′ (unlabeled competitor probes)]. To perform binding reactions, probes (10 pmol/μl) were incubated with 0 ng, 150 ng and 300 ng rlj-RHD purified protein, the rlj-ANK purified protein (300 ng) was used as a control. All experiments were performed at least three times.

2.6. Construction of the transfection vectors

The lj-RHD and lj-ANK (described in materials and methods 2.4) were amplified with their specific primers which attached with a Flag tag or a HA tag, respectively. Their PCR products were introduced into the pIRES2 vector (TaKaRa, Biotechnology, Dalian, China) or pcDNA3.1 vector (Invitrogen, San Diego, CA, USA), which were termed pIRES2-AcGFP1-Flag-RHD and pcDNA3.1-HA-ANK, respectively. For the study of the role of the NLS in lj-NF-κB, the truncated mutant of lj-RHD was inserted into the pIRES2 vector, which was called pIRES2-AcGFP1-Flag-RHD△.

2.7. Semi-quantitative RT-PCR

Total RNAs were separated from HEK293T cells, which were transfected with pIRES2-AcGFP1-Flag-RHD or pcDNA3.1-HA-ANK, respectively, using RNAiso solution (TaKaRa, Biotechnology, Dalian, China) according to the manufacturer's specifications. For removing genomic DNA, total RNAs were incubated with DNase I (TaKaRa, Biotechnology, Dalian, China). Then the reverse transcription was

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