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Short communication

Molecular characterization and expression analysis of IκB from *Haliotis discus discus*

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ABSTRACT

Innate immune system relies on the recognition of pathogen associated molecular patterns present in the microbes by the pattern recognition receptors leading to the activation of signaling cascade and subsequent synthesis of cytokines. NF-κB is a major stimulus activated transcription factor, which regulates the expression of a diverse array of genes. IκB is an inhibitor of NF-κB, retaining NF-κB in an inactive state in the cytoplasm. In this study, we have reported the characterization of first abalone *IκB (HdIκB)*. The cDNA possessed an ORF of 1200 bp coding for a protein of 400 amino acids with molecular mass of 45 kDa and isoelectric point of 4.7. HdIκB protein possessed a conserved phosphorylation site ^{58}DSGIFS^{63} in the N-terminal region, six ankyrin repeats, and a PEST sequence in the C-terminal region. A casein kinase II phosphorylation site could also be observed in the PEST sequence. Constitutive expression of *HdIκB* revealed its physiological significance since NF-κB is known to be activated by various stimuli. Elevated expression of *HdIκB* transcripts could be observed in abalones challenged with various mitogens and live microbes. This novel characterization of abalone *IκB* would further be a positive approach in the affirmation of evolutionary conservation and significance of this protein as a repressor/inhibitor of a pleiotropic transcription factor like NF-κB.

1. Introduction

Innate immune system is an ancient and universal form of host armament against pathogenic invasion. Invertebrates rely solely on innate immune system, for the containment and elimination of the pathogens unlike the vertebrates which possess adaptive immunity [1]. Innate immune system has evolved antecedently to the adaptive immunity. It executes its role through the circumscribed germ-line encoded receptors, which prevail universally in all forms of life and responsible for the recognition of pathogens through the pathogen associated molecular patterns (PAMPs). The recognition of different PAMPs by the designated receptors leads to the stimulation of the downstream signaling cascades resulting in the activation of numerous but appropriate stimulant specific cytokines, resulting in the elimination of the invading pathogens [2,3]. Many of these conserved pathways converge at the activation of a significant transcription factor, NF-κB which regulates the transcription of many different genes.

NF-κB is a stimuli-activated transcription factor that regulates a diverse array of genes involved in development, immunity, apoptosis, homeostatic mechanisms, and cellular differentiation in a multitude of cell types [4]. In immunity, NF-κB is known to regulate the expression of cytokines, cyclo-oxygenase-2, anti-microbial peptides, inducible nitric oxide synthase, apoptosis inhibitors [5,6]. NF-κB is also known to involve in cellular homeostasis and development of tissues in central nervous system in mammals [7–9]. Higher activation of NF-κB has been demonstrated to cause deleterious effects in the cells like pathogenesis of chronic inflammation, auto immunity, and cancers [10]. The expression and activation of such a highly pleiotropic transcription factor is tightly regulated by the inhibitory protein IκB (*I*nhibitor of NF-*κB*) delivering a significant role in the NF-κB signaling module.

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IκB sustains the NF-κB in an inactive state by interacting with Rel-homology domain (RHD) (a highly conserved domain, characteristic of the NF-κB family members) and by masking the nuclear localization signal (NLS) present in the RHD of NF-κB [11]. NF-κB is activated by two pathways namely canonical (or classical) and non-canonical (or alternative) pathways [12]. In both the pathways, IκB is phosphorylated by IκB kinases at two serine residues (S) leading to the lysine (K) ubiquitination and degradation by the proteasome.

In mammals, NF-κB family is comprised of five transcription factors namely, p50 (derived from larger precursor p105), p52 (derived from larger precursor p100), RelA (p65), c-Rel which form homo- or hetero-dimers and RelB which can only form hetero-dimers, resulting in functional NF-κB [13,14]. IκB homolog from *Drosophila* is called "cactus". Similarly, there are different IκB proteins namely, IκB α, IκB β, IκB γ, and IκB ε which are characterized by the ankyrin repeats and they possess varying affinities for the individual NF-κB dimers [14,15]. NF-κB is largely involved in innate and adaptive immunity in a diverse range of organism from invertebrates to mammals. In response to a variety of extracellular stimuli like proinflammatory cytokines, viral infection, bacterial lipopolysaccharide, phorbol esters, oxidants, and UV light, IκB is inducibly phosphorylated at the conserved serine residues. Once they are phosphorylated, the lysine residues present N-terminal to the serine residues are ubiquitinated and proteolytically degraded by the proteasome. The free NF-κB translocate from the cytoplasm to the nucleus, where it initiates the transcription of target genes, inclusive of IκB gene. Thus, translocation of NF-κB to the nucleus results in the synthesis of new IκB, which in turn binds to the NF-κB and sequesters it back in the cytoplasm in an inactive state to terminate the transcription process (a process called the post induction repression) and making it available for the rapid reactivation by another external stimulus. This generates an autonomous feedback loop, resulting in the transient expression of NF-κB [16–18].

The NF-κB pathway has been extensively studied in mammals and other model organisms like *Drosophila*, whereas great strides has been made in identifying new participants in the pathway conducing the research on the molecular evidence of the similar genes in other lower vertebrates and invertebrates as well [19]. With regard to the IκB gene identification and characterization, progress has been made in oyster *Pinctada fucata* [20], Pacific oyster *Crassostrea gigas* [21,22], Asiatic hard clam *Meretrix meretrix* [23], bay scallop *Argopecten irradians* [24], squid *Euprymna scolopes* [25], *Carcinoscorpius rotundicauda* [26], thus standing as an averment for the evolutionary existence of IκB as a modulator of innate immunity.

Abalones are major nutritional source of food and abalone aquaculture is a well-developing socio-economic industry in many parts of the world. The increase in demand for abalones in the food market, not only reflects the development of aquaculture industry but also the responsibility of the farmers to provide the customers with energy-rich, disease free food. Here comes the necessity of understanding the physiological system of abalones which will benefit the betterment of industry as a disease free, profitable property. In particular, unraveling the participants in the immune system, their expression, regulation, response to the heterogenous challenges/pathogens encountered will definitely help in innovation of novel strategies to achieve the goal of disease-free state of art in abalone aquaculture industry. In this regard, we have created a cDNA and BAC library and analyzing the valuable genes involved in the immune defense of the organism. In this report, we have characterized an *IκB* homolog from disk abalone, *Haliotis discus discus* at the molecular level and also analyzed its expression pattern post immune challenges.

2. Materials and methods

2.1. cDNA synthesis, cDNA library construction and HdIκB identification

A cDNA library was constructed from the disk abalone digestive gland, using a cDNA library construction kit (Creator SMART, Clontech, USA). The cDNA was then normalized via a Trimmer-Direct normalization kit, according to the manufacturer's recommendations (Evrogen, Russia). The library was sequenced and the individual ESTs were assembled into sequence groups [27]. A single EST homologous to the earlier reported IkB sequences was obtained by comparing the assembled ESTs against the non-redundant protein database from NCBI, using the BLASTX program. The identified cDNA clone was named as *HdIκB* and was further investigated.

2.2. In silico analysis

A sequence homologous to *IκB* was identified by NCBI BLAST program. The deduced HdIκB protein was subjected to conserved motif analysis in simple modular architecture research tool (SMART) (http://www.smart.emblheidelberg.de/). The PESTfind software available at EMBnet (European Molecular Biology Network, (http://www.at.embnet.org/toolbox/pestfind/) was used to predict the potential PEST sequence (proline (P), glutamic acid (E), serine (S), and threonine (T)). Pairwise and multiple sequence alignments of HdIκB were generated using a ClustalW version 2 program [28]. Phylogenetic evolutionary analysis of HdIκB was performed with the full length amino acid sequences from GenBank and reconstructed using the maximum likelihood method and MEGA 5 program [29] with bootstrapping values taken from 5000 replicates. The amino acid identity percentages were calculated by MatGAT program, using default parameters [30].

2.3. Tissue sampling and challenge experiments

Disk abalones with individual body weight of approximately 50 g, average width and height of 8 cm and 5 cm, respectively were obtained from the Dongjin abalone farm (Jeju Island, Republic of Korea). They were adapted to laboratory conditions in 250 L tanks filled with aerated seawater at 18 ± 1 °C for one week prior to the experiments and also during the experiment. To avoid overcrowding, around 35–40 animals were maintained in each tank. Healthy abalones were selected based on the general appearance and foot movement. To cognize the presence of *HdIκB* in various tissues, gill, mantle, male gonad, brain, female gonad, hepatopancreas, adductor muscle and digestive tract tissues harvested from such selected abalones. Hemocytes were collected and immediately centrifuged at 3000 × g for 10 min at 4 °C. The supernatant was removed and cells were collected. All the collected tissue samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C until use.

To decipher the defense responses of *HdIκB*, we performed a time course experiment with immunostimulants, including poly I:C, lipopolysaccharide (LPS), *Listeria monocytogenes, Vibrio parahemolyticus* and viral hemorrhagic septicemia virus (VHSV).

For intact bacterial challenge *L. monocytogenes* and *V. parahemolyticus* were obtained from the Korean Collection for Type Cultures. *L. monocytogenes* (KCTC3710) was cultured on LB agar plate at 30 °C overnight and inoculated in LB broth and maintained at 30 °C for 16 h. *V. parahemolyticus* (KCTC2729) was cultured in marine broth for 16–20 h at 25 °C. Then, the bacterial cells were harvested by centrifugation at 7000 × *g* at 4 °C for 5 min. The supernatant fluid was discarded and the pellets were resuspended in saline to make an original stock of 1×10^5 CFU/ml. Each abalone was injected with 100 μL suspension from the original stock