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Molecular characterization of an inhibitor of NF-κB in the scallop *Argopecten purpuratus*: First insights into its role on antimicrobial peptide regulation in a mollusk

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ABSTRACT

Inhibitors of nuclear factor kappa B (IκBs) are major control components of the Rel/NF-κB signaling pathway, a key regulator in the modulation of the expression of immune-related genes in vertebrates and invertebrates. The activation of the Rel/NF-κB signaling pathway depends largely in the degradation of IκB proteins and thus, IκBs are a main target for the identification of genes whose expression is controlled by Rel/NF-κB pathway. In order to identify such regulation in bivalve mollusks, the cDNA sequence encoding an IκB protein was characterized in the scallop *Argopecten purpuratus*, *Ap*IκB. The cDNA sequence of *ApIκB* is comprised of 1480 nucleotides with a 1086 bp open reading frame encoding for 362 amino acids. Bioinformatics analysis showed that *Ap*IκB displays the conserved features of IκB proteins. The deduced amino acid sequence consists of a 39.7 kDa protein, which has an N-terminal degradation motif, six ankyrin repeats and a C-terminal phosphorylation site motif. Phylogenetic analysis revealed a high degree of identity between *Ap*IκB and other IκBs from mollusks, but also to arthropod cactus proteins and vertebrate IκBs. Tissue expression analysis indicated that *ApIκB* is expressed in all examined tissues and it is upregulated in circulating hemocytes from scallops challenged with the pathogenic Gram-negative bacterium *Vibrio splendidus*. After inhibiting *ApIκB* gene expression using the RNA interference technology, the gene expression of the antimicrobial peptide big defensin was upregulated in hemocytes from non-challenged scallops. Results suggest that *Ap*IκB may control the expression of antimicrobial effectors such as big defensin via a putative Rel/NF-κB signaling pathway. This first evidence will help to deepen the knowledge of the Rel/NF-κB conserved pathway in scallops.

1. Introduction

The nuclear factor-κB (NF-κB) signaling pathway plays an essential role in regulating many physiological processes of vertebrates and invertebrates such as development, inflammation, apoptosis, cell proliferation, differentiation and immune responses [1,2]. NF-κB transcription factors from class II are characterized by a DNA binding region, a dimerization domain, a nuclear localization signal and a region of interaction with the IκB inhibitor protein family (IκBs), as class I possess their own inhibitory domain [1]. In unstimulated cells, NF-κB dimers are retained inactive in the cytoplasm by IκBs, proteins that display several ankyrin repeat motifs which serve to mask the nuclear localization signals of NF-κB proteins [3]. IκBs are also characterized by an N-terminal regulatory region that includes a degradation motif, and some IκBs display a C-terminal PEST domain with a casein kinase II phosphorylation motif [3]. A variety of extracellular stimuli activate cell surface receptors, which induce the activation of signal pathways

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that allow the phosphorylation of IκB proteins at specific serine residues located in the degradation motif. This phosphorylation triggers IκB degradation via ubiquitin-linked 26S proteasome [1]. Consequently, the active NF-κB dimer is then translocated into the nucleus, where it can promote the transcription of target genes.

As an evolutionary conserved pathway from invertebrates to higher vertebrates, the function of the NF-κB transcription factors have been studied extensively in the regulation of mammalian and arthropod immune-related genes [4]. In mammals, genes encoding cytokines, cell adhesion molecules, acute phase proteins, stress response proteins, cell surface receptors, growth factors, early response effectors and antimicrobial peptides, to name a few, have been described as regulated by NF-κB [5]. In invertebrates, the self-defense response against microbial infection is similar to the innate immune response in higher vertebrates [6]. The vast research on *Drosophila* immune response propose that NF-κB signaling represents an evolutionarily conserved pathway employed by diverse species in the self-defense mechanism. For instance, *Drosophila* has two independent immune signaling pathways, both of which lead to the activation of NF-κB transcription factors, such as Relish (Class I NF-κB) and Dorsal and DIF (Class II NF-κB) [7]. As in mammals, the activation of NF-κB in *Drosophila* involves the inducible expression of immune genes, with special emphasis on the regulation of antimicrobial peptides [7].

Despite the great progress made on the NF-κB regulatory mechanisms in mammals and *Drosophila*, many processes remain unknown in other non-model invertebrates such as mollusks. Like all invertebrates, the immune response of mollusks depends exclusively on innate immune mechanisms, mediated by cellular and humoral components. Not surprisingly, many of these components are well conserved between invertebrates, such as various cell receptors, signal transduction pathways and antimicrobial effectors [8].

A number of components related to Rel/NF-κB signaling pathway have been identified in scallops, including *Chlamys farreri* Toll-like receptor, Rel, IκB [9], MyD88 [10], TRAF6 [11], and IκB in *Argopecten irradians* [12]. Moreover, the expression levels of TLR, MyD88, and TRAF6 in hemocytes of *C. farreri* are upregulated by stimulation with LPS, suggesting their participation in the immune response [13]. In addition, recent data highlight the implication of these molecules in the Rel/NF-κB pathway. In the scallop *C. farreri*, the expression of a Rel homologue decreased significantly after its co-expression with the scallop IκB, determined by NF-κB luciferase reporter assays [14]. Similarly, the *Cg*IκB3 from the oyster *Crassostrea gigas* can inhibit the NF-κB activation [15]. Thus, the Rel/NF-κB signaling pathway has been well described in bivalve mollusks such as the oyster *C. gigas* and could display homologous functions as in arthropod and mammals. Nevertheless, it still lacks functional evidence of their implication in immune gene regulation.

The scallop *Argopecten purpuratus* represents one of the most economically important cultured bivalve mollusk on coastal provinces of northern Chile. However, scallop production has gradually declined due to the emergence of mass mortality events, which has been associated to the Gram-negative bacterium *Vibrio splendidus* [16]. Because the development of infectious diseases is a constraint to aquaculture sustainability, the health of the animals has been one of the major concerns in the intensification of aquaculture management methods [17]. Research efforts has been made in the last years to characterize important traits such as fast growth, reproduction and stress tolerance in *A. purpuratus* [18–22]. This approach has required an increased knowledge of the genetic factors involved in these traits which has motivated the characterization of scallop immunity (reviewed in Ref. [23]).

In order to understand the mechanisms underlying the immune regulation in scallops, we focus our attention in the Rel/NF-κB conserved pathway. The activation of the Rel/NF-κB signaling pathway depends largely in the degradation of IκB proteins and thus, IκBs are a main target for the identification of genes whose expression is controlled by Rel/NF-κB pathway [24]. In this study, an IκB homologue was cloned and characterized from *A. purpuratus*, designated as *Ap*IκB. The basal *ApIκB* gene expression was addressed from different tissues as well as the modulation of its expression in response to a bacterial challenge. Finally, the RNA interference technology was used for silencing *ApIκB* expression to assess the effect of the *Ap*IκB in the regulation of the Big defensin antimicrobial peptide gene expression.

2. Material and methods

2.1. Animals, bacterial challenge and tissue collection

US National Research Council guidelines for the care and use of laboratory animals were strictly followed during this research [25]. Adult scallops (70–80 mm shell height) were collected at the Tongoy bay, Chile (30°16′ S, 71° 35′W). Scallops were transferred to the wet laboratory at the Universidad Católica del Norte, Coquimbo Chile. Two hundred scallops were acclimatized for 1 week in 1000 L tanks supplied with filtered, aerated, running seawater (~16 °C), and fed with a diet of 50% *Isochrysis galbana* and 50% *Nannochloris* sp (6×10^6 cells/mL/day). Following acclimation, 100 μL of (i) heat-attenuated *V. splendidus* VPAP16 [16] (10^7 CFU/scallop) or (ii) sterile seawater, as injury control, were injected in the scallop adductor muscle. *V. splendidus* was heat-attenuated in order to expose the scallops to a pathogen associated molecular pattern (PAMP) immune stimulus and eliminate the virulent component of the strain that could inhibit the immune response activation. Four groups of four scallops were considered in each condition and no mortality was observed during the experimental challenge. Hemolymph from scallops was collected at 12 h, 24 h and 48 h from the pericardial cavity. Hemocytes were isolated by centrifugation to discard plasma (600×*g* for 5 min at 4 °C). Hemocytes, gills and mantle tissues from injected scallops were harvested by dissection and kept in RNAlater at −80 °C until total RNA extraction. In parallel, muscle, gills, mantle, digestive gland, gonad and hemocytes tissues were extracted from sixteen naïve adult scallops and kept in RNAlater at −80 °C until total RNA extraction for evaluation of *ApIκB* basal gene expression.

2.2. Total RNA extraction and reverse transcription

Total RNA was extracted from *A. purpuratus* tissues using TRIzol® reagent according to manufacturer instructions (Thermo Scientific). RNA was then treated with DNAse I (Thermo Scientific), 15 min at room temperature and inactivated by heat, 10 min at 65 °C, followed by a second precipitation with sodium acetate 0.3 M (pH 5.2) and isopropanol (1:1 v:v). Then, quantification and quality of total RNA were determined using a NanoDrop spectrophotometer (NanoDrop Technologies) and agarose gel electrophoresis, respectively. Following heat denaturation of 1 μg of total RNA (65 °C for 5 min), first strand synthesis was carried out using 50 ng oligo-(dT) 12–18 (Thermo Scientific), 1 mM dNTPs (Promega), 1 U Rnasin (Promega) and 200 U M-MLV reverse transcriptase in reverse transcriptase buffer (Promega) following the manufacturer protocol.

2.3. Molecular cloning of ApIκB cDNA

Two primers (Table 1) were designed from the *A. irradians* IκB sequence (GenBank no. FJ824733) to clone the complete cDNA sequence of *A. purpuratus* IκB. PCR reactions were carried out in