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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

Table 1
Nucleotide sequence of primers.

Primer name	Forward primer (5′–3′)	Reverse primer (5′–3′)	GenBank
Primers for molecular cloning			
c*ApIκB*	CGAACACGAGAGAACTCAGTG	AAGCTATCCCTTCGTACTTGTG	FJ824733
Primers for RT-qPCR			
q*ApActin*	CACTGCTCTTGCTCCACAAAC	GAAGGTGGACAGAGATGCCAA	ES469330
q*ApIκB*	GCGTTGATGGTGTATGGTAC	TCTGCCGTAATTCGTCGTTG	FJ824733
q*ApBD1*	TCAGTAGGTCTAGGGACAAC	ACAAACGCCTAAGTTCCCAC	KU499992
Primers for RNAi			
dsGFP	T7-GAGCAAGGGCGAGGAGCTGT	T7-CCTCCTTGAAGTCGATGCCC	HM640279
ds*ApIκB*	T7-TATGGCTCTGGTTCGTTGTC	T7-ATATCAAGCCAGTCTGCTGC	FJ824733

T7-tag sequence: TAATACGACTCACTATAGG.

25 μL-volume reaction with GOTaq polymerase according to manufacturer instructions (Promega), using 1 μL of synthesized cDNA under the following conditions: 10 min at 95 °C, then 35 cycles at 95 °C for 30 s, 57 °C for 30 min, 72 °C for 2 min and a final elongation step at 72 °C for 7 min. PCR product was gel purified using a E.Z.N.A.® Gel Extraction Kit (Omega Biotek) and cloned using TOPO TA with the pCR 2.1 TOPO® vector (Thermo Scientific). Plasmids were transformed into DH5α *Escherichia coli* cells and plasmids were purified using a E.Z.N.A.® Plasmid Mini Kit I (Omega Biotek). Plasmid constructs were verified by PCR and sequencing (Macrogen Inc., Seoul, Korea).

2.4. Sequence analysis, prediction of three-dimensional structure and phylogenetic analysis

The retrieval of IκB nucleotide sequences from vertebrates and invertebrates were performed using the BLAST algorithm at the National Center for Biotechnology Information [26]. The multiple alignments were generated using the MAFFT alignment program [27]. Domain analysis over the IκB protein sequence were performed using three different web portals as comparison, CDD (Conserved Domain Database, NCBI) [28], SMART (Simple Modular Architecture Research Tool, EMBL) [29] and MOTIF GenomeNet from the Kyoto University Bioinformatics Center, using the default parameters. Proscan (http://pbil.ibcp.fr/) was used to deduce the casein kinase II phosphorylation site. The epestfind software available at The European Molecular Biology Open Software Suite was used to predict potential PEST domains [30]. Homology modeling of the protein were perform using 4 different servers, RaptorX [31], Phyre2 [32], M4T [33] and IntFOLD2 [34] and the resultant structures where analyzed using the QMEAN server for quality estimation of each individual model, penalizing the incomplete models [35].Construction of phylogenetic trees was performed using the Neighbor-Joining method with bootstrap values calculated from 1000 trees, included in the MEGA package [36].

2.5. Reverse transcription polymerase chain reaction (RT-PCR) and real-time quantitative PCR (qPCR) analysis of gene expression

The expression stability of the β-actin gene (GenBank no. ES469330) was assessed by RT-PCR for each tissue. RT-PCR reactions were carried out with GOTaq polymerase according to manufacturer instructions (Promega), using specific primers (Table 1) and 1 μl of synthesized cDNA under the following conditions: 10 min at 95 °C, then 20 cycles at 95 °C for 30 s, 57 °C for 1 min, 72 °C for 2 min and a final elongation step at 72 °C for 7 min. RT-PCR products were analyzed by agarose gel electrophoresis. For the analysis of the gene expression, 10 μl-volume reaction consisted in 1 × Brilliant II SYBR Green QPCR master mix (Stratagene), 0.5 μM of each primer and 1 μL of cDNA diluted at 1/8 in sterile ultra-pure water. Primers are listed in Table 1. RT-qPCR assays were performed in triplicate in a Biorad C1000 Touch Thermocycler CFX96, and primer pair efficiencies (E) were calculated from six serial dilutions of pooled cDNA for each primer pair. Primer pair efficiencies were calculated from the given slopes in the BioRad CFX software according to the equation: $E = 10^{[-1/slope]}$. Assays were submitted to an initial denaturation step of 10 min at 95 °C followed by an amplification of the target cDNA (40 cycles of denaturation at 95 °C for 5 s, annealing at 58 °C for 5 s and extension time at 72 °C for 15 s) and fluorescence detection. After an initial 10 s denaturation step at 95 °C, a melting curve was obtained from a start temperature of 65 °C to a final temperature of 95 °C, with an increase of 0.06 °C/s. Relative expression was calculated using the $-2^{\Delta\Delta Cq}$ method [37] using the measured quantification cycle (Cq) values of the constitutively expressed gene β-actin to normalize the measured Cq values of target genes. Calculations of means, standard deviations and statistical analysis using Kruskal-Wallis test for expression analysis were carried out using GraphPad Prism software version 6.01 (significant value: $P < 0.05$). Statistical difference between conditions were based on the selection criteria upon a cut-off value of >3 fold-change over the control group ($P < 0.05$).

2.6. RNAi experiments

Sequence specific primers (Table 1) were designed to amplify specific sequences from plasmid constructs and to add the T7 promoter sequence TAATACGACTCACTATAGGG. The control plasmid used in this study contained the sequence for the green fluorescent protein (GFP) gene (GenBank no. HM640279) which is not present in *A. purpuratus*. The PCR products were verified on agarose gel and then purified using E.Z.N.A.® Gel Extraction Kit (Omega Biotek). T7 RiboMAX® Express RNAi System was used to synthesize the dsRNA in accordance with manufacturer's instructions. The quantity and quality of the RNAi were determined using a NanoDrop spectrophotometer (NanoDrop Technologies) and agarose gel electrophoresis, respectively. The dsGFP and the dsIκB were diluted in saline solution (10 mM Tris, 330 mM NaCl, pH 7.4) and 10 μg in 100 μL were injected into the adductor muscle of scallops. Two groups of 16 scallops were used in each experimental condition and no mortality was observed during the experiment. Hemocytes from dsGFP and dsIκB injected scallops were collected at 24 h and 48 h from the adductor muscle, as described above. Total RNA was extracted from hemocytes from each scallop individually and RT-qPCR was performed to assess the effectiveness of the RNAi experiment and the modulation of the *A. purpuratus* Big defensin *Ap*BD1 gene expression (GenBank no. KU499992).

3. Results

3.1. cDNA cloning and sequence analysis of ApIκB

A 1480 bp nucleotide sequence was amplified by PCR from

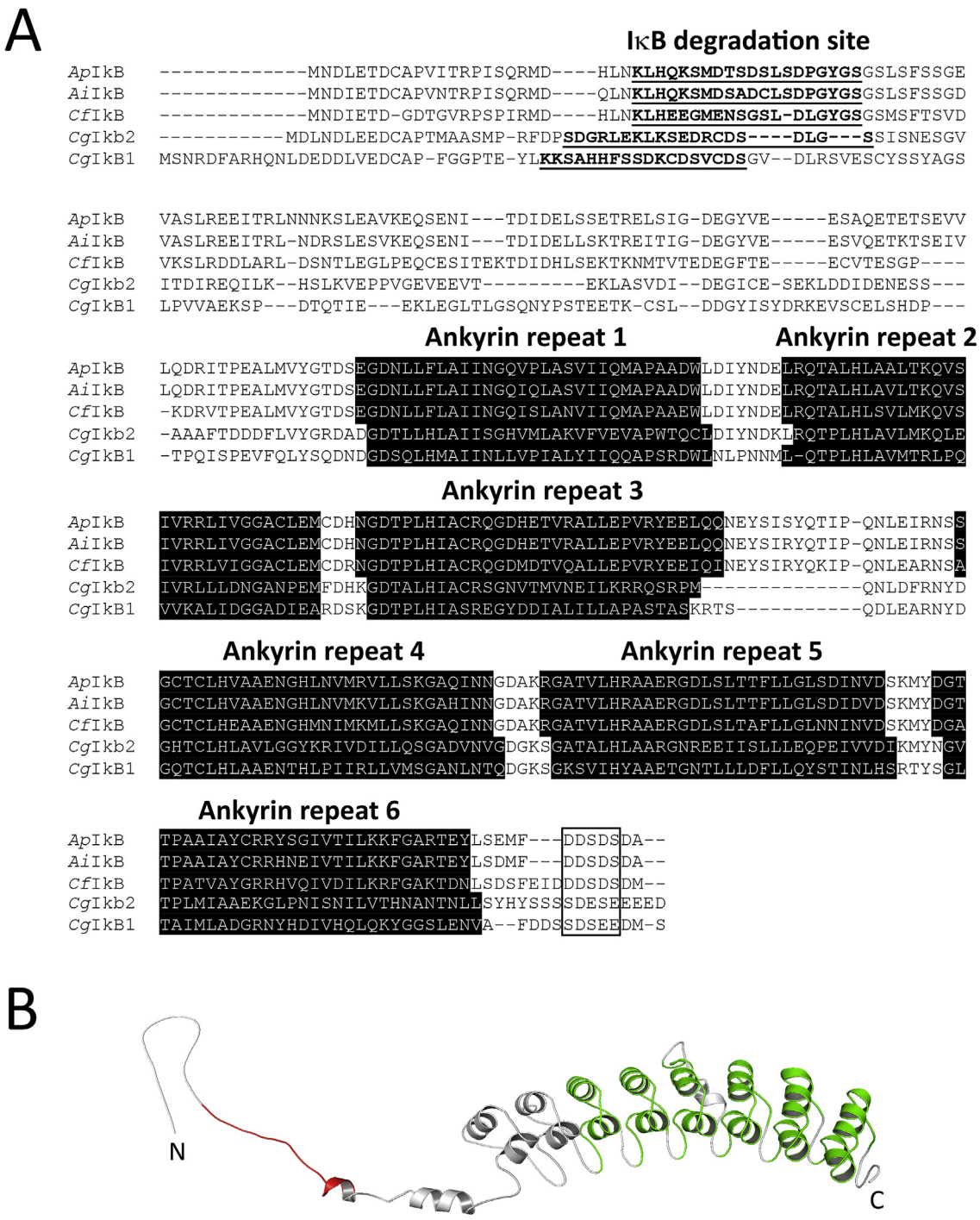

Fig. 1. Molecular characterization of the inhibitor of NF-κB from the scallop *Argopecten purpuratus*, *Ap*IκB. A. Amino acid sequence alignment of *Ap*IκB with other bivalve IκBs, the scallops *A. irradians* (*Ai*IκB: ACZ34178) and *Chlamys farreri* (*Cf*IκB: ABI37009) and the oyster *Crassostrea gigas* (*Cg*IκB1: ABB52821; *Cg*IκB2: ADX06856). The underlined region indicated the N-terminal degradation domain. The black regions indicated the ankyrin domains. Boxes indicated the casein kinase II phosphorylation domain. Dashes indicated gaps. **B**. Predicted three-dimensional structure of *Ap*IκB. The six ankyrin domains are shown in green, each containing two antiparallel helices and a beta-hairpin. The N-terminal degradation domain is shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A. purpuratus cDNA using primers designed from the *A. irradians* IκB sequence (GenBank no. FJ824733). The complete cDNA obtained sequence was designated as *ApIκB* (GenBank no. KU170642). *ApIκB* consisted in a 5'terminal untranslated region (UTR) of 160 bp, an open reading frame (ORF) of 1086 bp, encoding a protein of 362 amino acids, and a 3′UTR of 231 bp. Predicted molecular mass of the deduced protein sequence is 39724 Da and displays a theoretical isoelectric point of 4.64.

The multiple sequence alignment revealed a high identity of the typical conserved motifs of the IκB protein family, such as the IκB degradation sequence motif located at the N-terminal region and the six ankyrin repeats at the C-terminal region (Fig. 1A). The N-

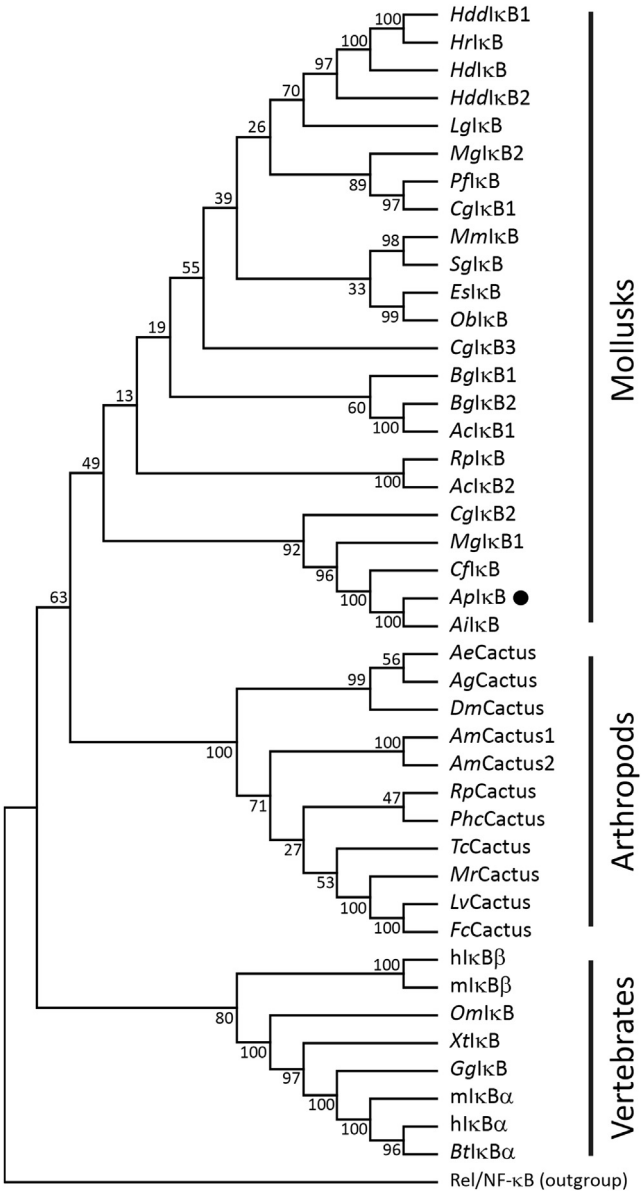

Fig. 2. Phylogenetic tree of IκB proteins from invertebrates and vertebrates. The tree was constructed using the neighbor-Joining method with bootstrap values calculated from 1000 trees. Sequences included in analyses were the following: (i) IκB proteins from mollusks: scallops *Argopecten purpuratus* (*Ap*IκB: ALV13262), *A. irradians* (*Ai*IκB: ACZ34178) and *Chlamys farreri* (*Cf*IκB: ABI37009), oysters *Crassostrea gigas* (*Cg*IκB1: ABB52821; *Cg*IκB2: ADX06856; *Cg*IκB3: ALF45441) and *Pinctada fucata* (*Pf*IκB: ACF93446), mussel *Mytilus galloprovincialis* (*Mg*IκB1: AHI17300; *Mg*IκB2: AHI17301), clams *Ruditapes philippinarum* (*Rp*IκB: AEB92230), *Solen grandis* (*Sg*IκB: AEW43453) and *Meretrix meretrix* (*Mm*IκB: ADK74377), abalones *Haliotis diversicolor* (*Hd*IκB: AHM27300), *H. discus discus* (*Hdd*IκB1: AFO64973; *Hdd*IκB2: AHX37217) and *H. rufescens* (*Hr*IκB: AGZ03662), snail *Biomphalaria glabrata* (*Bg*IκB1: ABL74452; *Bg*IκB2: XP_013067082), owl limpet *Lottia gigantea* (*Lg*IκB: ESO85665), sea hare *Aplysia californica* (*Ac*IκB1: XP_005111546; *Ac*IκB2: XP_005100816), squid *Euprymna scolopes* (*Es*IκB: AAY27980) and octopus *Octopus bimaculoides* (*Ob*IκB: KOF82090); (ii) Cactus proteins from arthropods: fruit fly *Drosophila melanogaster* (*Dm*Cactus: AAA85908), mosquitoes *Anopheles gambiae* (*Ag*Cactus: EAA12805) and *Aedes aegypti* (*Ae*Cactus: EAT48252), honey bee *Apis mellifera* (*Am*Cactus1: 001157184; *Am*Cactus2: XM_394485), body louse *Pediculus humanus corporis* (*Phc*Cactus: EEB15048), red flour beetle *Tribolium castaneum* (*Tc*Cactus: NP_001157183), blood-sucking bug *Rhodnius prolixus* (*Rp*Cactus: AJV90964), penaeid shrimps *Litopenaeus vannamei* (*Lv*Cactus: AFO38331) and *Fenneropenaeus chinensis* (*Fc*Cactus: AFU60550) and freshwater prawn *Macrobrachium rosenbergii* (*Mr*Cactus: AET34918); (iii) vertebrate IκB proteins: rainbow trout *Oncorhynchus mykiss* (*Om*IκB: CAC85086), frog *Xenopus* (*Silurana*) *tropicalis* (*Xt*IκB: CAJ83355), chicken *Gallus gallus* (*Gg*IκB: Q91974), cattle *Bos taurus* (*Bt*IκBα: AAI05485), mouse *Mus musculus* (mIκBα: AAH46754; mIκBβ: AAH21938) and human (hIκBα: AAP35754; hIκBβ: AAP36616). A scallop Rel/NF-κB sequence from *C. farreri* (GenBank: ADD25211) was used as outgroup.

terminal region of *Ap*IκB contained a D_{39}PGYGS$_{44}$ amino acid sequence that showed homology to the conserved DSGXXS degradation motif in other IκBs. Furthermore, two lysine residues presenting potential ubiquitination are found upstream of the consensus sequence within the degradation motif (Fig. 1A). No PEST motif was found by the *in silico* analysis. Nevertheless, the C-terminal region appears to contain a putative conserved casein kinase II phosphorylation site (DDSDS$_{359}$) (Fig. 1A). The IκB degradation motif, the ankyrin region and the casein kinase II phosphorylation site are highly conserved among IκBs from scallops and to a lesser extend among oyster IκBs (Fig. 1A).

3.2. Three-dimensional structure and phylogenetic analysis of *ApIκB*

The deduced amino acid sequence of *Ap*IκB was analyzed by the three-dimensional structure modeling. The structural analysis showed that each ankyrin repeat contains two antiparallel helices and a beta-hairpin (Fig. 1B). Additionally, the cleavage domain (between K_{25} to S_{44}) corresponding to the IκB degradation motif was detected close to the N-terminal region.

The deduced amino acid sequences of a number of IκB isoforms from vertebrates and invertebrates (including arthropod and mollusk) were aligned and used to build a phylogeny (Fig. 2). The Rel/NF-κB sequence from *C. farreri* (GenBank no. ADD25211) was included as an outgroup. The phylogenetic tree showed a clearly structured phylogeny where sequences clustered in distinct clades, separating vertebrate IκBs from invertebrate IκBs. Contained in the clade of invertebrate IκBs, *Ap*IκB clustered with the IκBs from bivalve mollusks. *Ap*IκB was shown to be highly related with IκBs from the scallops *A. irradians* (GenBank no. ACZ34178) and *C. farreri* (GenBank no. ABI37009), followed by *Mg*IκB1 from the mussel *Mytilus galloprovincialis* (GenBank no. AHI17300) and *Cg*IκB2 from the oyster *C. gigas* (GenBank no. ADX06856) (Fig. 2).

3.3. Tissue distribution of *ApIκB* mRNA expression

The relative expression of *ApIκB* was evaluated in six particular organs and tissues to determine the specific distribution of *ApIκB* mRNA. Thus, cDNA from muscle, gills, mantle, digestive gland, gonad and hemocytes of *A. purpuratus* was synthesized and *ApIκB* expression was determined by RT-qPCR using actin as reference gene due to its high expression stability among all analyzed tissues (Fig. 3). Results showed that *ApIκB* was constitutively expressed in all examined tissues, but at different relative levels. The highest expression level of *ApIκB* was found in the muscle and gill, while the lowest was found in hemocytes.

3.4. Temporal expression of *ApIκB* and *ApBD1* after *V. splendidus* challenge

The mRNA expression of *ApIκB* and the antimicrobial peptide Big defensin (*ApBD1*) was assessed from scallops injected with heat-attenuated *V. splendidus* or sterile seawater. The expression of Big defensin gene *ApBD1* was considered as a control of immunostimulation due to its well-known upregulation after an immune challenge in scallop [38]. Therefore, mRNA expressions from both genes were analyzed in hemocytes, gills and mantle tissues by RT-qPCR (Fig. 4). Results showed a significant increase in the *ApIκB* transcription specifically in hemocytes post *V. splendidus* injection at 48 h ($P < 0.05$) (Fig. 4, upper panel). The relative expression of *ApIκB* increased up to four-fold in *V. splendidus* injected scallops, while no significant change in the relative expression of *ApIκB* was observed in sterile seawater injected scallops during the examined period. No significant modulation of *ApIκB* was detected in the gills

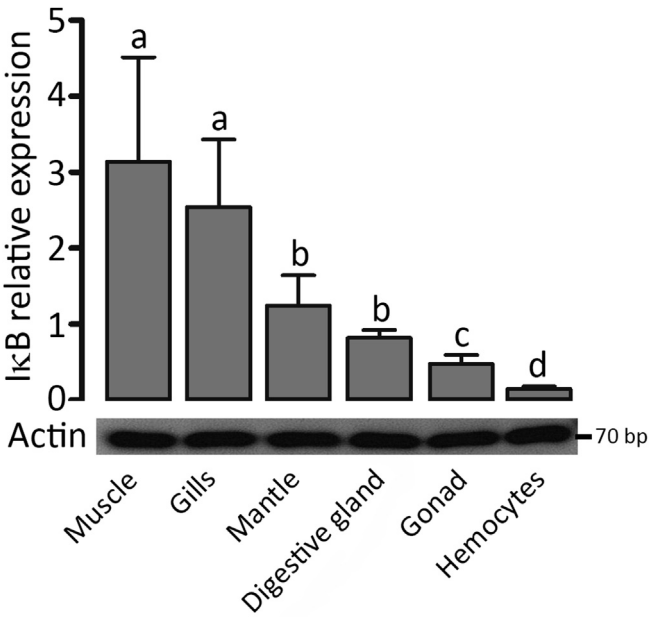

Fig. 3. Basal gene expression of *ApIκB* in different tissues from naïve adult scallops. Relative expression levels of *ApIκB* were obtained by RT-qPCR according to the $2^{-\Delta\Delta Cq}$ method [37]. The scallop β-actin gene was used as reference gene and its expression stability was assessed by RT-PCR product electrophoresis for each tissue (lower panel). Muscle, gills, mantle, digestive gland, gonad and hemocytes tissues were collected from sixteen individual scallops. Results are expressed as mean values ± SD. Significant differences of relative expressions between tissues are indicated by different lowercase letters ($P < 0.05$).

or mantle tissues in the *V. splendidus* and sterile seawater injected conditions. Interestingly, the *ApBD1* expression was upregulated in the three examined tissues post *V. splendidus* injection at 24 h and 48 h ($P < 0.05$) (Fig. 4, lower panel). In hemocytes, the relative expression of *ApBD1* increased between three and four-fold in *V. splendidus* injected scallops at 24 h and 48 h respectively, with the highest level detected at 48 h post-injection. In gills and mantle tissues the *ApBD1* expression was also upregulated close to three-fold, showing the highest level at 24 h post *V. splendidus* injection. No significant differences of *ApBD1* expression were found in sterile seawater injected scallops during the examined period.

3.5. Effect of ApIκB silencing by RNA interference on ApBD1 expression in A. purpuratus hemocytes in vivo

To assess the role of *Ap*IκB on the Rel/NF-κB signaling pathway in mollusks, a RNA interference approach was used in order to inhibit its expression *in vivo*. Thus, dsRNA for *ApIκB* gene and for the green fluorescent protein (GFP) gene were synthesized and 10 μg were injected in the adductor muscle of individual scallops. The group injected with the dsGFP was used as control. The expression of *ApIκB* was evaluated in hemocytes from scallops after 24 h and 48 h post-injection by RT-qPCR (Fig. 5). Results showed that the mRNA expression of *ApIκB* in the dsIκB injected group was suppressed to approximately 23% compared to the dsGFP injected group at both 24 h and 48 h after treatment (~77% of knockdown) (Fig. 5A).

To further investigate the effect of the *ApIκB* silencing on the antimicrobial peptide gene regulation, the mRNA expression of *ApBD1* was assessed in hemocytes from *A. purpuratus* injected with dsIκB or dsGFP at 24 h by RT-qPCR. Results showed that the expression of *ApBD1* in the dsGFP injected group display the normal basal level previously observed in hemocytes from naïve scallops. Nevertheless, the *ApBD1* expression on hemocytes from the dsIκB injected group showed a significant upregulation ($P < 0.05$), increasing up to five-fold at 24 h post-injection (Fig. 5B).

4. Discussion

This study reports the identification and characterization of a novel IκB gene from the scallop *A. purpuratus*, namely *ApIκB*. Results

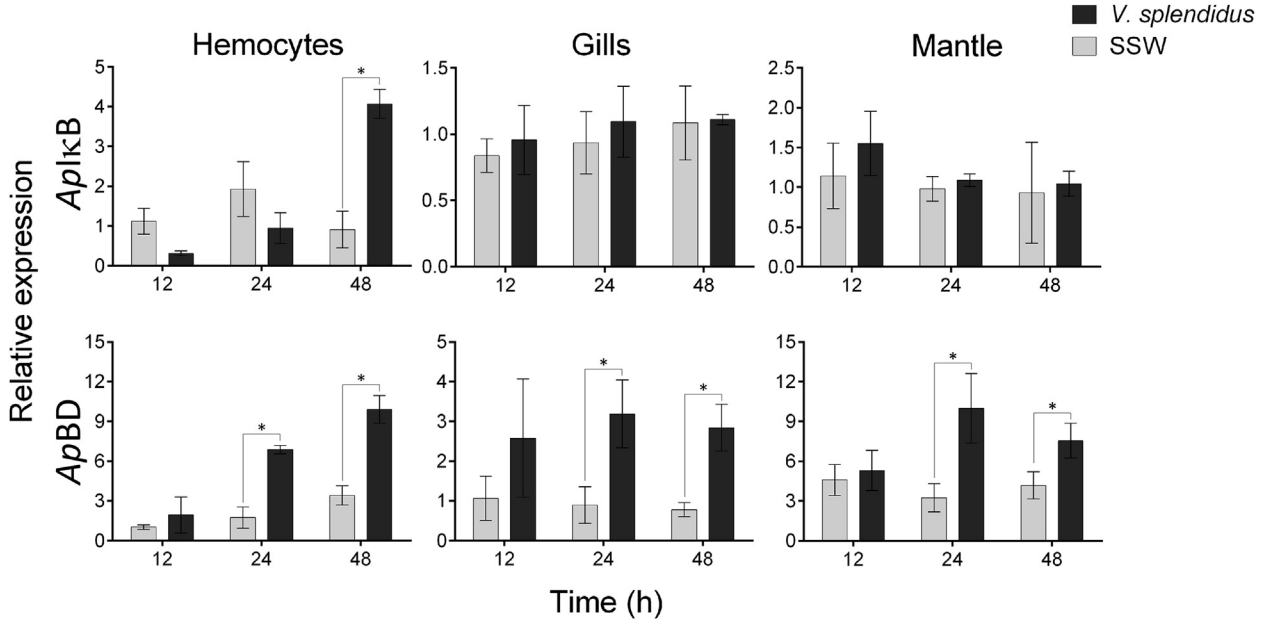

Fig. 4. Relative expression of *ApIκB* and *ApBD1* in the scallop *A. purpuratus* after a bacterial challenge. Relative expression of the immune genes *ApIκB* and *ApBD1* were obtained by RT-qPCR according to the $2^{-\Delta\Delta Cq}$ method [37]. The scallop β-actin gene was used as reference gene. Relative expressions were obtained from circulating hemocytes, gill and mantle tissues at 12 h, 24 h and 48 h from injury control scallops (SSW, gray bars) and *Vibrio* challenged scallops (*V. splendidus*, black bars). Upper panel; *ApIκB* expression. Lower panel; *Ap*BD1 expression. Results are expressed as mean values ± SD from 4 pools of 4 scallops by each condition. Asterisks indicate significant differences ($P < 0.05$).

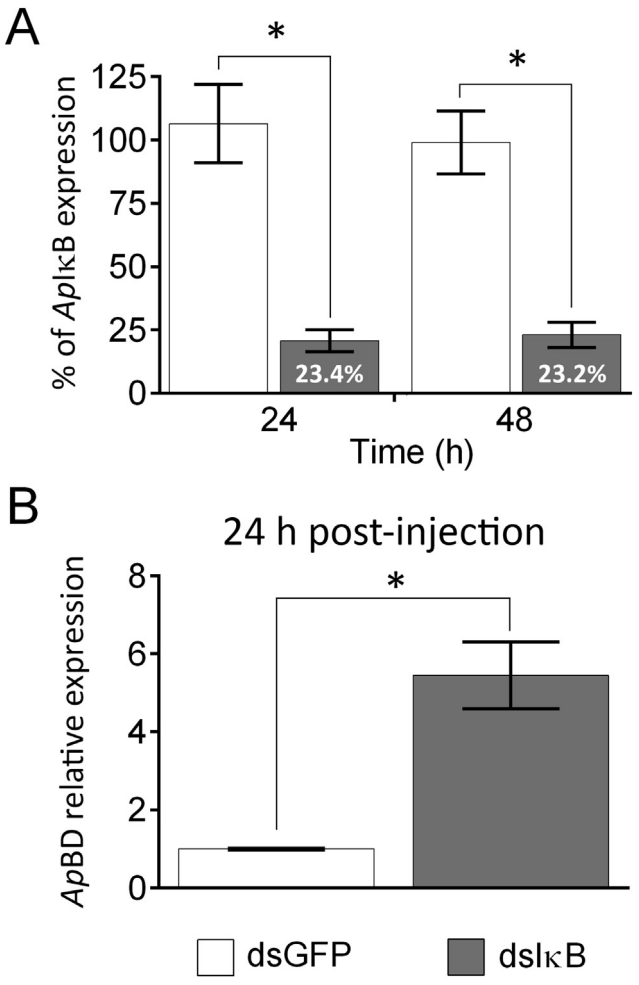

Fig. 5. *In vivo* effect of the knockdown of *ApIκB* mRNA by RNA interference on *ApBD1* gene expression. A. Expression level of *ApIκB* in dsGFP (white bars) and dsIκB (gray bars) injected scallops at 24 h and 48 h post dsRNA injection. Results are expressed as a percentage of *ApIκB* expression in dsGFP injected scallops. **B**. Relative expression of Big defensin *ApBD1* in dsGFP and dsIκB injected scallops at 24 h post RNAi injection obtained by RT-qPCR according to the $2^{-\Delta\Delta Cq}$ method [37]. Two groups of 16 scallops were used in each experimental condition. Asterisks indicate significant differences ($P < 0.05$).

showed that the predicted protein displays the typical features of invertebrate IκBs, such as the molecular mass and an isoelectric point, the conserved functional domains, and its mRNA upregulation in hemocytes after an immune challenge. Most importantly, the knockdown of *ApIκB* gene expression by RNAi suggests that this inhibitor of NF-κB may be implicated in the regulation of the expression of antimicrobial peptides such as Big defensin. To the best of our knowledge, whereas quite a few molluscan IκBs have been characterized, no such IκB has been associated to the regulation of antimicrobial peptide gene expression in a mollusk. Notably, further research related to the activation of NF-κB and its translocation into the nucleus is essential to fully understand the role of Rel/NF-κB pathway in scallops.

*Ap*IκB contains several conserved domains which were demonstrated to be essential for IκB function in mammals [39]. Ankyrin repeat motifs are known to mediate the protein-protein interaction in diverse protein families [40]. In mammalian IκBs and arthropod Cactus genes, they serve to interact with the Rel-homology domain of Rel/NF-κB transcription factors and mask the nuclear localization sequence [5]. The number and structure of ankyrin repeats in the C-terminal region of *Ap*IκB is similar to those found in all IκB proteins [5], which indicates the potential ability of Rel homology domain of class II Rel/NF-κB transcription factors inactivation by *Ap*IκB in *A. purpuratus*.

Following cell stimulation, IκB is phosphorylated at specific residues in the N-terminal degradation motif. Within this degradation motif, two serine residues found in the consensus sequence DSGXXS are critical to allow the degradation of the inhibitor [41]. Strikingly, the *Ap*IκB consensus sequence $D_{39}PGYGS_{44}$ displays a substitution in the second position which produced the mutation of a serine residue for a proline residue. Although serine residues have been described as essential for IκB degradation, the same mutation of this specific serine has been observed in other scallop IκB [12]. In addition, two lysine residues within the degradation domain are located upstream of the consensus sequence, which are most likely a target for ubiquitination [42]. Thus, results suggest the existence of a degradation motif diversity in molluscan IκBs which requires further research.

The PEST motif is defined as domain rich in proline, glutamic acid, serine and threonine residues which appears to function as a proteolytic signal for degradation of mammalian IκBs [43]. Certain phosphorylation sites have been described within the PEST domain, such as the casein kinase II phosphorylation motif [44]. Several IκBs from mammals and *Drosophila* Cactus display the PEST motif [43,44] but is not a common feature in most invertebrate IκBs [12,15,45,46]. Nevertheless, the casein kinase II phosphorylation motif is widely present in molluscan IκBs, suggesting that this site could actually play an important role in the degradation of *Ap*IκB.

Transcript expression analysis showed that *ApIκB* is constitutively expressed at variable levels in all analyzed tissues. *ApIκB* expression profile is in accordance with the expression described in the bay scallop *AiIκB*, where the lowest expression was found in hemocytes [12]. Nevertheless, *ApIκB* expression contrasts with the results described in clams and oyster IκBs, where the highest expression was found in hemocytes [45,47,48]. Remarkably, previous studies have identified three types of IκBs which are differentially upregulated in *C. gigas* tissues [15,49]. Thus, the expression variability of IκBs could be explained by the existence of different types of IκBs according to different tissues, related to the pleiotropic function of NF-κB pathway.

In vertebrates and arthropods, the Rel/NF-κB pathway is critical for the activation of immune responses and must be finely regulated to overcome the detrimental effects of a prolonged activation [39]. One of the self-regulatory mechanisms of the NF-κB pathway is the activation of *IκB* transcription along with the expression of immune genes, which promotes the IκB accumulation *de novo* and the inactivation of NF-κB [50]. The upregulation of *ApIκB* in the hemocytes at 48 h suggest that *ApIκB* undergoes *de novo* synthesis to sequestrate NF-κB. Indeed, the upregulation of the well-known inducible antimicrobial peptide *ApBD1* in hemocytes at 24 h post-challenge support the hypothesis of the *ApIκB* self-regulating loop. Noteworthy, *ApBD1* was overexpressed in the hemocytes, mantle and gills in response to the bacterial challenge whereas *ApIκB* was only overexpressed in hemocytes. Since Big defensins are exclusively expressed in hemocytes [38,51], it is tempting to speculate that the overexpression of *ApBD1* in mantle and gills is produced by hemocyte infiltration and it can be detected due to its strong expression level. The lack of detection of *ApIκB* overexpression in mantle and gills could be related to the *ApIκB* low mRNA levels from hemocytes found in the infiltrated tissues.

RNAi technology is an effective manner to induce a post-transcriptional gene-silencing by dsRNA that has been slightly employed to silence gene expression in non-model organisms such as mollusks [13,52–54]. In the present study, the RNAi approach was performed in order to assess the effect of *Ap*IκB in the

antimicrobial peptide *Ap*BD1 expression in naïve scallops. We have focused the expression analysis on the 24 h time point since big defensins from a number of bivalve mollusks have been shown to be upregulated in hemocytes after an immune challenge at that time point [38,51,55]. Therefore, considering that the silencing of *ApIκB* will increase the availability of NF-κB, we expected to observe the upregulation of big defensin expression at a similar time point. Accordingly, *ApIκB* was specifically knockdown by dsRNA and this resulted in the overexpression of the *ApBD1* gene. Considering the specificity of IκBs in the retention of inactive NF-κB, it is likely that one of the effects of the knockdown of *ApIκB* is the translocation of NF-κB into the nucleus. In this sense, *ApBD1* expression could depend on NF-κB or other expression product regulated by NF-κB. This is in accordance with several studies that describe the regulation of antimicrobial peptide expression by the Rel/NF-κB signaling pathway in mammalian and arthropod species [6,7,56]. Thus, the results obtained in this study suggest the existence of a conserved function of the NF-κB pathway in mollusks, with emphasis on the regulation of antimicrobial peptides as innate immune effectors. Further studies on the effect of *ApIκB* silencing in immune challenged scallops will provide new insights of the role of NF-κB during the immune response in *A. purpuratus*.

In conclusion, an inhibitor of NF-κB was identified and characterized in *A. purpuratus*. *Ap*IκB displays the conserved characteristics of IκB proteins and it is upregulated in hemocytes in response to an immune challenge. Besides, the knockdown of *ApIκB* gene expression by RNAi suggests that it may control the expression of a big defensin via a putative Rel/NF-κB signaling pathway. This first evidence will help to deepen the role of the Rel/NF-κB conserved pathway in scallops.

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