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Pf-Rel, a Rel/Nuclear Factor-κB Homolog Identified from the Pearl Oyster, *Pinctada fucata*

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Abstract Transcription factor Rel/nuclear factor-kappa B (NF-κB) has been the focus of many studies since its discovery in 1986. Different homologs of Rel/NF-κB have been found in both vertebrate and invertebrate. A cDNA clone encoding a putative Rel/NF-κB homolog (designated *Pf*-Rel) was isolated from the pearl oyster, *Pinctada fucata*. The sequence of *Pf*-Rel consists of the Rel homology domain, IPT NF-κB domain and C-terminal transactivation domain. Sequence analysis of *Pf*-Rel shows that it shares high similarity with other Rel/NF-κB family proteins, especially within the conserved domains. Reverse transcription-polymerase chain reaction analysis revealed that *Pf*-Rel mRNA was expressed ubiquitously. Further *in situ* hybridization analysis showed that *Pf*-Rel mRNA was expressed mainly at the outer epithelial cells of the middle fold and the inner epithelial cells of the outer fold. The identification and characterization of pearl oyster *Pf*-Rel help to further investigate the involvement of Rel/NF-κB in oyster immunity and other biological processes.

Keywords NF-κB; Rel; *Pinctada fucata*; pearl oyster

Nuclear factor-kappa B (NF-κB) was first identified as a binding protein that interacted with the specific DNA sequence within the immunoglobulin heavy chain and kappa light chain enhancers in B cells. It was also shown that NF-κB could be induced by bacterial lipopolysaccharide and active phorbol ester, and the process of induction did not require protein synthesis [1,2]. During the two decades since its discovery, Rel/NF-κB has been identified in most cell types and much progress on this subject has been made. It is now known that different kinds of biological factors can induce NF-κB, which then regulates the expression of a wide variety of genes that are involved in many cellular processes. All the Rel/NF-κB family proteins share the highly conserved region called Rel homology domain (RHD), which is essential in dimerization, nuclear location and DNA binding [3–5]. In the majority of cell types, Rel/NF-κB proteins are sequestrated in the cytoplasm as homodimers or heterodimers, binding to the inhibitory proteins IκB. The interactions between the ankyrin repeats of IκB and the RHD in Rel/NF-κB dimers inhibit the translocation of Rel/NF-κB proteins to the nucleus. However, with the effects of many extracellular stimuli, the serine kinase IκB kinase (IKK) is activated [6]. It then catalyzes the phosphorylation, ubiquitination and degradation of IκB proteins, which leads to the translocation of the released Rel/NF-κB dimers to the nucleus. The activated Rel/NF-κB proteins then bind to DNA and activate gene transcription.

Extensive studies have elucidated the important roles of the NF-κB signaling pathway in many aspects: ontogeny of the immune system; immune and inflammatory responses to pathogens; cell survival and apoptosis; oncogenesis; skin physiology and pathology; hemopoiesis; and neuronal survival [7–10]. Of these, the focus of most studies has been on the involvement of the NF-κB signaling pathway in both vertebrate and invertebrate immunity. Cytokines and growth factors (interleukin-1 and -2, and tumor necrosis factor-α and -β), bacterial products such as lipopolysaccharide and exotoxin, and viruses can all activate the cellular NF-κB signaling cascade [3], which can regulate many aspects of immune and inflammatory

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responses. Knockout and transgenic approaches in mice have shown the essential role of Rel/NF-κB family proteins in the function of the mammal immune system [11]. Striking similarities in Rel/NF-κB family members between vertebrate and invertebrate have also been shown. Dorsal, a Rel family transcription factor, plays a key function in the determination of dorsoventral polarity in *Drosophila melanogaster* [12]. The dorsoventral pathway is also involved in the immune response in *Drosophila*.

In mammals, the NF-κB signaling pathway also plays a key role in the generation and development of osteoclasts and bone formation. $p50^{-/-}$, $p52^{-/-}$ (p50 and p52 are two members of Rel/NF-κB family proteins) double knockout mice showed severe osteopetrosis due to failure of osteoclast formation and function [13,14]. Estrogen deficiency leads to NF-κB activation, an increase in osteoclast number and postmenopausal osteoporosis [15]. *In vitro* and *in vivo* studies in mice showed that NF-κB activates *BMP-2* gene expression, which regulates chondrogenesis and postnatal bone growth [16].

Compared to studies about the roles of the NF-κB signaling pathway in different cellular processes in mammals, little is known about that in mollusk. In recent years, the Rel/NF-κB homologs were identified from the Pacific oyster [17] and the abalone [18]. The functions of the NF-κB signaling pathway in mollusk development and immunity need to be further studied, particularly because of the great economic significance of these animals. Previous study into the cloning of cDNA encoding a mammalian IKK homolog (*Pf*-IKK) from the pearl oyster (*Pinctada fucata*) showed the presence of the NF-κB signaling pathway in the pearl oyster (unpublished data).

In this study, a cDNA clone encoding Rel/NF-κB homolog (*Pf*-Rel) was isolated from the hemocytes of pearl oyster, *P. fucata*. The gene, *Pf-rel*, was expressed ubiquitously in *P. fucata*. This element of the NF-κB cascade, together with *Pf*-IKK, helps to further investigate the function of the NF-κB signaling pathway in mollusk.

Materials and Methods

Preparation of total RNA from hemocytes

Adult *P. fucata* was obtained from the Guofa Pearl Farm in Beihai, China. Hemolymph was extracted from the posterior adductor muscle of oysters using a sterile 1 ml syringe and immediately centrifuged at 1000 *g* at 4 ºC for 10 min. Hemocyte pellets were used for RNA extraction. Total RNA was extracted from hemocyte pellets with the RNAzol RNA isolation kit (Biotecx, Houston, USA) according to the manufacturer's instructions. RNA integrity was determined by separation on 1.2% formaldehyde-denatured agarose gel and staining with ethidium bromide. The quantity of RNA was determined by measuring A_{260} with an Ultrospec 3000 ultraviolet/visible spectrophotometer (Amersham, Piscataway, USA).

First strand synthesis and polymerase chain reaction

Five micrograms of total RNA from hemocytes was used as the template for the reverse transcription (RT) reaction with SuperScriptII reverse transcriptase and oligo(dT) primers (Invitrogen, Carlsbad, USA). The cDNA first strand was synthesized and used as the template for further PCR. A pair of degenerate oligonucleotide primers was designed based on the two conserved domains within Rel/NF-κB family proteins: FP1 (5′-TGTGARGGNAGATC-WGCNGGHAG-3′, where R=A/G, W=A/T, H=A/C/T, N=A/G/C/T) corresponding to C-E-G-R-S-A-G-S; and RP1 (5′-AAACANAGACGVACHRCRTTVAGATC-3′, where V=A/G/C) corresponding to D-L-N-V/A-V-R-L-C-F. PCR was carried out according to the manufacturer's instructions on a TGradient Thermocycler (Biometra, Gottingen, Germany). PCR cycles were conducted according to the following conditions: denaturation at 94 ºC for 5 min, followed by 30 cycles at 94 ºC for 40 s, 43.5 ºC for 30 s, 72 ºC for 90 s. A final extension step was conducted at 72 ºC for 7 min. The PCR product with expected size was cloned into pGM-T Easy Vector (Tiangen, Beijing, China) and sequenced. Another PCR was then carried out. A pair of oligonucleotide primers were designed based on the known specific sequence and another conserved domain: FP (5′-ATACTATCACCT-TTCCACATTT-3′); and RP (5′-GGNGTNCKRAAN-ACAATWGC-3′, where K=G/T) corresponding to A-I-V-F-R-T-P. PCR cycles were conducted according to the following conditions: denaturation at 94 ºC for 5 min, followed by 30 cycles at 94 ºC for 40 s, 43 ºC for 30 s, 72 ºC for 90 s. A final extension step was conducted at 72 ºC for 7 min. The PCR product with expected size was cloned into pGM-T easy vector and sequenced.

Rapid amplification of cDNA ends (RACE)

5′-RACE and 3′-RACE were conducted according to the manufacturer's instructions, using the BD SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, USA). Single-stranded cDNA templates for both RACE reactions were prepared using the total RNA from hemocytes. The gene-specific primers for 5′-RACE were designed based on the nucleotide sequence of the cDNA fragments

amplified by previous PCR reactions: the gene-specific primer 5′-GSP1 (5′-TTATGGGGGTGGGGGTCGTA-TGGTGTGT-3′); and the nested primer 5′-GSP2 (5′-TCTTCTCATTTGTGCTGCTCTCC-3′). The first round of the 5′-RACE reaction was conducted according to the following conditions: denaturation at 94 ºC for 5 min, followed by 25 cycles at 94 ºC for 40 s, 60 ºC for 30 s, 72 ºC for 1 min. A final extension step was conducted at 72 ºC for 7 min. A secondary "nested" PCR was then carried out using the diluted primary PCR product as the template in place of the cDNA according to the following conditions: denaturation at 94 ºC for 5 min, followed by 30 cycles at 94 ºC for 40 s, 47 ºC for 30 s, 72 ºC for 1 min. A final extension step was conducted at 72 ºC for 7 min. The gene-specific primer for 3′-RACE was 3′-GSP (5′-GCTAAAGGTGGAGATGAGGTGTT-3′). The 3′-RACE reaction was conducted under the following conditions: denaturation at 94 ºC for 5 min, followed by 30 cycles at 94 ºC for 40 s, 47 ºC for 30 s, 72 ºC for 2 min. A final extension step was conducted at 72 ºC for 10 min. Both the 5′- RACE and 3′-RACE products were cloned into pGM-T Easy Vector and sequenced.

DNA sequence analysis

To confirm the accuracy of cloning and sequencing, the entire cDNA was re-amplified with high fidelity polymerase (*Ex Taq*; TaKaRa, Dalian, China), using a pair of gene-specific primers: sense (5′-AACAACACTGGAAAG-CCCAGACT-3′); and antisense (5′-ATGCCATTGAG-ATGAGGGGGAAT-3′) . The purified PCR product was cloned into pGM-T Easy Vector and re-sequenced. The entire nucleotide sequence was analyzed using the BLAST program available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Primer Premier 5.0 (PRIMER Biosoft International, Palo Alto, USA) was used to identify its encoding protein. Multiple alignments were created using the DNAMAN program (Lynnon, Vaudreuil-Dorion, Canada).

Gene expression analysis by RT-PCR assay

Tissue expression of the pearl oyster Rel/NF-κB mRNA was investigated by RT-PCR. Total RNA was isolated using the method described above from mantle, gill, adductor muscle and gonad of the adult individual of *P. fucata*. Equal quantities (2 μg) of total RNA from different tissues were reverse-transcribed into the cDNA first strand with Quant Reverse Transcriptase (Tiangen) following the manufacturer's instructions. The synthesized cDNA was used as the template for the following PCR. PCR conditions were as follows: 94 ºC for 5 min, 30 cycles at 94 ºC for 30 s, 48 ºC for 30 s and 72 ºC for 45 s, followed by 1 min incubation at 72 ºC. Primer pairs designed for the amplification of *Pf-rel* were RT-FP (5′-AGCAGCACAAA-TGAGAAGAA-3′) and RT-RP (5′-TCCCAAATAGT-ATCACCAGAGC-3′). A pair of primers to amplify the gene encoding glyceraldehyde 3-phosphate dehydrogenase (*Pf*-GAPDH) was GAPDHF (5′-GATGGTGCCGAGTATG-TGGTA-3′) and GAPDHR (5′-CGTTGATTATCTT-GGCGAGTG-3′). Negative controls were carried out in the absence of cDNA template to examine the cross contamination of the samples.

In situ hybridization

The mantle was removed from the adult *P. fucata* and immediately fixed in 4% paraformaldehyde containing 0.1% DEPC (Sigma-Aldrich, St. Louis, USA) overnight. *In situ* hybridization of *Pf-rel* mRNA was carried out on frozen sections of the mantle. Digoxigenin-labeled RNA probes were synthesized from the linearized plasmid with the insertion fragment encoding *Pf*-Rel using DIG RNA Labeling Mix (Roche, Mannheim, Germany) and T7 and SP6 RNA polymerase (Promega, Madison, USA). *In situ* hybridization was conducted at 50 ºC using the Enhanced Sensitive ISH Detection Kit II (AP) (Boster, Wuhan, China) according to the manufacturer's instructions.

Results

Amplification of the full-length cDNA of *Pf-rel*

The amplification of a 374 bp product was conducted by PCR, using cDNA reverse-transcribed from the total hemocytes RNA as the template, and a pair of degenerate primers (FP1 and RP1). Further PCR was conducted using a gene-specific primer based on the known sequence (FP) and a degenerate primer (RP). A 460 bp PCR product was amplified. The two PCR reactions resulted in the identification of 671 bp cDNA sequence. Based on the sequence, three gene-specific primers were designed for 5′-RACE (5′-GSP1 and 5′-GSP2) and 3′-RACE (3′-GSP). 5′-RACE and 3′-RACE reactions were conducted and the 5′-end and 3′-end nucleotide sequences were obtained. All the PCR and RACE reactions resulted in the identification of a 2737 bp sequence. To confirm the sequence obtained, a pair of gene-specific primers (sense and antisense) was designed and RT-PCR was carried out.

Sequence analyses of *Pf-rel* and *Pf*-Rel

As shown in **Fig. 1**, the complete cDNA sequence of

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                                                                GAGGGGCTTTTCGCCGTGTAGATCA      25
ACAACACTGGAAAGCCCAGACTTACTAAAATAAACCAAAACTTTAGGTAAACCATATATCTGTTTATACCAAGTT     100
ATGGCATCAACTAAGCCATCAGGGAACTTAACCGGTCTTGATGAAGAAATGGCAAATTTAAGTAGTGCAGACTTG     175
 M  A  S  T  K  P  S  G  N  L  T  G  L  D  E  E  M  A  N  L  S  S  A  D  L      25
CAGCGTTTCATCCTAGGAGATACTGATGACACTATTATCCAGCAATTAGATTTCACTGGACCCACGGATAATGAG     250
 Q  R  F  I  L  G  D  T  D  D  T  I  I  Q  Q  L  D  F  T  G  P  T  D  N  E      50
TACTCCTATGTACAGAATTTAATAGCCCACTCCCAGCCTGCGCCTCAGCCTGTTCAGACATACAACAATCCTGGA     325
 Y  S  Y  V  Q  N  L  I  A  H  S  Q  P  A  P  Q  P  V  Q  T  Y  N  N  P  G      75
CCATCCCGTCAGAATAGATCGACTACAAAGCCAGGGGGAGGTAAAAGTATGCCTCAGACCACTCCAGACGGAATA     400
 P  S  R  Q  N  R  S  T  T  K  P  G  G  G  K  S  M  P  Q  T  T  P  D  G  I     100
TGGACTGAGATAATGGAACAGCCGAAACAGAGGGGCTTACGGTTCCGTTATGAGTGTGAGGGACGATCAGCTGGT     475
 W  T  E  I  M  E  Q  P  K  Q  R  G  L  R  F  R  Y  E  C  E  G  R  S  A  G     125
AGCATCCCCGGGGAGAGCAGCACAAATGAGAAGAAGACCTTCCCAACAATAAAGATTCACAACTACACTGGTACT     550
 S  I  P  G  E  S  S  T  N  E  K  K  T  F  P  T  I  K  I  H  N  Y  T  G  T     150
GCTGTAATCGTTGTGTCATGTGTCACCAAGGACACACCATACGACCCCCACCCCCATAATTTGGTGGGCAGGGAC     625
 A  V  I  V  V  S  C  V  T  K  D  T  P  Y  D  P  H  P  H  N  L  V  G  R  D     175
TGTAAGAGGGGAGTGTGCACACTCAAGGTCAAGGACACTAATACTATCACCTTTCCACATTTGGGCATTCAATGT     700
 C  K  R  G  V  C  T  L  K  V  K  D  T  N  T  I  T  F  P  H  L  G  I  Q  C     200
GCTAAAAAGAAGGATGTGACAGATAATCTAAGGCAGCGCAAAGAGATTAATGTTGATCCCTATCAAAGTGGATTT     775
 A  K  K  K  D  V  T  D  N  L  R  Q  R  K  E  I  N  V  D  P  Y  Q  S  G  F     225
AAGCATATGAATAAAGCTAACAACATAGATCTGAACGTAGTACGACTCTGTTTCCAAGTCTTCCTGCCGGATGAC     850
 K  H  M  N  K  A  N  N  I  D  L  N  V  V  R  L  C  F  Q  V  F  L  P  D  D     250
CAGGGTAAAATCACTCGCATCGTACCACCTGTAGTCTCCCATCCTATACATGACAAAAAATCTCTGAATGAGTTA     925
 Q  G  K  I  T  R  I  V  P  P  V  V  S  H  P  I  H  D  K  K  S  L  N  E  L     275
GTTATCTGTAGAGTGGACAGGTCCTCTGGTAAAGCTAAAGGTGGAGATGAGGTGTTCCTATTGTGTGAGAAAATA    1000
 V  I  C  R  V  D  R  S  S  G  K  A  K  G  G  D  E  V  F  L  L  C  E  K  I     300
AACAGAGATGACATAGGAGTGAGGTTTTACCAGGAGAACAGCTCTGGTGATACTATCTGGGAAGATTACGGTGAA    1075
 N  R  D  D  I  G  V  R  F  Y  Q  E  N  S  S  G  D  T  I  W  E  D  Y  G  E     325
TTCAGTGTTAATGACATCCATCGTCAGTACGCTATCGTGTTCAAAACACCCCCGTATAAAGACACCCTGATAAGT    1150
 F  S  V  N  D  I  H  R  Q  Y  A  I  V  F  K  T  P  P  Y  K  D  T  L  I  S     350
CAACCAGTAGAAGTCAGAATGCAGCTGAAACGACAAAATGACGCAGAGACAAGTGAAGCGATTCCTTTCATTTAT    1225
 Q  P  V  E  V  R  M  Q  L  K  R  Q  N  D  A  E  T  S  E  A  I  P  F  I  Y     375
ATGCCAGAAGATCCTGATCCAGACCGAATCATGGAGAAGAGAAAACGTAAGGCAGATCAACTGAAAAGCTGGGGA    1300
 M  P  E  D  P  D  P  D  R  I  M  E  K  R  K  R  K  A  D  Q  L  K  S  W  G     400
TTCAACTTGGATTCTTCCAGCATGTCTGGAGATGACATCAAACAGAGACTGAAGCTCAAAGCCACTAAATCAAGG    1375
 F  N  L  D  S  S  S  M  S  G  D  D  I  K  Q  R  L  K  L  K  A  T  K  S  R     425
ATTAAAGCAGAAGCCCATACACCTGACATACCGCTATGGGACAACATAGATAATGTACCAGGTCCTAGCCAATCA    1450
 I  K  A  E  A  H  T  P  D  I  P  L  W  D  N  I  D  N  V  P  G  P  S  Q  S     450
GCTGTCATTGAATCATCCTCATCCTCTGTGAACGTAAGGTCCAGTGACAACACCGCCATGGGTAACGTTACAATC    1525
 A  V  I  E  S  S  S  S  S  V  N  V  R  S  S  D  N  T  A  M  G  N  V  T  I     475
ACTGCCTCGGAAAATGTGCAAAGGCAACTAGCTCTATTACCAGAACAAATTCAAAATCAAATTCTTCAACAGTTG    1600
 T  A  S  E  N  V  Q  R  Q  L  A  L  L  P  E  Q  I  Q  N  Q  I  L  Q  Q  L     500
GCCATGCAGAAACTACGTGACCAAGCGAGACTCCGTCAAATTGAGGATAGCCTCTCTCAGCCTCCTCCTACCTCC    1675
 A  M  Q  K  L  R  D  Q  A  R  L  R  Q  I  E  D  S  L  S  Q  P  P  P  T  S     525
TCTAATTTCTTAGGTGATTTCAATTCCCAGAATTCACAATTCACAATGGCTCAGCAGCAGACTGCATCAGGAGGG    1750
 S  N  F  L  G  D  F  N  S  Q  N  S  Q  F  T  M  A  Q  Q  Q  T  A  S  G  G     550
CCCCTGATAGGTGATGTGTCGATGCAGCAGGCAGCTGCTAACAATCAAAATGACATCTCGCACGCTGTTGACTTG    1825
 P  L  I  G  D  V  S  M  Q  Q  A  A  A  N  N  Q  N  D  I  S  H  A  V  D  L     575
AATATGGTACAAACTTACTTAGGTGACCAAGGACAAAATGTGTCTTTCGATAGTTTCGGTTCCCTGACGGGGAAC    1900
 N  M  V  Q  T  Y  L  G  D  Q  G  Q  N  V  S  F  D  S  F  G  S  L  T  G  N     600
ATTATGAACATAGATTGTGATAGCAATGTGGCAGCTAATGTTAACATCAGCAGTACGGACACTTCACAGTCCCAC    1975
 I  M  N  I  D  C  D  S  N  V  A  A  N  V  N  I  S  S  T  D  T  S  Q  S  H     625
CAGTACACGATGCAGCAGAATGAGGTCGATGCAGCAATCCAATCTCTACAGTCTCAATCCTGAGGTTGTTATAGC    2050
 Q  Y  T  M  Q  Q  N  E  V  D  A  A  I  Q  S  L  Q  S  Q  S  *
ATTTATTGTTATGTATATAGATGTAAATATTCCCCCTCATCTCAATGGCATGTAATGCATTACTTCATCACAAAC    2125
TGCCAATTGGATTACAATTTGTACACACAAACTGCTTTGTACATAAGGATCATGTGTACAGTATATATTCATTTT    2200
ATTGATGGTAGTGCTTTACAAATATGAAACTGTGATTTCCTAACTTAGTAGCTACATGTTAGTTGAATGCTGATT    2275
GGTTGCTGGATATTCTGCAAGCATTGAACTATTATTTTTATTTTACTAGGCAATTTCATATTTTTTACATGGCAC    2350
CTATATCTGTCCCAGAACTTCTCAACAAAAACTGCTAATAAATCCATGTTCTAGCAATATTTCTTGTGTTGTTGT    2425
TATTTTATATTAAGAGTATTGTGTAACTTTTTCTAAAATTGTTGTAATTCTATGAAACTGAAAGTGAAACTAAAC    2500
TTCATTCTTAATCATAGTGCTATTTATATCTATTCTAAGTGATTCTAAGAATAGGTTGACTGAAGGTTTTATTCA    2575
TGCATACATTATGTAACATGTGGATCATGATATTTTAAAGATTGTTCAATTTGTTGTGTACATTGTGTGTTTTGA    2650
AGTAGTAGAGCATTGATATACACAACTTGTTACCATGAATCTATTAATCAATTTAAGAATAAAATTTGAAAAAGT    2725
GAAAAAAAAAA
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Fig. 1 cDNA sequence and deduced amino acid sequence of nuclear factor (NF)-κB from the pearl oyster, *Pinctada fucata*

The start codon and stop codon are boxed, the latter indicated by an asterisk at the end of the amino acid sequence. The putative polyadenylation signal (AATAAA) is underlined. Two conserved domains (Rel homology domain and IPT domain of the transcription factor NF-κB) and the nuclear localization signal are shaded, consecutively.

Pf-rel including the poly(A) tail is 2737 bp. It contains a 100 bp 5′-untranslated sequence, an open reading frame consisting of 1938 bp, a TGA stop, a 688 bp 3′-untranslated sequence and a poly(A) tail of 11 nucleotides. A putative polyadenylation signal (AATAAA) is recognized at the position 2708, which is located in the upstream of the poly(A) tail separated by 13 nucleotides. The cDNA sequence has been submitted to GenBank with the accession number EF121959.

The open reading frame of the cDNA clone encodes a protein consisting of 645 amino acid residues. Amino acid sequence analysis (BLAST) shows that *Pf*-Rel possesses the characteristic primary structure of Rel/NF-κB family proteins. Two putative conserved domains have been detected (**Fig. 1** and **Fig. 2**): RHD of 167 amino acid residues (103–269); and IPT_NFκB (IPT domain of the transcription factor NF-κB) of 103 amino acid residues (275–377). The *Pf*-Rel also contains a nuclear localization signal (NLS) of five amino acid residues (388–392). The C-terminal region of *Pf*-Rel, which shares low similarity with other Rel/NF-κB proteins, is likely to be a trans-

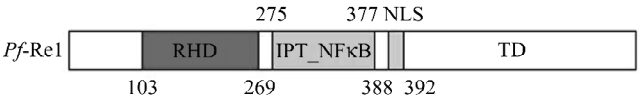

Fig. 2 Amino acid sequence analysis of *Pinctada fucata* Rel (*Pf*-Rel), a cDNA clone encoding transcription factor Rel/nuclear factor-κB homolog

Characteristic domains, including the Rel homology domain (RHD), IPT domain of the transcription factor NF-κB (IPT_NFκB), nuclear localization signal (NLS) and C-terminal transactivation domain (TD), are indicated.

activation domain (TD). It does not contain polyalanine, polyglutamine or polyasparagine, the characteristic structures in the C-terminal of *D. melanogaster* Dorsal. Nor does it contain the glycine-rich region and ankyrin repeat, which p100 and p105 possess.

The highest sequence identity of *Pf*-Rel with other Rel/NF-κB family proteins is 59%; however, the highest sequence identity of the conserved regions (including RHD, IPT domain and NLS) is up to 85% (**Fig. 3**). Other than the homologs from mollusk, *Pf*-Rel shares the highest

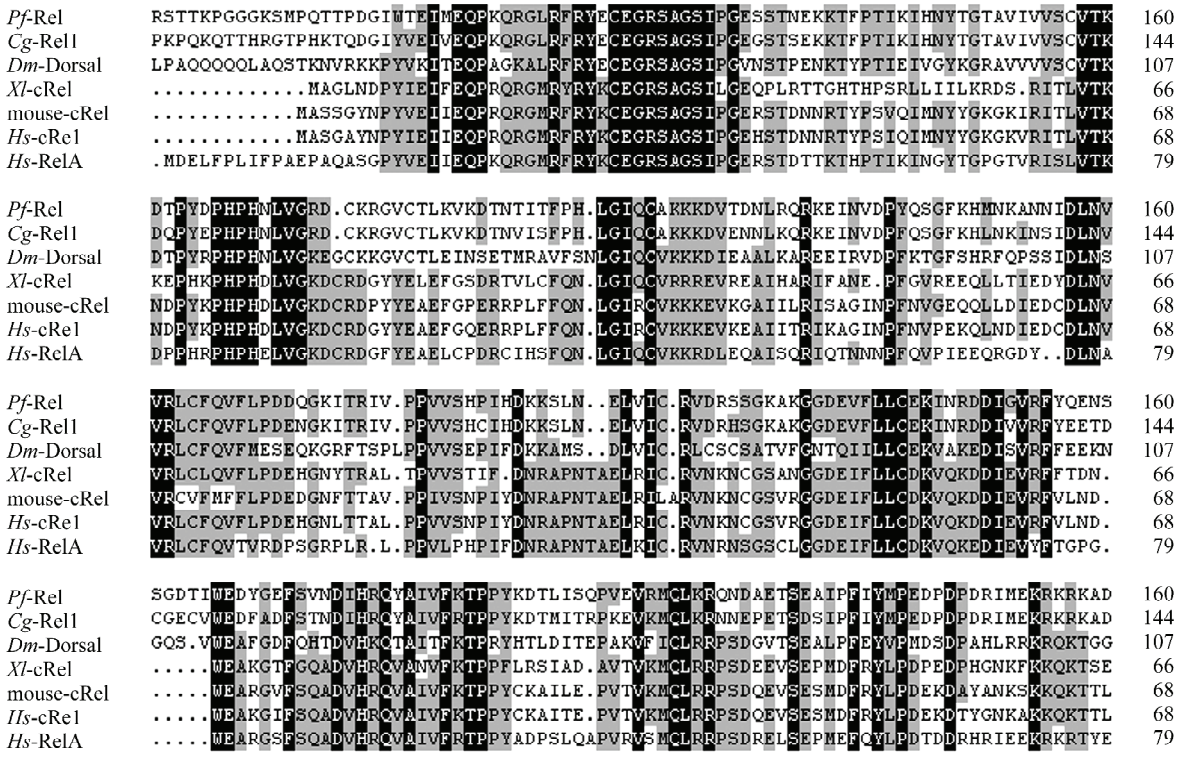

Fig. 3 Comparison of amino acid sequences at the conserved domains of *Pinctada fucata* Rel (*Pf*-Rel) and other Rel/NF-κB proteins

Cg-Rel1, *Crassostrea gigas* Rel1 (GenBank accession No. AAK72690); *Dm*-Dorsal, *Drosophila melanogaster* Dorsal (GenBank accession No. AAA28479); *Hs*-cRel, *Homo sapiens* cRel (GenBank accession No. CAA52954); *Hs*-RelA, *Homo sapiens* RelA (GenBank accession No. AAA36408); mouse-cRel, *Mus musculus* cRel (GenBank accession No. CAA33843); *Xl*-cRel, *Xenopus laevis* cRel (GenBank accession No. AAC18087);

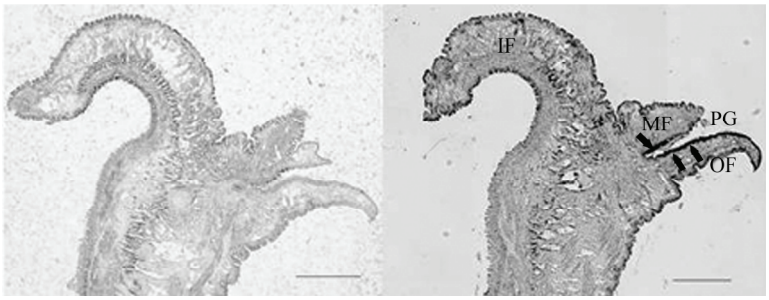

Fig. 5 Detection of *Pf-rel* mRNA distribution in mantle of *Pinctada fucata* by *in situ* hybridization

A frozen section stained with a sense probe was used as the control (left frame). Strong hybridization signals are indicated by arrows (right frame). IF, inner fold; MF, middle fold; OF, outer fold; PG, periostracal groove. Bar=0.5 mm.

identity with the Dorsal protein from *Tubifex tubifex* (sludge worm) (GenBank accession No. BAD60879). It also shares high identity with *D. melanogaster* Dorsal.

Gene expression analyses of *Pf-rel*

To examine whether *Pf-rel* mRNA was ubiquitously expressed, a pair of gene-specific primers was designed and RT-PCR was carried out (**Fig. 4**). The mRNA for *Pf-rel* was expressed in all the four tissues analyzed, which suggested that the *Pf-rel* gene was ubiquitously expressed in adult *P. fucata.*

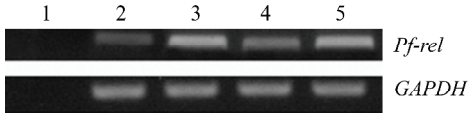

Fig. 4 Reverse transcription–polymerase chain reaction for analysis of *Pf-rel* gene expression in four tissues from *Pinctada fucata*

1, negative control; 2, mantle; 3, gill; 4, adductor muscle; 5, gonad. The house-keeping gene encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was included as a positive control.

In situ hybridization was carried out to locate the precise site of *Pf-rel* mRNA in mantle (**Fig. 5**). Strong signals were detected in the outer epithelial cells of the middle fold near the base of the periostracal groove and the inner epithelial cells of the outer fold.

Discussion

In this study, we report the identification and characterization of a Rel/NF-κB homolog from pearl oyster, *P. fucata*. A cDNA clone was isolated based on RT-PCR and amino acid sequence analysis was conducted. The putative *Pf*-Rel sequence consists of the RHD, IPT_NFκB domain, NLS and TD. The RHD and IPT_NFκB domain, which are two characteristic domains in Rel/NF-κB family proteins, function in DNA binding, dimerization and ankyrin protein binding. The putative *Pf*-Rel sequence shows high similarity in the two conserved domains with other Rel/NF-κB proteins. Nevertheless, the TD, which is essential in the transactivating ability of Rel/NF-κB, shows no similarity with known proteins. The TD of *Pf*-Rel is also rich in serine, one characteristic of this domain [3].

The gene expression analyses help to investigate the pattern and location of *Pf-rel* expression. As expected, mRNA of *Pf-rel* was expressed in all the tissues studied (also including hemocytes), which is the case for most of the Rel/NF-κB family proteins. This expression pattern suggests the involvement of *Pf*-Rel in diverse cellular processes. To investigate the precise expression site of the *Pf-rel* gene, *in situ* hybridization was carried out. Strong hybridization signals were detected in the outer epithelial cells of the middle fold near the base of the periostracal groove and the inner epithelial cells of the outer fold. In previous studies, the two sorts of cells were considered to be involved in the formation of the prismatic layer [19–22] and periostracum [23]. *Pf*-Rel might function in these processes.

Rel/NF-κB proteins have been identified in many organisms: human, mouse, chicken, frog, fruit fly, sludge worm and mollusk, including abalone and oyster. In these organisms, Rel/NF-κB proteins function differently and regulate diverse processes. For example, in *D. melanogaster*, the Rel-like transcription factor Dorsal is involved in the determination of dorsoventral polarity.

Recently, there has been increased interest in solving questions about the molecular mechanisms of the

biomineralization processes of mollusk, including shell formation and nacre production. The extracellular organic matrix-mediated mollusk shell formation hypothesis has been dominant for many years. Mount *et al*. [24] proposed a new statement which suggested the involvement of hemocytes in shell mineralization. This shift of investigation focus from extracellular matrix to cells provides a new perspective for further studies. The evidence from clinical experiments shows that the mechanisms that direct the formation of bone and nacre might be homologous. Nacre powder mixed with blood then injected into human tissues not only fails to evoke inflammatory responses, but also leads to the activation of osteoclasts and formation of new bone [25]. It is suggested that, although there are great differences between bone and nacre, their formation mechanisms might be similar and the signal molecules involved in the formation process might be conserved. It is shown that the NF-κB signaling pathway plays an important role in vertebrate bone formation and development [13–16]. Thus the investigation of the NF-κB signaling pathway in mollusk might be an alternative method to study the processes of biomineralization.

In conclusion, a putative NF-κB homolog *Pf*-Rel was identified from pearl oyster, *P. fucata*, and it was expressed ubiquitously. Further structural and functional investigations could help to understand the involvement of the NF-κB signaling pathway in mollusk immunity and biomineralization.

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