Oyster IKK-like protein shares structural and functional properties with its mammalian homologues

Jean-Michel Escoubas[a,*], Laurence Briant[b], Caroline Montagnani[a], Stephanie Hez[a], Christian Devaux[b], Philippe Roch[a]

[a] *Laboratoire de Défense et Résistance chez les Invertébrés Marins (DRIM), Université de Montpellier II, place Eugène Bataillon, CC80, 34095 Montpellier, France*

[b] *Laboratoire Infections Rétrovirales et Signalisation Cellulaire, Institut de Biologie, 4 Blvd Henri IV, 34060 Montpellier, France*

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Abstract In our search for genes involved in oyster immunity we isolated a cDNA encoding a polypeptide closely related to the mammalian IκB kinase (IKK) family. IKK proteins play a central role in cell signaling by regulating nuclear factor-κB (NF-κB) activation. We report here the cloning of an oyster IKK-like protein (oIKK) which possesses the characteristic organization of the mammalian IKK proteins, namely an amino-terminal kinase domain followed by a leucine zipper region and a carboxyl-terminal helix-loop-helix motif. When transfected into human cell lines, oIKK activated the expression of NF-κB-controlled reporter gene, whereas transfections with mutants of oIKK deleted within the kinase domain or within the helix-loop-helix motif respectively abolished and greatly reduced reporter gene activation. These results indicate that oIKK can replace the hIKK-α in catalyzing NF-κB nuclear translocation, and in triggering gene expression. Our results sustain the concept of an evolutionarily conserved signaling machinery in which IKK plays a major role.

Key words: Nuclear factor κB cascade; Bivalve mollusk; IκB kinase; *Crassostrea gigas*

1. Introduction

The mammalian nuclear factor-κB (NF-κB) signaling pathway coordinates the induction of numerous genes notably involved in inflammatory response and immune function [1]. The NF-κB family of transcription factors is composed of a set of structurally related and evolutionarily conserved DNA binding proteins [2]. NF-κB proteins are sequestered in the cytoplasm by tightly bound inhibitory proteins called IκBs [3–5]. Many of the signals known to activate NF-κB result in phosphorylation, ubiquitination, and degradation of the IκBs, allowing NF-κB to translocate into the nucleus and activate target genes. Recently two serine kinases termed IκB kinase (IKK) α and IKK-β, which are part of a large multiprotein complex known as the IKK signalsome, have been cloned and demonstrated to phosphorylate IκB in response to cytokines and signals known to activate the NF-κB cascade [6–11].

In the past 10 years it has become evident that the NF-κB signaling pathway was not the privilege of mammals. The most convincing demonstration comes from works in insects that revealed remarkable parallels and conservation with the mammalian cytokine-induced NF-κB signaling pathway [12]. Indeed, the antifungal response in *Drosophila* is controlled by the dorsoventral regulatory gene cassette (Spätzel-Toll-Cactus) which shows striking structural and functional similarities with the mammalian NF-κB signaling pathway [13]. There is currently increased interest in resolving questions about the molecular mechanisms of resistance in invertebrates, including mollusks which represent the second largest group of the invertebrate phylum after insects.

The economic consequences of infectious disease in aquaculture has led research groups to develop efforts in the field of marine mollusk pathology and immunology [14]. The internal defense mechanisms of bivalve mollusks can be separated into cell-mediated and humoral types; it has become increasingly apparent that both are interrelated and closely associated with hemocytes. Cellular immunity in bivalves can be conveniently described as phagocytosis and is subdivided into several successive processes: chemotaxis, which is still poorly known, recognition involving opsonins such as lectins [15], endocytosis [16], intracellular degradation by lysosomal enzymes [17], and production of toxic reactive oxygen intermediates (ROIs) involved in pathogen killing [18]. Bivalve mollusks also possess humoral factors including agglutinins [19], cytotoxic proteins [20] and antimicrobial peptides [21,22]. With the exception of antimicrobial peptides, most of the work concerning humoral defenses in bivalve mollusks is based on indirect evidence and molecular features of humoral effectors remain unknown.

To characterize genes involved in the immune response of the Pacific oyster *Crassostrea gigas* we used the mRNA differential display method (DD-RT-PCR) [23]. We report here the characterization of an oyster cDNA encoding a serine kinase structurally and functionally homologous to the mammalian IKKs. These results strongly suggest the existence of an NF-κB signaling pathway in mollusks and sustain the concept of an evolutionarily conserved signaling machinery in immune response [24–26].

2. Materials and methods

2.1. Animal challenge and RNA extraction

Three to four year old oysters, *C. gigas*, were collected from Palavas-France (Gulf of Lion) and kept in sea water at 15°C. Oysters were challenged by injection in the pericardial cavity of 5×10^7 heat-killed bacteria (*Escherichia coli* D31, *Micrococcus luteus* and *Vibrio alginolyticus*). Hemolymph was withdrawn from the pericardial cavity 1 or 2 days after injection and hemocytes were collected by centrifugation. Total RNAs were extracted from hemocytes, gill, mantle or labial palps using TRIzol (Gibco-BRL) kit.

*Corresponding author. Fax: (33) 467144622.
E-mail: jmescoub@ifremer.fr

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2.2. mRNA differential display

DD-RT-PCR experiments were carried out with the RNAmap kit (GenHunter Corporation). Briefly, total RNAs from hemocytes of challenged or unchallenged oysters were first treated with DNase RQ1 (Promega) for 30 min at 37°C and the reaction was stopped by heating at 65°C for 10 min. Reverse transcriptions were performed with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Gibco-BRL) in the presence of 1.2 μg of DNase-treated total RNA and one of the four anchored primers from the RNAmap kit. PCR reactions were made with the AmpliTaq DNA polymerase (Perkin-Elmer) in the presence of 2 μl of reverse transcription reactions (equivalent of 2 ng of total RNA), the anchored primer used for the reverse transcription, ^{33}P-dATP and one of the arbitrary primers provided in the kit. The PCR steps consisted of 30 s at 94°C, 2 min at 40°C, 30 s at 72°C for 40 cycles followed by 5 min at 72°C. PCR products were analyzed on 6% sequencing gels. Differentially expressed products were extracted from the gel, amplified, cloned into pCR-Script (Stratagene), and sequenced.

2.3. cDNA cloning

A cDNA library was constructed in the λ ZAP Express vector (Stratagene) using poly(A)$^+$ RNA extracted from *C. gigas* hemocytes. One million plaque-forming units were screened with the radiolabelled D9 cDNA fragment (405 bp) isolated by DD-RT-PCR. Hybridizations of nylon filters (Amersham Hybond-N) were performed overnight at 65°C in 5×Denhardt's, 0.1% SDS, 5×SSPE, and 100 μg/ml denatured salmon sperm DNA. Filters were first rinsed in 2×SSC, 0.1% SDS at room temperature, then washed at 65°C for 15 min twice in each of the following solutions: 1×SSC, 0.1% SDS; 0.2×SSC, 0.1% SDS, and 0.1×SSC, 0.1% SDS. A secondary screening was performed to purify the positive plaques. Phagemids were obtained by in vivo excision according to the manufacturer's instructions and sequenced on both strands.

2.4. Northern blot analysis

Poly(A)-rich RNAs were extracted with Dynabeads Oligo(dT)$_{25}$ (DYNAL) at 0, 24, and 48 h after oyster challenge. Each of the poly(A)-rich RNAs (10 μg) was fractionated by denaturing 1.2% agarose/formaldehyde gel electrophoresis and blotted onto Hybond-N membrane. The membranes were prehybridized for 2 h at 65°C in 50% formamide, 5×SSC, 8×Denhardt's, 50 mM NaH_2PO_4 pH 6.5, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. Hybridizations were performed overnight at 42°C in the same buffer containing the radiolabeled probe. Filters were washed twice for 15 min at room temperature in a solution of 2×SSC containing 0.1% SDS and then twice 20 min at 60°C in a solution of 1×SSC containing 0.1% SDS.

2.5. RT-PCR analysis

To eliminate DNA contaminants, RNAs (10 μg total RNA) were treated with DNase RQ1 (Promega) in the presence of RNasin (Promega) for 30 min at 37°C. Reverse transcriptions were performed with M-MLV reverse transcriptase (Gibco-BRL) in the presence of oIKK specific primer (primer II, 5′-TTTGTGGAGGTACTCTACAGC-3′; Fig. 3). One tenth of this reaction was amplified by PCR with Taq DNA polymerase (Promega) using another oIKK-specific primer (primer I, 5′-CAGCCAGAGCAGAGAGGTG-3′). The amplification program started with a denaturation step of 5 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 53°C, 1 min at 72°C and 1 cycle of 5 min at 72°C. PCR products were analyzed on 2.5% agarose gels.

2.6. Plasmid constructs

Myc epitope-tagged oIKK expression vector was constructed by inserting oIKK cDNA into the pCS3 plasmid in frame with the N-terminal Myc epitope recognized by the 9E10 monoclonal antibody [27]. Truncation mutants of oIKK were derived from the pCS3-oIKK plasmid using internal restriction sites or a PCR-based method. All mutations were confirmed by DNA sequencing. Mammalian expression vector (N-flag-CHUK) encoding human IKK-α was kindly provided by Tularik Inc. [6].

2.7. Transient transfections and reporter gene assays

Transient transfections of lymphoblastoid CEM cells (5×10^6 cells) were carried out by electroporation [28]. 293 cells (10E7) were transfected using the calcium phosphate precipitation procedure [29]. Each transfection was done with 10 μg of reporter vector (pHIV1-LTR-Luc or pNF-κB-Luc from Clontech), 5 μg β-galactosidase expression vector and various amounts of each oIKK or hIKK-α expression constructs. Total DNA concentration was kept constant by adding pCS3 to the mixture. Cells were harvested after 72 h, washed twice in PBS buffer divided into two aliquots and lysed in appropriate buffer for luciferase and β-galactosidase assays. The luciferase activities were normalized for transfection efficiency on the basis of β-galactosidase expression.

2.8. Electrophoretic mobility shift assays

Nuclear and cytoplasmic extracts were performed as previously described [30]. NF-κB mobility shift assays were performed using 2 μg protein of nuclear or cytoplasmic extracts, 10^5 cpm of double-stranded oligonucleotide probe containing the HIVLTR5′-1 κB-site (underlined), 5′-GCTGGGGACTTTCCAGGGAGGCGT-3′, in the appropriate buffer [30]. After 20 min incubation at room temperature, the mixture was run in a 5% non-denaturing acrylamide gel and protein-DNA complexes were visualized by autoradiography.

3. Results

3.1. Molecular cloning of oIKK cDNA

To characterize genes involved in oyster immunity, we used a DD-RT-PCR approach [23] performed with total RNAs extracted from hemocytes of bacterial-challenged or unchallenged oysters. A short (405 bp) cDNA fragment, named D9, was isolated, cloned and sequenced. The search for sequence homology using the FASTA program [31] indicated similarities between the D9 sequence and a portion of genes encoding the IKK family of serine-threonine kinases (59% identity over 365 bp). To confirm the possibility that the oyster genome encodes an IKK-like transcript, we used D9 as a probe to screen an oyster hemocyte cDNA library. The 3234 bp cDNA clone isolated (GenBank accession number AF051320) contained an open reading frame of 2199 bp predicted to encode a 732 amino acid protein. Amino acid sequence analysis revealed that oIKK has the same molecular organization as mouse and human IKK-α and IKK-β subunits, namely an amino-terminal kinase domain (KD), a leucine zipper amphipathic α-helix (LZ) and a helix-loop-helix (HLH) domain at the carboxy-terminus (Fig. 1A). Sequence alignment of the murine IKK-α (mIKK-α) and oIKK proteins showed 43% amino acid identity over the entire length of the protein chains. Interestingly, the highest protein sequence homology was observed within the kinase domain (56% identity and 71% homology; Fig. 1A,B) where the signature pattern of serine threonine kinase and the MAP kinase kinase activation loop (Ser-X-X-X-Ser) targeted by NF-κB-inducing kinase (NIK) [32] are conserved as compared with mammalian IKK proteins [10,33]. Comparable percentages of homology were observed between oIKK and the two subunits of IKK proteins (64% for IKK-α and 62% for IKK-β). Nevertheless, it is worth noting that oIKK and IKK-α have the same calculated molecular weight (85 kDa) and isoelectric point (6.3) suggesting that oIKK may be the oyster equivalent of IKK-α.

3.2. Constitutive and ubiquitous expression of oIKK

According to the DD-RT-PCR technique used to isolate oIKK, it was supposed that oIKK was differentially expressed during bacterial challenge of oysters. However, it is known that members of the IKK family are constitutively expressed in mammals [8,34]. One of the problems intrinsic to the DD-RT-PCR technique is the rather high rate of false positive clones. Clones isolated by this method frequently turn out

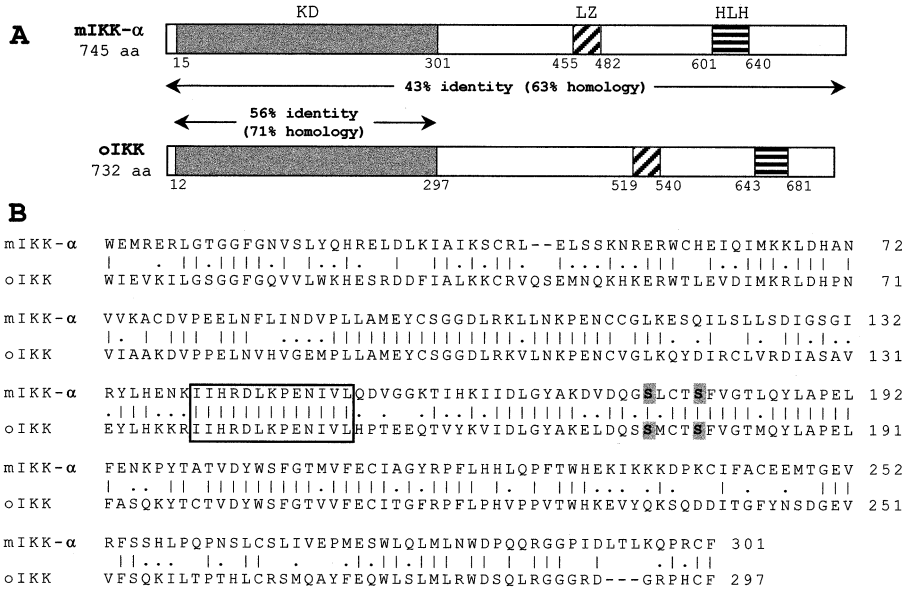

Fig. 1. Comparison of mouse IKK-α (mIKK-α) and oyster IKK (oIKK). A: Schematic representation of mIKK-α and oIKK protein organization. The kinase domain (KD), leucine zipper (LZ) and helix-loop-helix (HLH) are indicated as well as the first and the last amino acid of each domain. Degrees of identity and homology are indicated for the total amino acid sequences and for the kinase domains. B: Amino acid sequence alignment of kinase domains from mIKK-α and oIKK. Gaps were introduced to optimize the alignment. Vertical lines indicate identities and dots represent similarities between the corresponding pair of amino acid residues. Boxed areas correspond to the serine/threonine protein kinases active site signature. Bold highlighted amino acids indicate the canonical MAPKK activation loop motif (Ser-X-X-X-Ser, where X is any amino acid).

to be constitutively expressed in cells when analyzed by Northern blot [35]. To examine the expression pattern of oIKK, a 2.5 kb fragment prepared from the oIKK cDNA was used to probe Northern blot containing poly(A)$^+$ RNA from hemocytes of challenged and unchallenged oysters. In all cases, a 4.9 kb transcript was detected with the oIKK probe (Fig. 2). The blot was stripped and probed with a 1.5 kb actin restriction fragment used as internal control. After each hybridization, blots were quantified using the Molecular Dynamics Storm system for each challenge conditions (0, 24 and 48 h) and oIKK transcripts were expressed as a function of the expression level of actin transcripts. This analysis revealed that oIKK mRNA expression was unaffected by bacterial challenge, indicating that oIKK gene was constitutively expressed in hemocytes.

We investigated tissue expression of the oIKK transcript by RT-PCR. Total RNAs from gill, labial palps, mantle and hemocytes were reverse transcribed and amplified using oIKK-specific synthetic oligonucleotide primers (primers I and II Fig. 3B). These primers generated a 396 bp product when the full length oIKK cDNA was used as template (Fig. 3A, lane C3). Similarly, a 396 bp fragment was obtained with the four different tissues analyzed (Fig. 3A, lanes G, LP, M

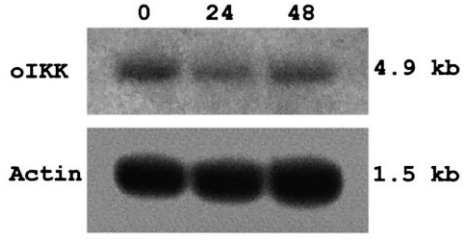

Fig. 2. Northern blot of poly(A)$^+$ RNAs from oyster hemocytes. RNAs (10 μg per line) from unchallenged oysters (lane 0) or bacterial-challenged oysters for 24 h (lane 24) and 48 h (lane 48) were separated on denaturing 1.2% agarose/formaldehyde gel, blotted onto nylon membrane and hybridized with a radiolabeled oIKK-specific cDNA probe. Internal control was performed by hybridization with a radiolabeled actin-specific cDNA probe.

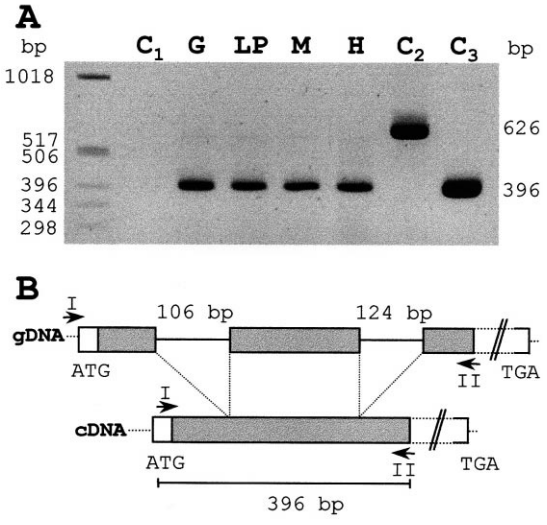

Fig. 3. Tissue expression of oIKK gene analyzed with a RT-PCR-based technique. A: RNAs extracted from oyster labial palps (LP), gill (G), mantle (M) and hemocytes (H) were reverse transcribed and amplified with oIKK-specific primers. DNA-free control is shown in lane C1. Positive control was performed by amplification of oIKK cDNA (C3). Partial amplification of genomic oIKK is shown in lane C2. B: Schematic representation of the oIKK genomic (gDNA) and complementary DNA (cDNA) in the region amplified during RT-PCR reactions. Introns and exons are respectively represented by lines and boxes (gray boxes represent part of the oIKK kinase domain). Primers I and II used for reverse transcription and PCR amplification are indicated by arrows.

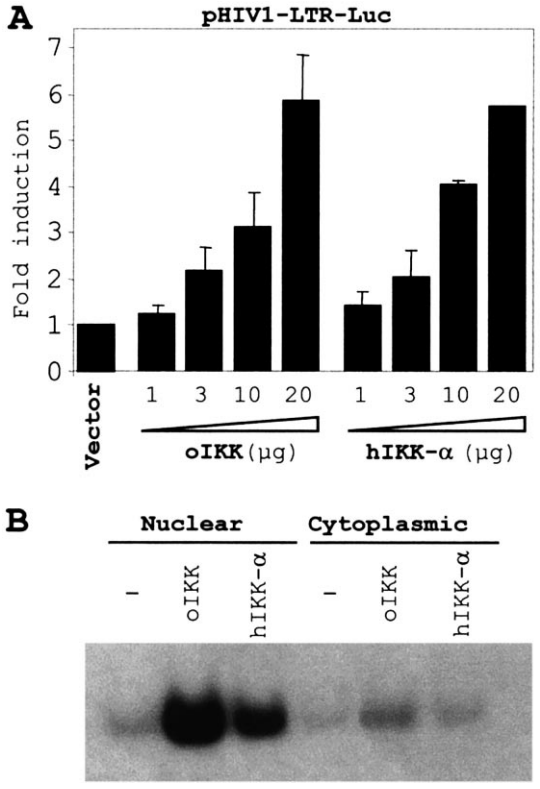

Fig. 4. Overexpression of oIKK stimulated the expression of NF-κB-controlled luciferase genes. A: CEM cells were transiently co-transfected with an LTR luciferase reporter construct and β-galactosidase expression plasmid, together with variable amounts of vector control (pCS3) and the indicated amount of oIKK or hIKK-α expression vectors. Luciferase activity was normalized according to the level of β-galactosidase expression. The values shown are average induction (mean ± S.E.M.) of at least three independent transfection experiments. B: Electrophoretic mobility shift assays were carried out with nuclear and cytoplasmic extracts prepared from 293 cells untransfected (−) or transfected with oIKK or hIKK-α expression vectors. Double-stranded oligonucleotide containing the HIV LTR 5′-1 κB site was used as probe.

and H). This amplification could not be ascribed to genomic DNA contamination since PCR amplification of *C. gigas* genomic DNA generated a 626 bp fragment (Fig. 3A, lane C2). The complete sequencing of this larger PCR fragment indicated that the corresponding genomic sequence contains two introns in this region of the oIKK gene (Fig. 3B). These results demonstrated that oIKK, like other members of the IKK family, was ubiquitously expressed.

3.3. Overexpression of oIKK in mammalian cells stimulated the expression of NF-κB-controlled reporter gene and induced NF-κB translocation

To investigate the functional properties of oIKK and its relationship to mammalian IKKs, we examined whether the transient overexpression of the oIKK protein might activate the expression of an NF-κB-controlled reporter gene. Given the lack of bivalve mollusk cell lines, transfection experiments were performed in a human T cell line (CEM). CEM cells were transfected with a luciferase (Luc) reporter gene construct driven by the human immunodeficiency virus (HIV-1) long terminal repeat (LTR) which includes two tandemly repeated κB consensus sequences. The function of oIKK was evaluated by cotransfecting a Myc-tagged oIKK expression vector together with the pHIV1-LTR-Luc reporter gene construct. Expression of the recombinant oIKK protein in the CEM cells was probed with anti-myc antibodies and was compatible with the calculated molecular weight of the recombinant protein (data not shown). Overexpression of oIKK was found to activate the reporter gene in a concentration-dependent manner with a maximal increase of 6-fold in luciferase activity compared to the control vector (Fig. 4A). Under similar experimental conditions, human IKK-α (hIKK-α) vector increased the reporter gene expression in the same order of magnitude (Fig. 4A).

Nuclear translocation of NF-κB was examined by electrophoretic mobility shift assay (Fig. 4B). In untransfected 293 cells, a faint amount of nuclear NF-κB was detected whereas in cells transfected by hIKK-α or oIKK an important nuclear translocation of NF-κB was observed. These results demonstrate that oIKK triggers the reporter gene expression by activating the human NF-κB transcription factor and suggest that oIKK can functionally replace hIKK-α in human cells.

3.4. Role of kinase domain and HLH motif in biological activity of oIKK

To define which domain of the molecule was responsible for oIKK biological activity two mutants were constructed by deleting either the helix-loop-helix motif (HLH$^-$) or part of the kinase domain (KD$^-$). The HLH motif was suppressed by introducing a stop codon at position 649, this mutation in-

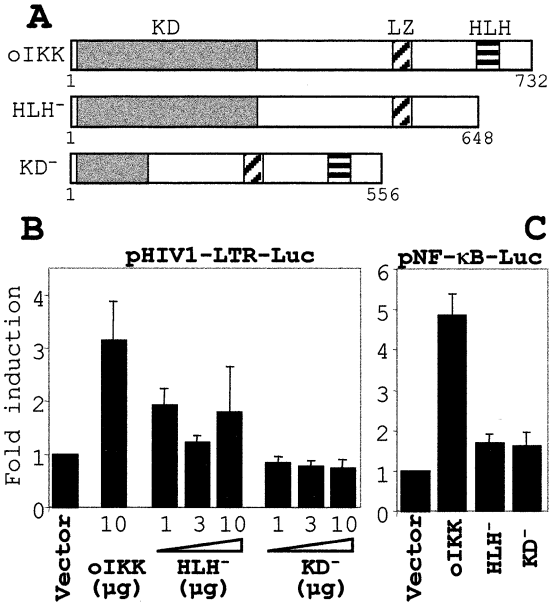

Fig. 5. Role of kinase domain and HLH motif in biological activity of oIKK. A: Schematic representation of wild-type oIKK and oIKK mutants lacking either the HLH motif (HLH$^-$) or part of the kinase domain (KD$^-$). B: CEM cells were transiently cotransfected with an LTR luciferase reporter construct and β-galactosidase expression plasmid, together with variable amounts of vector control (pCS3) and the indicated amount of wild-type oIKK or with oIKK mutants (HLH$^-$ or KD$^-$). Luciferase activity was normalized according to the level of β-galactosidase expression. The values shown are average induction (mean ± S.E.M.) of at least three independent transfection experiments. C: CEM cells were transiently cotransfected with pNF-κB-Luc reporter construct instead of pHIV1-LTR-Luc, β-galactosidase expression plasmid and 10 μg of the indicated plasmid.

duced the deletion of the last 84 amino acids in the carboxy-terminal part of oIKK (Fig. 5A). Deletion in the kinase domain extended between amino acids 94 and 269 and included the serine/threonine protein kinase active site signature as well as the canonical MAPKK activation loop motif (Fig. 5A). Overexpression of the oIKK HLH$^-$ mutant still activated the reporter gene but with a weak induction of luciferase activity whereas overexpression of the oIKK KD$^-$ mutant, lacking 60% of the kinase domain, was unable to activate the NF-κB-dependent reporter gene (Fig. 5B). Similar results were obtained when the reporter vector pHIV1-LTR-Luc was replaced by pNF-κB-Luc in which four tandemly repeated copies of the κB enhancer are fused to the herpes simplex virus thymidine kinase (HSV-TK) promoter driving the luciferase reporter gene (Fig. 5C). Therefore luciferase expression was controlled by κB motifs and not by other regulatory elements carried by the HIV1 LTR. Our results demonstrated that activation of κB-dependent reporter genes by oIKK was dependent on the integrity of the oIKK kinase domain and greatly reduced or even abolished by deletion of the HLH motif.

4. Discussion

We report here the isolation of an oyster cDNA encoding a 732 amino acid protein which presents a molecular organization close to that of serine protein kinase from the IKK family. We found that *oIKK* gene was equally expressed in challenged and unchallenged oysters and that oIKK mRNAs were present in all oyster tissues tested. These results revealed that oIKK is expressed constitutively and ubiquitously, like the other members of the IKK family [8,34]. oIKK protein is composed of an amino-terminal kinase domain (KD), a leucine zipper amphipathic α-helix (LZ), and a helix-loop-helix (HLH) motif. Overall, the sequences of oIKK and IKK-α are 43% identical, with the NH_2-terminal kinase domains sharing 56% identity and the COOH-terminal regions, which contain the LZ and HLH domains, having 31% sequence identity. It is worth noting that the homology between kinase domains of oyster IKK and mouse IKK-α (56% identity), two species separated by over 800 million years of evolution, is quite high compared to the homology observed intra-species between kinase domains of IKK-α and β subunits in human and mouse (63% identity) [9,33]. We demonstrated the ability of oIKK to induce NF-κB activation by showing that its overexpression induces NF-κB nuclear translocation and stimulates NF-κB-dependent reporter gene expression in a dose-dependent manner. Removal of the HLH motif of oIKK abrogated NF-κB reporter gene activation, this result is in perfect agreement with the recent data showing the essential role played by the HLH motif in the regulation of IKK activity [36].

Evidence for conservation of the NF-κB activation pathway between mammals, insects, and plants is well documented. The NF-κB signal transduction pathway in mammals shows striking functional and structural similarities with the Toll pathway in *Drosophila* and to a lesser extent with some proteins involved in plant disease resistance. These pathways use homologous receptors, kinases, transcription factors and inhibitors. Toll-like receptors (TLR) are evolutionarily conserved transmembrane proteins involved in the innate immunity of insects and mammals [37]. The Toll protein was originally identified and described in *Drosophila* as a receptor required for dorsal-ventral patterning during development, and was recently shown to be involved in the defensive response to fungal infection in adult flies [13]. In human, five TLRs have been identified and two of them have been demonstrated to mediate innate and adaptive immune responses via NF-κB activation [38,39]. In *Drosophila*, downstream signaling in the Toll cascade requires an adapter protein known as Tube [40]. In mammals, a Tube homologue has not yet been identified; however, MyD88 appears to play a role similar to that of Tube by coupling a serine/threonine protein kinase to the interleukin-1 receptor complex [41,42]. Structural homologies have also been reported between serine/threonine kinases Pelle (from *Drosophila*) and IRAK (from mammals) which respectively interact with adapter proteins Tube and MyD88 [43].

In mammals the IRAK, NIK and IKK kinase cascade leads to the activation of the Rel family of transcription factors via phosphorylation of IκB inhibitors. In insects, four Rel proteins (Dif, Cif, Gambif, and Relish) in addition to dorsal have been described and all of them are involved in immune response [44–47]. In *Drosophila*, the Cactus protein, an IκB homologue, is rapidly degraded and resynthesized after activation of the Toll pathway, indicating that the *cactus* gene, like *IκB* genes, is autoregulated [48]. The major effector mechanism of the *Drosophila* immune response is a rapid induction of a variety of antimicrobial peptides [49]. Some of the genes encoding antimicrobial peptides contain in their promoter regions sequences similar to NF-κB binding motifs of mammalian immune responsive genes (reviewed in [12]). Experiments with transgenic flies have shown that these motifs are mandatory for immune inducibility of the insect antimicrobial peptide genes [50,51]. A large number of antimicrobial peptides have been characterized in vertebrates and their expression is either constitutive or inducible [52]. Although very little is known about antimicrobial gene regulation in vertebrates, sequence analysis of the promoter region of a mammalian antimicrobial gene revealed the presence of a NF-κB binding site [53].

Interestingly, some components of the NF-κB pathway have also been identified in plants. Several disease resistance genes encode proteins belonging to the TLR family, such as the tobacco *N* gene that mediates resistance to tobacco mosaic virus [54], or the flax *L6* gene involved in rust fungus resistance [55]. The rice disease resistance protein Xa21 constitutes a two-component receptor system resembling a transmembrane TLR domain and an intracellular serine/threonine kinase domain [56]. Evidence for an intracellular kinase cascade involved in activation of plant defense response has been obtained with isolation of the *Pto* gene from tomato encoding a serine/threonine kinase, related to IRAK, that confers resistance to bacterial speck disease [57] and which phosphorylates a second serine/threonine kinase, Pti, equally involved in plant defense [58]. To our knowledge, no plant defense or resistance genes encoding Rel homologous proteins have been identified to date. However, two *Arabidopsis* genes (*NPR1* and *NIM1*), which play a critical role in disease resistance, have been characterized and found to be homologous to IκB [59,60]. These functional and structural similarities among evolutionarily divergent organisms suggest that insect and mammalian innate immunity and the plant pathogen defense pathways may be inherited from a common ancestor. It is therefore

very likely that similar pathways in will be found in other phyla or groups.

Here we report, for the first time, the characterization of a NF-κB pathway component in a mollusk. Similarities in the signaling pathway between vertebrates and bivalve mollusks have been previously reported by Hughes and collaborators. They showed that human interleukin-1 and tumor necrosis factor-α induced a size increase of mussel hemocytes, and these responses are dose-dependent and can be blocked by antibodies specific for either monokine [61]. Such a response tends to imply that a recognition mechanism for tumor necrosis factor-α and interleukin-1 exists in mussel hemocytes. Nevertheless, no molecular data have appeared to sustain this hypothesis. Altogether, our results open a new way to investigate the mollusk cell signaling network possibly relevant to defense mechanisms. The next step will consist of characterizing, in mollusks, other members of the NF-κB signaling cascade such as receptor molecules, other components of the IKK signalsome, and genes controlled by this signaling pathway.

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