Cg-Rel, the first Rel/NF-κB homolog characterized in a mollusk, the Pacific oyster *Crassostrea gigas*

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Abstract **We report here the identification and functional characterization of *Cg*-Rel, a gene encoding the *Crassostrea gigas* homolog of Rel/NF-κB transcription factors found in insects and mammals. Sequence and phylogenetic analysis showed that *Cg*-Rel shares the structural organization of Rel/NF-κB transcription factors of class II. It includes a Rel homology domain as well as a C-terminal transactivation domain (TD). Overexpression of *Cg*-Rel in the *Drosophila* S2 cell line activated the expression of a NF-κB-dependent reporter gene, whereas transfection with a *Cg*-Rel construct containing a C-terminal deletion of the TD or using a reporter gene with mutated κB binding sites failed to activate expression. These results suggest that *Cg*-Rel is a functional member of the Rel family of transcription factors, making this the sixth structurally homologous component of the Rel/NF-κB pathway characterized in *C. gigas*. Based on homology to other invertebrates' Rel/NF-κB cascade, the function of the oyster pathway may serve to regulate genes involved in innate defense and/or development. These findings serve to highlight a potentially important regulatory pathway to the study of oyster immunology, hence allowing comparison of the immune system in vertebrates and invertebrates, an important key issue to understand its evolution.**

Key words: Bivalve mollusk; Rel/nuclear factor-κB signaling pathway; Oyster *Crassostrea gigas*

1. Introduction

The innate immune system, which is the first line of defense against infectious microorganisms, appeared early in evolution, and the basic mechanisms of pathogen recognition and activation of the response are conserved throughout much of the animal kingdom [1]. Recent studies have revealed striking similarities in the signaling pathway used by mammals and dipterans to activate their innate immune responses. In both cases, infection leads to the induction of these intracellular signaling cascades, namely the nuclear factor NF-κB pathway, in which the most significant feature is the central role of the Rel/NF-κB family of transcriptional activator proteins [2,3].

The Rel/NF-κB family of transcription factors is composed of a set of structurally related and evolutionarily conserved DNA binding proteins [4]. In most cell types, Rel/NF-κB transcription complexes are present as latent, cytoplasmic forms that can be induced to enter the nucleus and activate gene expression [5]. The cytoplasmic sequestration of Rel/NF-κB is regulated by a family of IκB inhibitors [6,7]. Many of the signals known to activate Rel/NF-κB also lead to the activation of Toll-like receptors, which in turn initiate intracellular signaling cascades that culminate in phosphorylation, ubiquitination and degradation of IκB. Serine kinases termed IKKs (IκB kinases), which are part of a large multiprotein complex known as the IKK signalsome, have been shown to phosphorylate IκB in response to signals known to activate the NF-κB cascade [8–11]. Once released, NF-κB is free to translocate into the nucleus and activate target genes through binding to 10 bp DNA sites (κB sites) as dimers [12].

All the Rel/NF-κB proteins are related through a highly conserved 300 amino acid region called the Rel homology domain (RHD) that is involved in DNA binding and dimerization [5]. The RHD also contains the nuclear localization site (NLS) that consists of a stretch of four or five basic residues and is responsible for the nuclear translocation of the protein. Further, Rel/NF-κB proteins can be divided into two classes based on sequences C-terminal to the RHD. Members of class I (p100 and p105 in mammals and Relish in *Drosophila*) have long C-terminal domains that contain multiple copies of ankyrin repeats (similar to those present in IκB proteins), which act to inhibit themselves. Members of class II (cRel, RelA, RelB in mammals; Dorsal, Dif in *Drosophila* and Gambif in *Anopheles*) contain C-terminal activation domains, which are often not conserved at the primary sequence level across species, even though they can activate transcription [13].

There is currently increased interest in resolving questions about the molecular mechanisms of defense in invertebrates, including mollusks, which represent the second largest group of the invertebrate phylum after insects. This is of particular interest for marine organisms with economic importance, in particular the Japanese oyster, *Crassostrea gigas*, which represents 97% of the worldwide oyster production. The mollusk's internal defense mechanisms involve cell-mediated and humoral reactions that interplay to recognize and eliminate pathogens. It has become increasingly evident that the hemocytes play a central role in the bivalve internal defense through chemotaxis, recognition involving opsonins such as lectins [14], phagocytosis and antimicrobial peptide production [15,16]. Recently, oyster hemocytes have also been shown to participate in homeostatic mechanisms, including apoptosis

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and neuroendocrine signaling [17,18]. Previously reported results using mRNA differential display reverse transcription polymerase chain reaction (RT-PCR) allowed us to characterize a gene encoding an IKK-like protein (oIKK) which shares structural and functional properties with its mammalian homologs [19]. This finding strongly suggested the presence of a Rel/NF-κB signaling pathway in the Pacific oyster.

We report here the cloning and functional characterization of the *C. gigas* Rel protein (*Cg*-Rel), the first mollusk Rel family member to be identified. Sequence and phylogenetic analysis showed that *Cg*-Rel shares the structural organization of class II Rel/NF-κB transcription factors. Transient transfection experiments performed in a *Drosophila* cell line showed that *Cg*-Rel was able to trigger Rel-controlled reporter gene expression. This capacity required the integrity of the C-terminal domain of *Cg*-Rel and the presence of functional κB binding sites in the reporter gene promoter. Our results confirm the existence of a Rel/NF-κB pathway in a mollusk and support the concept of an innate immune response that is common to all metazoans.

2. Materials and methods

2.1. PCR cloning of Cg-rel

Degenerate primers were designed on a consensus sequence of the RHD and used for PCR amplification from a cDNA library of *C. gigas* hemocytes [19]. The sense primer (KB1: 5′-TTYCGITAYGGITGYGAIGG-3′ where I = inosine, Y = C/T) corresponds to the peptide FRYGCE which is the consensus pattern of the Rel protein family (F-R-Y-X-C-E where X is any amino acid). The antisense primer (KB2: 5′-RAAIGCYTGRAARCAIAGICGIACIAC-3′ where R = A/G) corresponds to the peptide VVRLCFQAF, a consensus sequence from a well conserved area within the RHD. PCR was performed as follows: initial denaturation at 94°C for 5 min; followed by 40 cycles at 94°C for 1 min; 50°C for 1 min; 72°C for 1 min; using 40 pmol of each primer and 2.4×10^6 pfu (plaque forming units) of the cDNA library. The expected 392 bp PCR amplicon was cloned in pCR-Script™ (Stratagene), sequenced and radiolabelled to screen the cDNA library for full-length clone characterization. Screening of the cDNA library and obtaining of phagemids were performed as described before [19]. The phagemids obtained by in vivo excision were sequenced on both strands.

2.2. Phylogenetic tree constructions

A list of all known sequences of the members of Rel/NF-κB was obtained from GenBank and EMBL databases using the BLAST program. Phylogenetic trees were computed using the MEGA 2 program (Molecular Evolutionary Genetics Analysis, version 2.1). The tree was built by the neighbor-joining method based on the alignment of the sequences using ClustalX (alignment was improved using the Seaview software). Bootstrap values (%) of 1000 replicates were calculated for each node of the consensus tree obtained.

2.3. Oyster challenge and RNA extraction

Three to four year old oysters (*C. gigas* Thunberg) were collected from a commercial farm (Palavas, Gulf of Lyon, France), and kept in seawater at 15°C. At least 10 oysters were used in each experimental condition. Oysters were challenged by filing the shell and injecting into the adductor muscle either 100 μl of saline peptone water (SPW: peptone 15 g/l, NaCl 15 g/l), Gram-positive bacteria (*Micrococcus luteus*), Gram-negative bacteria (*Escherichia coli* D31) or a mixture of four pathogenic *Vibrio* strains (*V. anguillarum*, *V. metshnikovii*, *V. tubiashii* and *Vibrio* sp. S322). The bacterial strains were grown separately overnight, in SPW at 25°C for *Vibrio* strains, or LB for *M. luteus* (30°C) and *E. coli* (37°C). Bacteria were then collected by centrifugation ($4000\times g$, 5 min) and resuspended in fresh growth medium. Bacterial concentration was calculated from the optical density at 550 nm (1 unit OD_{550} corresponds to 5×10^8 bacteria/ml) and the concentration verified by plating the bacteria. The different strains were then injected at a concentration of 10^8 bacteria in 100 μl. Hemolymph from unchallenged or challenged oysters was collected at different times post challenge with a syringe from the pericardial cavity, through the adductor muscle, and immediately centrifuged at $1000\times g$ for 10 min at 4°C. Hemocyte pellets were used for RNA extraction.

Hemocyte pellets were resuspended in Trizol reagent (Gibco BRL), 1 ml/10^7 cells. Oyster tissues (adductor muscle, mantle margin, mantle inner surface, heart, gills, labial palps, digestive gland, and stomach) were collected from at least 10 oysters. Tissues (100–300 mg) were washed twice in sterile seawater (50 ml), cut into small pieces and incubated overnight at 4°C in Trizol reagent (1 ml/100 mg of tissue). Total RNAs were extracted following the manufacturer's instructions. Poly(A)-rich RNAs from hemocytes were extracted with Dynabeads Oligo $(dT)_{25}$ (Dynal). For real-time PCR assays, RNA was extracted using the High Pure RNA Isolation Kit (Roche Diagnostics).

2.4. Northern blot analysis

RNAs were separated on a 1.2% agarose/formaldehyde gel and blotted onto nylon membranes (Hybond-N, Amersham Pharmacia Biotech). Membranes were pre-hybridized for 4 h at 65°C in 50% deionized formamide, 5×sodium saline citrate (SSC), 0.1% sodium lauroylsarcosine, 0.02% sodium dodecyl sulfate (SDS), 2.5% blocking reagent (Roche Diagnostics). Hybridization using radiolabelled probes was performed overnight at 42°C in the same buffer. Membranes were washed twice for 15 min at room temperature in 2×SSC/0.1% SDS and twice for 20 min at 60°C in 1×SSC/0.1% SDS. Membranes were exposed to autoradiographic films for 2–3 days at −80°C using an intensifying screen.

2.5. Probe preparation

PCR fragments overlapping the RHD of *Cg-rel* (891 bp) were radiolabelled by [α-^{32}P]dCTP using the Prime-it® II Random Labelling Kit (Stratagene) following the manufacturer's instructions. For PCR reactions, a mix of the following reaction components was prepared to the indicated end concentration: 1×Thermopol buffer, 1 μM primers, 200 μM dNTPs, 1 U of Vent DNA polymerase (New England Biolabs), 150 ng full-length cDNA template and amplification was performed for 30 cycles using the following parameters: 94°C for 30 s; 60°C for 30 s; 72°C for 1 min. *Cg-timp* and rRNA probes were prepared as described in [20].

2.6. Real-time PCR assay

2.6.1. Primer design and reverse transcription. For *Cg-rel* the forward and reverse primers were RelF (5′-GCTACGAGTGTGAGGGGAGATCA-3′) and RelR (5′-GGGAAACTGATGACGTTGGTGTC-3′) respectively. The gene encoding the elongation factor (*e.f.*) was used as internal control (this gene was isolated during an EST program [21]). For *e.f.* the forward and reverse primers were EFF (5′-ATGCACCAAGGCTGCACAGAAAG-3′) and EFR (5′-TCCGACGTATTTCTTTGCGATGT-3′) respectively.

2.6.2. LightCycler real-time PCR. First strand cDNA was synthesized using poly(dT) primers $(Oligo(dT)_{12-18}$, Invitrogen). Reverse transcription was performed on 1 μg total RNA using the SuperScript™ RNase H^- reverse transcriptase (Invitrogen) according to the manufacturer's instructions. PCR amplifications were carried out with the LightCycler™ (Roche) in the presence of SYBR-Green™ (Master SYBR Green™). Briefly, the following components were mixed to the indicated end concentrations: 5 mM $MgCl_2$, 0.5 μM of each primer, 1 μl of reaction mix (LC Fast Start Master SYBR Green I; Roche Diagnostics) in a final volume of 9.5 μl. 0.5 μl reverse-transcribed RNA was added as PCR template to the LightCycler master mix and the following run protocol used: initial denaturation at 95°C for 8 min; 95°C for 15 s; 62°C for 5 s; 72°C for 10 s with a single fluorescence measurement; melting curve program (73–95°C with a heating rate of 0.1°C/s and a continuous fluorescence measurement) and finally a cooling step to 30°C. For further expression level analysis, the crossing points (CP) are determined for each transcript using the LightCycler software. Specificity of RT-PCR product was analyzed on agarose gel and melting curve analysis. The copy ratio of each analyzed cDNA was determined as the mean of three replicates.

2.6.3. Relative quantification. The relative expression ratio of *Cg-rel* was calculated based on the CP deviation of each RT-PCR product of RNA extracted from stimulated oyster versus the appropriate control sample (RT-PCR product of RNA from oyster stimulated with specific growth medium), and expressed in comparison to the

reference gene, *e.f.* The relative expression ratio of *Cg-rel* was calculated based on the delta-delta method for comparing relative expression results developed by PE Applied Biosystems (Perkin Elmer) and is defined as: ratio $= 2^{-[\Delta CP_{sample} - \Delta CP_{control}]} = 2^{-\Delta\Delta CP}$.

2.7. Plasmid constructs

Cg-Rel expression vector was constructed by inserting a restriction fragment overlapping the entire open reading frame (ORF) of *Cg-rel* into the pPac expression vector containing the *Drosophila* 5C actin promoter (described previously in [22]). *Cg*-Rel was Myc epitope-tagged by inserting a *myc* tag (GEQKLISEEDLQL) in frame with the *Cg*-Rel ORF. This construct is predicted to encode a 70.4 kDa protein containing the *Cg*-Rel protein bearing the Myc tag in its N-terminus, between the fourth and fifth amino acid. The truncation mutant (*Cg-rel* ΔTD) was constructed by inserting a stop codon 36 amino acids downstream of the NLS resulting in a C-terminally truncated mutant protein lacking the TD. The pPac-*Cg-rel* plasmid was hydrolyzed by *Tth*111I restriction enzyme, and then blunt-ended with the *Pfu* DNA polymerase before ligation. The resulting construct was predicted to encode a 48.5 kDa truncated protein of 425 amino acids lacking 88% of the C-terminal domain.

The *Drosophila* expression vector encoding the dorsal transcription factor (pPac-dorsal) was used as a control for activation of the reporter gene expression. The reporter plasmid 8κB-*luc* utilized in the transfection assays was constructed as described previously [23]. Briefly, this plasmid contains the firefly luciferase reporter gene driven by the HSV *thymidine kinase* gene promoter situated downstream of eight copies of the oligonucleotide 5′-ATCGGGGATTCCTTTT-3′ which contains the κB motif (underlined) from the diptericin antimicrobial peptide gene promoter. In the plasmid 8κBmut-*luc*, the κB motifs from the diptericin gene promoter were mutated to: 5′-ATCGATTATTCCTTTT-3′ (mutations are underlined).

2.8. Cell culture, transfection experiment and luciferase detection

Embryonic *Drosophila melanogaster* Schneider 2 cells were grown at 25°C in Schneider's medium (Sigma) complemented with 10% fetal calf serum (Gibco BRL), 10^5 U/l penicillin and 100 mg/l streptomycin. 3×10^6 cells were transfected using Effectene™ Transfection Reagent (Qiagen) and 0.1 μg of reporter plasmid, 0.1 μg of the β-galactosidase expression vector (pACH110) as internal control for the transfection efficiencies, and 1 μg of a specific *Cg*-Rel expression vector. After 2 days of incubation (23°C), cells were washed in phosphate-buffered saline and lysed during 20 min in the reporter lysis buffer (Promega). Luciferase activity was measured in a luminometer after addition of the luciferin substrate (Promega). The β-galactosidase activity was measured using *o*-nitrophenol-β-D-galactoside as a substrate and values were used to normalize luciferase activities for variability in transfection efficiency.

3. Results

3.1. Isolation and sequence analysis of a C. gigas Rel family member, Cg-Rel

Degenerate oligonucleotides were designed based on sequence analysis of RHDs from vertebrate and insect Rel proteins. PCR amplification, using a *C. gigas* hemocyte cDNA library as template, resulted in the amplification of a 392 bp product. The amplicon was sequenced and homology search revealed 60% identity with Rel proteins. Using the PCR fragment as a probe to screen the cDNA library, we isolated two independent cDNA clones *Cg-rel*1 (2665 bp) and *Cg-rel*2 (2647 bp) (GenBank accession numbers AY039648 and AY039649).

The majority of the differences between the two cDNAs occur in the 5′ untranslated region (UTR) (39 bp including four gaps) and 3′ UTR (9 bp including four gaps). *Cg-rel*1 contains an ORF of 1848 bp predicted to encode a 615 amino acid protein whereas the ORF present in *Cg-rel*2 is 1845 bp long and encodes a 614 amino acid protein. The two predicted proteins differ also by five amino acid residues, of which only

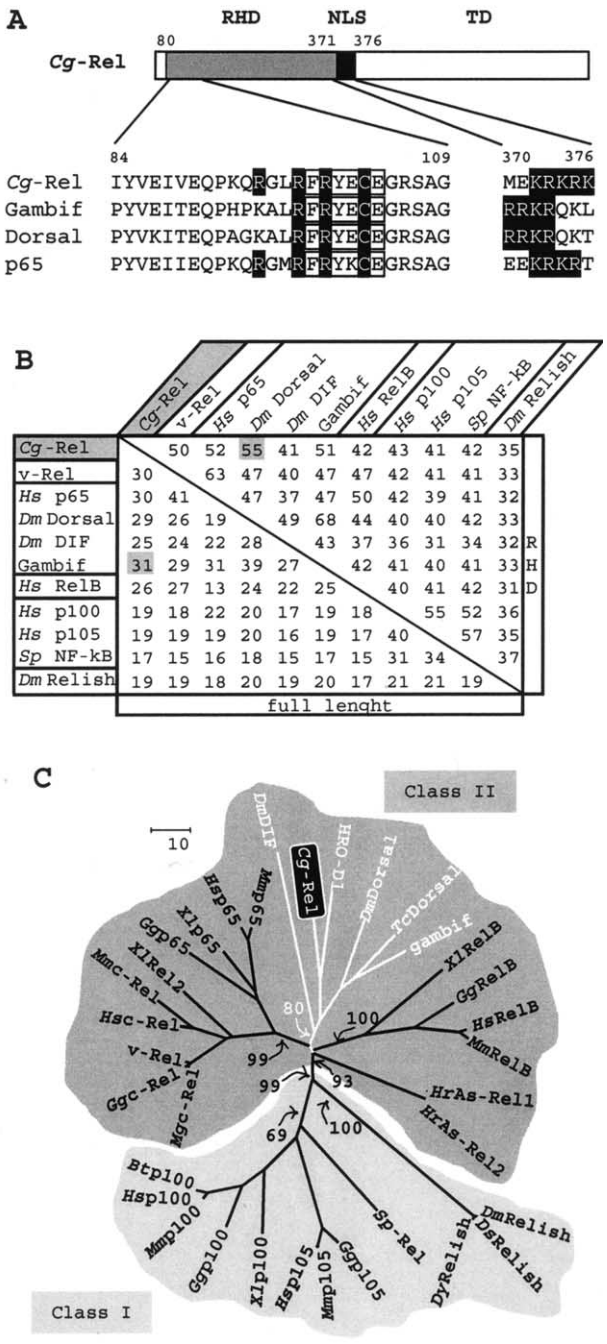

Fig. 1. Sequence analysis of *Cg*-Rel and phylogenetic tree of Rel proteins. A: Schematic representation of *Cg*-Rel and sequence alignment with the characteristic motifs of other Rel proteins. The RHD, the NLS and TD are indicated as well as the first and last amino acids of the RHD and NLS. Amino acid sequences of *Cg*-Rel, Gambif (*A. gambiae*), dorsal (*D. melanogaster*) and human p65 were aligned, for the area surrounding the Rel protein signature F-R-Y-X-C-E, the DNA binding motif R-XX-R-X-R-XX-C and the NLS. B: Matrix of percentage amino acid identity in the aligned full length and the RHD domain between representative members of Rel protein families of class I and II. Maximum identity between *Cg*-Rel and other sequences is in gray background. C: Unrooted phylogenetic tree of Rel proteins. The tree was built by the neighbor-joining method based on the alignment of the sequences using ClustalX. The class I and II Rel protein clades are indicated by different gray backgrounds. The dorsal-like clade is in white. Bootstrap values of 1000 replicates (%) correspond to nodes indicated by arrows.

two correspond to conservative substitutions. These results suggest the existence of a polymorphism within oyster *Cg-rel* genes that may have resulted from the fact that the cDNA library was constructed with hemocytes collected from several oysters. Since the two cDNAs were highly similar and hardly distinguishable by any nucleic acid hybridization-based methods, and since the differences did not introduce drastic transformations in the predicted gene product, we focused the study on *Cg-rel*1 (referred to hereafter as *Cg-rel*).

Amino acid sequence analysis (using ProfileScan and BLAST programs) revealed that *Cg*-Rel possesses the characteristic organization of Rel proteins. Specifically, *Cg*-Rel contains an N-terminal RHD of 292 amino acids (residues 80–371) and a NLS constituted of a stretch of five basic residues (residues 372–376) (Fig. 1A). The *Cg*-Rel RHD contains two domains conserved in all Rel family proteins: (i) the Rel protein signature F-R-Y-X-C-E (where X is any amino acid), and the DNA binding motif R-XX-R-X-R-XX-C (Fig. 1A). Sequences flanking the RHD show no similarity to known proteins and do not contain any recognizable protein motifs. In particular, the C-terminal region, which by analogy to other Rel/NF-κB proteins was likely to be a TD, contains neither the polyglutamine, polyalanine, or polyasparagine stretches characteristic of the C-termini of *D. melanogaster* Dorsal, Dif and Gambif proteins, nor the mini-leucine zipper domain and conserved acidic motifs characteristic of vertebrate RelA and c-Rel proteins [24,25].

The overall sequence identity of *Cg*-Rel with Rel proteins ranged from 17 to 31%, while the identity in the most conserved region (RHD) ranged from 35 to 55% (Fig. 1B). Interestingly, *Cg*-Rel was most similar to Dorsal-like proteins from insects. In particular, *Cg*-Rel shares highest sequence identity (31%) with Gambif, a Rel protein characterized in the human malaria vector *Anopheles gambiae* [26].

The availability of vertebrate and invertebrate (mostly insect) Rel protein sequences in databases allowed us to perform a phylogenetic analysis of the Rel family to confirm the relationship between *Cg*-Rel and the other Rel transcription factors. To this purpose, we listed all Rel protein sequences available in databases: (i) members of the class I subfamily which includes vertebrate and invertebrate p100, p105 and Relish proteins, and (ii) members of the class II subfamily which include p65 proteins, RelB proteins, c-Rel/v-Rel proteins and dorsal-like proteins [4,27,28]. Based on the RHD multisequence alignment of these proteins built with ClustalX, we constructed an unrooted phylogenetic tree using the neighbor-joining method (Fig. 1C).

Two subfamilies can be defined, supported by high bootstrap values: class I Rel proteins (bootstrap of 99%) and class II Rel proteins (bootstrap of 93%). The class I Rel proteins include (i) the p100/p105 homolog group (69%) and (ii) Relish homologs (100%). Class II Rel proteins comprise three groups: (i) a dorsal-like protein cluster (80%), (ii) the RelB group (100%), (iii) a large group (99%) composed of two subfamilies, c-Rel/v-Rel and RelA/p65. Interestingly *Cg*-Rel clearly clustered with all the insect class II Rel transcription factors. This result is consistent with the previous sequence analysis where *Cg*-Rel shared most similarities with insect Dorsal-like sequences. The same topology was obtained when we constructed a phylogenetic tree based on the alignment of full-length protein sequences or using the maximum parsimony method (data not shown).

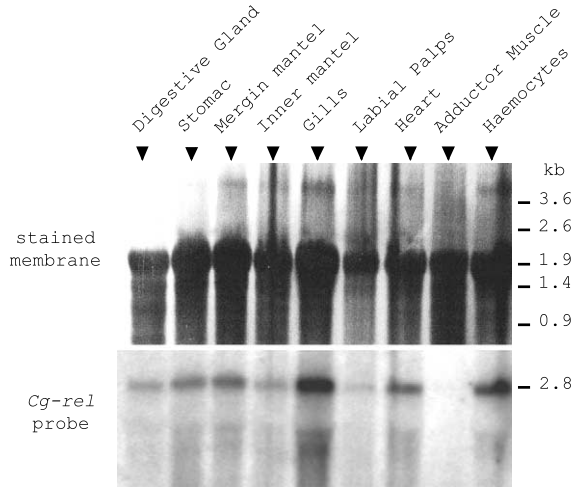

Fig. 2. Tissue expression of *Cg-rel*. Total RNAs were extracted from several tissues: digestive gland, stomach, mantle (margin and inner parts), gills, labial palps, heart, adductor muscle and hemocytes. RNAs (30 μg per lane) were electrophoresed through a denaturing 1.2% agarose/formaldehyde gel and blotted onto nylon membranes. The membrane was stained with methylene blue to visualize total RNA (upper panel), then destained and hybridized with a radiolabelled probe specific to the *Cg-rel* RHD sequence (lower panel).

Using a PCR-based approach we identified a Rel/NF-κB-like sequence in the Pacific oyster. Moreover, sequence similarity and phylogenetic analysis both confirmed that *Cg*-Rel is a member of the class II Rel proteins closest to insect Dorsal-like proteins.

3.2. *Tissue expression of* Cg-rel

To examine whether *Cg-rel* was ubiquitously expressed, as is the case for most of the *rel* genes, a 891 bp fragment of *Cg-rel*, containing the RHD, was used to probe a Northern blot of total RNAs extracted from several oyster tissues (Fig. 2). In nearly all tissues analyzed, a 2.8 kb transcript was detected, suggesting that *Cg-rel* is ubiquitously expressed in adults. Moreover, the rather high amount of RNA needed for hybridization detection (30 μg per lane) strongly suggests that *Cg-rel* was weakly expressed.

3.3. Cg-rel *expression during bacterial challenge*

In order to determine the expression pattern of *Cg-rel* during bacterial challenge we used two batches of oysters. In the first batch, oysters were injected with SPW whereas in the second batch, oysters were injected with a mixture of four pathogenic *Vibrio* strains (see Section 2). Northern blots were performed with total RNA extracts from hemocytes collected at several time points post challenge (3, 6, 9, 12, and 24 h). The same Northern blot was hybridized sequentially with *Cg-rel*, *Cg-timp* and *e.f.* probes (Fig. 3A). Hybridization signals obtained in two independent experiments were quantified using the Molecular Dynamic Storm system, *Cg-rel* transcripts were normalized to *e.f.* for each time point and expressed as a function of the signal obtained for SPW-challenged oysters (Fig. 3B).

No striking discrepancies were observed for *Cg-rel* expression between SPW- and bacteria-challenged oysters at the different time points analyzed. In contrast, the expression pattern of *Cg-timp*, a bacterially inducible gene [20], was strongly

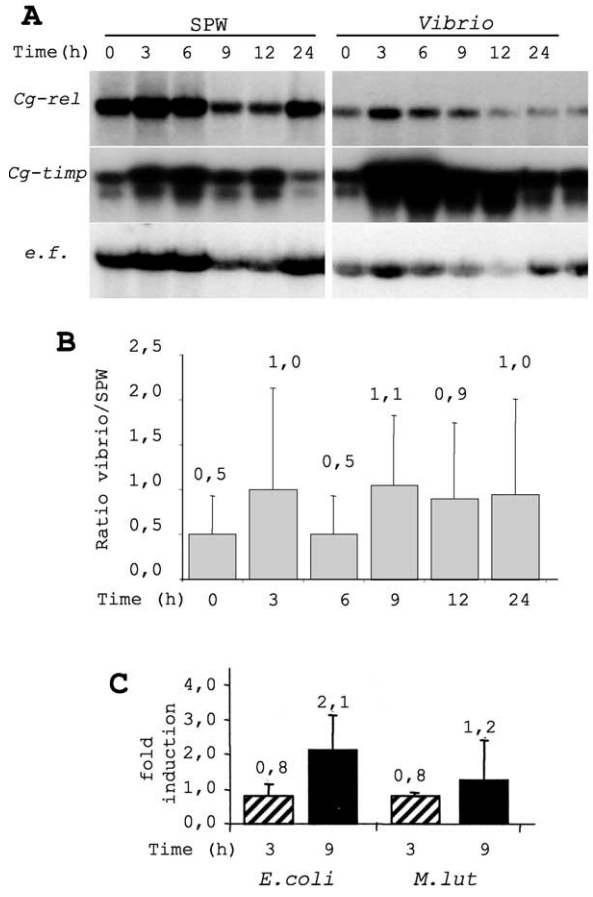

Fig. 3. *Cg-rel* expression from hemocytes of bacterially challenged oysters. A: Northern blot analysis of *Cg-rel* gene expression in challenged oysters. Hemocytes were collected 3, 6, 9, 12, and 24 h after the oysters were treated by injection of SPW or a mixture of four *Vibrio* strains. Ten oysters were used for each time point. Blot containing 35 μg of total RNA per lane was successively hybridized with *Cg-rel*, *Cg-timp* (to verify challenge effectiveness) and *e.f.* (loading control). B: Signals obtained with *Cg-rel* and *e.f.* probes were quantified using the Molecular Dynamics Storm system, *Cg-rel* mRNA expression was normalized according to the level of *e.f.* and expressed as a function of the signal obtained for SPW-treated oysters at the corresponding times. Values indicated above the histograms represent the means of three independent experiments. C: Real-time PCR analysis of *Cg-rel* transcript accumulation after bacterial challenge. Fold induction measured 3 and 9 h after challenge is shown as hatched and black histograms, respectively. The values are average induction (±S.E.M.) of at least two independent experiments done in triplicate.

modified, attesting to the proficiency of the challenge conditions. The strong variations observed between the two experiments may be explained by the low abundance of *Cg-rel* transcripts or by natural variability between the populations sampled. Experiments were performed using individuals originating from natural populations characterized by large genetic diversity. Nevertheless, this analysis implies that *Cg-rel*, unlike *Cg-timp*, was unaffected by bacterial challenge.

However, we may not exclude the possibility that, due to their pathogenicity, the *Vibrio* strains used for the challenge did not trigger *Cg-rel* activation. In fact, in vertebrates as well as in *Drosophila*, NF-κB signaling pathways may be differentially activated depending on pathogens used for infections, i.e. Gram-positive versus Gram-negative bacteria [3]. In order to determine if *Cg-rel* expression was regulated by other bacterial strains, we challenged oysters with Gram-negative (*E. coli*) or Gram-positive (*M. luteus*) bacteria. Oysters were injected with bacterial culture or with bacteria-specific growth medium as a control. Due to the low abundance of *Cg-rel* transcripts observed during the previous experiments, we chose to monitor *Cg-rel* expression by real-time RT-PCR using RNAs extracted from hemocytes as a template. For each time point (3 and 9 h) and for each condition (bacteria or growth medium), *Cg-rel* transcript abundance was normalized according to the level of *e.f.* transcripts and expressed as a function of *Cg-rel* transcript abundance in the control experiments (injection of growth medium). Fig. 3C shows that neither the injection of *E. coli* nor the injection of *M. luteus* induced a significant accumulation of *Cg-rel* transcripts in hemocytes. Altogether these results strongly suggest that *Cg-rel* was constitutively expressed in oyster hemocytes.

3.4. *Functional analysis of* Cg-*Rel*

Phylogenetic analysis of the *Cg*-Rel protein showed strong similarities between Rel proteins in oyster and insect. Therefore, given the lack of bivalve mollusk cell lines, it appeared appropriate to investigate the *Cg*-Rel functional properties by performing transient transfection experiments in *D. melanogaster* embryonic S2 cell line. In these experiments, we examined whether overexpression of *Cg*-Rel might activate the expression of a Rel-controlled reporter gene.

S2 cells were transfected with a luciferase reporter gene driven by a multimerized κB-diptericin sequence consisting of eight tandemly repeated κB binding sites from the antimicrobial diptericin gene. The function of *Cg*-Rel protein was evaluated by cotransfecting the reporter vector with a vector expressing either the Myc-tagged wild type *Cg*-Rel protein or the Myc-tagged mutant *Cg*-Rel ΔTD, deleted within the C-terminal domain (Fig. 4A). Expression of the recombinant *Cg*-Rel and *Cg*-Rel ΔTD proteins in S2 cells was probed with anti-myc antibodies and the protein sizes observed were compatible with the calculated molecular weight of the recombinant proteins (data not shown). Overexpression of *Cg*-Rel was found to activate the luciferase reporter gene with an increase of 25-fold in luciferase activity compared to the control vector (pPac) (Fig. 4B). The same range of increase was obtained when we overexpressed the *D. melanogaster* Dorsal protein (35-fold). Overexpression of *Cg*-Rel ΔTD did not induce luciferase expression showing that the integrity of the carboxy-terminal domain of the protein was essential for reporter gene activation. The same results were obtained when we used a reporter vector driven by a multimerized κB-cec sequence consisting of tandemly repeated κB binding sites from the antimicrobial cecropin gene (data not shown).

We have asked here whether the increase in luciferase activity resulted from a specific binding of *Cg*-Rel to the κB sites. To this end we cotransfected *Cg-rel* expression plasmid with another reporter vector in which the wild type κB binding sites (GGGGATTCC) were replaced by mutated sites (GATTATTCC). This mutation has been shown to completely abolish specific binding of Rel proteins to these sites [23]. This experiment showed that overexpression of *Cg*-Rel failed to induce any luciferase activity (Fig. 4C).

These results demonstrate that *Cg*-Rel is able to trigger a Rel-controlled reporter gene expression. This capacity relied primarily on the integrity of the C-terminal domain of *Cg*-Rel

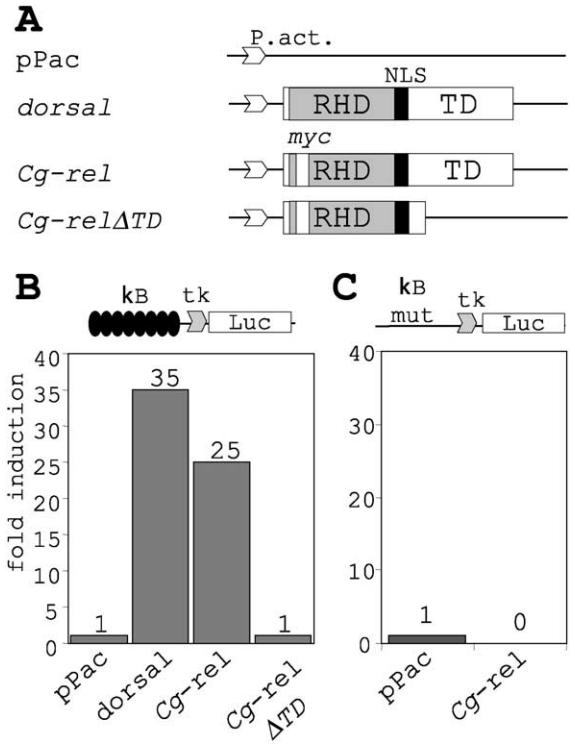

Fig. 4. Transfection of *Cg*-Rel expression vector, in a *Drosophila* cell line, induced the expression of a κB-dependent reporter gene. A: Schematic representation of the vectors used in transfection experiments. pPac, an empty vector containing only the *Drosophila* actin 5C constitutive promoter (P. act.), whereas *dorsal*, *Cg-rel* and *Cg-rel ΔTD* respectively contain the *Drosophila dorsal* gene, the wild type *Cg-rel* gene and the mutant *Cg-rel* gene lacking most of the transactivation domain. B: S2 cells were transiently cotransfected with a Rel-dependent luciferase reporter construct (eight tandemly repeated κB binding sites from the diptericin gene promoter) and a β-galactosidase expression plasmid, together with pPac, *dorsal*, *Cg-rel* or *Cg-rel ΔTD* expression vector. Luciferase activity was normalized according to levels of β-galactosidase expression (three independent experiments). C: S2 cells were transiently cotransfected with a Rel-independent luciferase reporter construct (eight mutated κB binding sites) and a β-galactosidase expression plasmid, together with the indicated expression vectors.

which can therefore be considered a functional TD, and on the presence of functional κB binding sites in the reporter gene promoter. Therefore, functional analysis of *Cg*-Rel revealed that this protein was an efficient NF-κB-like activating transcription factor.

4. Discussion

Over the past decade, the Rel/NF-κB signal transduction pathway appeared to be a key component of the immune response owing to its involvement in both the innate immunity in multicellular organisms and adaptive immunity in vertebrates. Several years ago, in our search for genes involved in oyster immunity, we characterized a protein structurally and functionally homologous to a member of the Rel/NF-κB signal transduction pathway. This discovery led us to propose that this cell signaling network was conserved in mollusks [19]. In this report we present the isolation of an oyster homolog of NF-κB. We isolated two cDNAs called *Cg-rel*1 and *Cg-rel*2 (*C. gigas* Rel) encoding polypeptides that share the characteristic organization of Rel proteins, namely a RHD and a NLS. Both sequence homology and phylogenetic analysis suggest that *Cg*-Rel is a Rel protein of class II more related to the Dorsal-like protein family.

In order to better understand the biological role of *Cg*-Rel, we analyzed gene expression. We found that *Cg-rel* mRNAs were present in all oyster tissues studied, suggesting that *Cg-rel* was ubiquitously expressed. This result is not unlike that for mammals, where most Rel proteins are ubiquitously expressed, and this pattern of expression strongly implicates their role in numerous physiological processes [29]. In *C. gigas*, the fact that *Cg*-Rel is ubiquitously expressed also suggests that it might have a pleiotropic function. Moreover, we found that *Cg-rel* was equally expressed in challenged and unchallenged oysters suggesting that expression is constitutive. The regulation of *rel* expression differs between class I and II genes. Most Rel proteins of class I are regulated by the NF-κB pathway in response to infection, whereas most Rel proteins of class II (RelA, RelB, Gambif and Dif) are constitutively expressed [26,30]. Nevertheless, there are notable examples of *rel* genes that are controlled by the NF-κB signaling pathway such as *dorsal*, which was strongly induced by bacterial challenge. Our results suggest that transcriptional control plays a minor role in regulating expression of *Cg-rel*.

Functional properties of *Cg*-Rel were investigated by in vitro experiments using the embryonic *Drosophila* Schneider 2 cell line. These experiments showed that *Cg*-Rel was able to induce a NF-κB-dependent reporter gene in binding specifically to κB sites. This induction was comparable to that obtained when we overexpressed the endogenous protein Dorsal. This functional homology may result from primary sequence similarities observed within the DNA binding domain of Dorsal (LRFRYECE) and *Cg*-Rel (LRFRYECE) (Fig. 1A). Transfection experiments using the *Cg*-Rel C-terminal deletion construct showed (i) that *Cg*-Rel was directly responsible for the transcriptional activation and (ii) that the C-terminal part of *Cg*-Rel was a transactivation domain. However, in *Cg*-Rel we did not find a transactivation domain similar to that of vertebrate or insect proteins. This might suggest that the *Cg*-Rel transactivation capacities, as in the cases of RelB or Gambif, rely on other motifs within the C-terminal domain that remain to be identified [26]. Taken together, these results confirm that *Cg*-Rel is an effective transcription factor belonging to the Rel protein family of class II.

We also report here, for the first time in a bivalve mollusk, the characterization of a Rel/NF-κB transcription factor structurally related to insect Rel proteins that is able, in vitro, to activate the expression of NF-κB-controlled reporter gene. *Cg*-Rel was the second protein characterized in oyster, together with oIKK (oyster IκB kinase-like protein), which shares structural and functional properties with an element of the Rel/NF-κB pathway and suggests the existence of a NF-κB signaling pathway in the oyster [19]. Furthermore, we have recently isolated, in an EST library, four cDNAs highly homologous (*E*-value ranging from $7e^{-7}$ to $4e^{-53}$) to other components of the Rel/NF-κB pathway including the adapter proteins MyD88, ECSIT and TRAF, and the *Drosophila* homolog to IκB (cactus) [21]. With regard to the components isolated so far, the oyster pathway appears to be most similar to Toll or TLR/IL-1 signaling pathways from *Drosophila* and mammals, respectively (Fig. 5) [3]. To our knowledge, it is the first time that six genes related to the Rel/NF-κB pathway

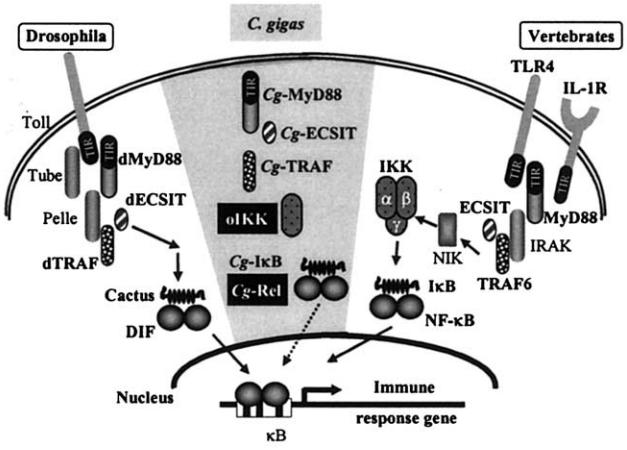

Fig. 5. Conservation of Rel/NF-κB pathways in vertebrates, *Drosophila* and oyster (from [1,3,44]). In vertebrates and in *Drosophila* the Rel/NF-κB pathways are conserved at the molecular level from transmembrane receptors (Toll, TLR for Toll-Like receptor, IL-1R for interleukin-1 receptor) to Rel proteins (Dif, NF-κB). Ligand binding to TLR leads to the activation of adapter proteins (MyD88, Tube) then to the activation of kinases (Pelle, IRAK, IKK) and to the phosphorylation and degradation of Rel inhibitory proteins (cactus, IκB). The phosphorylated inhibitors are degraded to release Rel transcription factors which are then translocated to the nucleus to activate target genes, notably those involved in immune responses. The different components characterized in oyster are shown (*Cg*-MyD88, *Cg*-TRAF, *Cg*-ECSIT, oIKK and *Cg*-IκB). DD, death domain; KD, kinase domain; TIR, Toll/IL-1 receptor homology domain.

have been characterized together from an invertebrate other than a dipteran. Even if these components have not yet been proven to belong to the same cascade, these findings further support the concept of a Rel/NF-κB signaling pathway in *C. gigas*. This finding also raises the important issue as to the role of this putative transduction pathway in this organism.

Albeit some of the signal pathways that regulate Rel family members are highly conserved, the fine regulation of expression of individual components of the Rel family might have diverged and adapted to specific physiological needs. Extensive studies of the Rel/NF-κB signal transduction pathway revealed its pivotal role in innate immunity in both *Drosophila* and mammals (reviewed in [2,3]). In both systems, this pathway also has an essential role in a wide variety of physiological processes, including cell division, cell survival, apoptosis, differentiation and development processes ([31,32] and reviewed in [29,33]). In *Drosophila* for example, nuclear translocation of Dorsal, following infection, is regulated by the same intracellular genetic pathway as in dorso-ventral polarization during embryonic development [34].

Rel proteins have also been identified in birds, fishes, an amphibian, an ascidian, an echinoderm and a leech [35–38]. The latter Rel proteins characterized, as well as proteins found in insects, are mostly known to take part in the innate immune response and development, but a few of them have been functionally characterized or shown to be associated to a NF-κB-like pathway [39–42] with one notable exception. In the nematode *Caenorhabditis elegans*, four genes homologous to elements of the *Drosophila* Toll pathway have been identified, and of the four genes, only one (*tol-1*) is required for nematode development and none of them for the resistance of *C. elegans* to pathogens [43].

Further progress in functional analysis of the oyster Rel/NF-κB signaling pathway requires the further development of methods currently only applied to the study of model organisms (genetic, transgenesis and gene knockout). An epigenetic gene disruption technique like RNA interference seems a very attractive alternative. It is anticipated that this approach will not only help investigators to characterize genes controlled by the oyster Rel/NF-κB cascade, but also provide insight into the function of this signaling pathway. To extrapolate, we expect that further study of the *C. gigas* Rel/NF-κB cascade will provide clues not only on system regulation during development and immunity, but also on the relationships between both systems during ontogenesis.

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References

[1] Hoffmann, J.A., Kafatos, F.C., Janeway, C.A. and Ezekowitz, R.A. (1999) Science 284, 1313–1318.
[2] Silverman, N. and Maniatis, T. (2001) Genes Dev. 15, 2321–2342.
[3] Hoffmann, J.A. and Reichhart, J.M. (2002) Nat. Immunol. 3, 121–126.
[4] Baldwin, A.S.J. (1996) Annu. Rev. Immunol. 14, 649–683.
[5] Ghosh, S., May, M.J. and Kopp, E.B. (1998) Annu. Rev. Immunol. 16, 225–260.
[6] Thompson, J.E., Phillips, R.J., Erdjument-Bromage, H., Tempst, P. and Ghosh, S. (1995) Cell 80, 573–582.
[7] Whiteside, S.T., Epinat, J.-C., Rice, N.R. and Israel, A. (1997) EMBO J. 16, 1413–1426.
[8] Régnier, C.H., Song, H.Y., Gao, X., Goeddel, D.V., Cao, Z. and Rothe, M. (1997) Cell 90, 373–383.
[9] Woronicz, J.D., Gao, X., Cao, Z., Rothe, M. and Goeddel, D.V. (1997) Science 278, 866–869.
[10] Cohen, L., Henzel, W.J. and Baeuerle, P.A. (1998) Nature 395, 292–296.
[11] Lu, Y., Wu, L.P. and Anderson, K.V. (2001) Genes Dev. 15, 104–110.
[12] Chen, F.E. and Ghosh, G. (1999) Oncogene 18, 6845–6852.
[13] Cramer, P., Varrot, A., Barillas-Mury, C., Kafatos, F.C. and Muller, C.W. (1999) Struct. Fold. Des. 7, 841–852.
[14] Vasta, G.R., Cheng, T.C. and Marchalonis, J.J. (1984) Cell. Immunol. 88, 475–488.
[15] Hubert, F., Nöel, T. and Roch, P. (1996) Eur. J. Biochem. 240, 302–306.
[16] Mitta, G., Vandenbulcke, F., Hubert, F. and Roch, P. (1999) J. Cell Sci. 112, 4233–4242.
[17] Lacoste, A., De Cian, M.C., Cueff, A. and Poulet, S.A. (2001) J. Cell Sci. 114, 3557–3564.
[18] Lacoste, A., Cueff, A. and Poulet, S.A. (2002) J. Cell Sci. 115, 761–768.
[19] Escoubas, J.M., Briant, L., Montagnani, C., Hez, S., Devaux, C. and Roch, P. (1999) FEBS Lett. 453, 293–298.
[20] Montagnani, C., Le Roux, F., Berthe, F. and Escoubas, J.-M. (2001) FEBS Lett. 500, 64–70.
[21] Gueguen, Y. et al. (2003) Gene 303, 139–145.
[22] Reichhart, J.-M., Georgel, P., Meister, M., Lemaitre, B., Kappler, C. and Hoffmann, J. (1993) C.R. Acad. Sci. Paris 316, 1218–1224.
[23] Georgel, P., Kappler, C., Langley, E., Gross, I., Nicolas, E., Reichhart, J.-M. and Hoffman, J.A. (1995) Nucleic Acids Res. 23, 1140–1145.
[24] Schmitz, M.L. and Baeuerle, P.A. (1991) EMBO J. 10, 3805–3817.
[25] Govind, S., Drier, E., Huang, L.H. and Steward, R. (1996) Mol. Cell. Biol. 16, 1103–1114.
[26] Barillas-Mury, C., Charlesworth, A., Gross, I., Richman, A.,

Hoffmann, J.A. and Kafatos, F.C. (1996) EMBO J. 15, 4691–4701.
[27] Blank, V., Kourilsky, P. and Israel, A. (1992) Trends Biochem. Sci. 17, 135–140.
[28] Gilmore, T.D. (1999) Oncogene 18, 6842–6844.
[29] Gerondakis, S., Grossmann, M., Nakamura, Y., Pohl, T. and Grumont, R. (1999) Oncogene 18, 6888–6895.
[30] Irving, P., Troxler, L., Heuer, T.S., Belvin, M., Kopczynski, C., Reichhart, J.M., Hoffmann, J.A. and Hetru, C. (2001) Proc. Natl. Acad. Sci. USA 98, 15119–15124.
[31] Pahl, H.L. (1999) Oncogene 18, 6853–6866.
[32] Dixit, V. and Mak, T.W. (2002) Cell 111, 615–619.
[33] Govind, S. (1999) Oncogene 18, 6875–6887.
[34] Lemaitre, B., Meister, M., Govind, S., Georgel, P., Steward, R., Reichhart, J.-M. and Hoffmann, J.A. (1995) EMBO J. 14, 536–545.
[35] Pancer, Z., Rast, J.P. and Davidson, E.H. (1999) Immunogenetics 49, 773–786.
[36] Schlezinger, J.J., Blickarz, C.E., Mann, K.K., Doerre, S. and Stegeman, J.J. (2000) Chem. Biol. Interact. 126, 137–157.
[37] Miele, R., Bjorklund, G., Barra, D., Simmaco, M. and Engstrom, Y. (2001) Eur. J. Biochem. 268, 443–449.
[38] Goldstein, B., Leviten, M.W. and Weisblat, D.A. (2001) Dev. Genes Evol. 211, 329–337.
[39] O'Neill, L.A.J. and Greene, C. (1998) J. Leukoc. Biol. 63, 650–657.
[40] Qiu, P., Pan, P.C. and Govind, S. (1998) Development 125, 1909–1920.
[41] Fallon, P.G., Allen, R.L. and Rich, T. (2001) Trends Immunol. 22, 63–66.
[42] Dangl, J.L. and Jones, J.D. (2001) Nature 411, 826–833.
[43] Pujol, N. et al. (2001) Curr. Biol. 11, 809–821.
[44] Khush, R.S., Leulier, F. and Lemaitre, B. (2001) Trends Immunol. 22, 260–264.