

The nucleic acid-binding protein *PcCNBP* is transcriptionally regulated during the immune response in red swamp crayfish *Procambarus clarkii*

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Abstract Gene family encoding cellular nucleic acid binding proteins (CNBP) is well conserved among vertebrates; however, there is limited knowledge in lower organisms. In this study, a CNBP homolog from the red swamp crayfish *Procambarus clarkii* was characterised. The full-length cDNA of *PcCNBP* was of 1257 bp with a 5'-untranslated region (UTR) of 63 bp and a 3'-UTR of 331 bp with a poly (A) tail, and an open-reading frame (ORF) of 864 bp encoding a polypeptide of 287 amino acids with the predicted molecular weight of about 33 kDa. The predicted protein possesses 7 tandem repeats of 14 amino acids containing the CCHC zinc finger consensus sequence, two RGG-rich single-stranded RNA-binding domain and a nuclear localization signal, strongly suggesting that *PcCNBP* was a homolog of vertebrate CNBP. The *PcCNBP* transcript was constitutively expressed in all tested tissues of unchallenged crayfish, including hepatopancreas, gill, eyestalk, haemocytes, intestine, stomach and cuticle with highest expression in haemocytes, intestine, gills and hepatopancreas. The mRNA expression of *PcCNBP* in haemocytes was modulated at transcriptional level by different immune challenges, suggesting its involvement

in the immune response of *P. clarkii* during both bacteria and viruses infection.

Keywords Nucleic acid-binding protein · Zinc finger motifs · Crayfish · Innate immunity · Gene expression pattern

Introduction

Cellular nucleic acid binding proteins (CNBPs) represent a highly conserved protein family among vertebrates; they harbour seven tandem zinc finger repeats CCHC type (Armas et al. 2004; Calcaterra et al. 2010) and have been described as transcriptional and translational regulator. CNBP, acting as a nucleic acid chaperone, binds single-stranded DNA and RNA molecules and promotes rearrangements of their secondary structure (Armas et al. 2008).

CNBP was first depicted as a DNA-binding protein acting as a negative transcriptional regulator of HMGCoA gene. Moreover, CNBP has been reported as a negative regulator of the expression of the JC virus early promoter-enhancer (Liu et al. 1998), as well as a myosin heavy chain gene (Flink and Morkin 1995) and transcription of *c-myc*, through its ability to promote the formation of G quadruplexes and bind it in the *c-myc* promoter (Michelotti et al. 1995; Borgognone et al. 2010). Because of its affinity for single-stranded nucleic acids (Pellizzoni et al. 1997), CNBP modulates the translation of several transcripts including *c-Myc* (Yasuda et al. 1995) and ribosomal proteins (Cardinali et al. 1993; Pellizzoni et al. 1997) by interacting with a G-rich RNA sequence present in these mRNAs. Recently, it has also been shown that CNBP modulates the transcription of Wnt signalling pathway components, thus regulating development, cell proliferation and apoptosis (Margarit et al. 2014).

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Through the years, several studies investigating *CNBPs* in vertebrates including human (Rajavashisth et al. 1989), mouse (Warden et al. 1994), rat (Yasuda et al. 1995), chicken (van Heumen et al. 1997), *Xenopus* (Flink et al. 1998), *Bufo arenarum* (Armas et al. 2001), zebra fish (Armas et al. 2004) and gibel carp (Liu and Gui 2005) have been reported.

Moreover, alternative splicing events have been described in these vertebrates as such human cells (Flink and Morkin 1995), rat (Yasuda et al. 1995), mouse (Warden et al. 1994) and *Xenopus* (De Dominicis et al. 2000). Thus, isoforms are often associated with the utilisation of different splicing donor sites.

To date, there is little characterisation of CNBP in invertebrates. Its structure and function have been analyzed solely in *Drosophila melanogaster* (Antonucci et al. 2014) where CNBP is involved in wing development by promoting IRES-dependent translation of dMyc.

However, no CNBP has been investigated in other arthropod systems. In an effort to isolate immune-related genes in *Procambarus clarkii*, a mRNA coding a partial zinc finger containing protein was found to be up-regulated during the response to white spot syndrome virus (WSSV) infection (Zeng and Lu 2009).

The red swamp crayfish *P. clarkii* represents an attractive animal model because of its tolerance to extreme environmental conditions and resistance to diseases (Barbaresi and Gherardi 2000). Thus, it has become an important crustacean model organism for virological and innate immune system studies (Chen et al. 2008).

It is well known that invertebrates lack adaptive immune systems homologous to those of vertebrates; thus, the innate immune response mainly relies on Toll, IMD and JAK/STAT pathways (Li and Xiang 2013).

The Toll pathway is usually responsible for Gram-positive bacteria, viruses and fungi challenges (Lemaitre et al. 1996; Rutschmann et al. 2002; Zamboni et al. 2005), whereas the IMD pathway is involved in defense against Gram-negative bacteria and virus infection (Avadhanula et al. 2009; Lemaitre et al. 1995). Additionally, the JAK/STAT pathway plays a role in antiviral defense (Dostert et al. 2005; Souza-Neto et al. 2009) and STAT has also been reported to serve as a transcription factor for the WSSV immediate early gene (*ie1*) (Liu et al. 2007).

In the last decades, the main components of Toll and NF- κ B, IMD and JAK/STAT pathways were identified in penaeids including shrimp, prawn and crayfish. Moreover, the pivotal roles of these pathways in innate immunity have been reviewed by several authors (Li and Xiang 2013; Tassanakajon et al. 2013; Wang and Wang 2013).

Although significant differences exist between vertebrates and invertebrates, it has been proposed that the immune signal pathways might be evolutionarily conserved from some invertebrates to vertebrates (Chen et al. 2008; Dostert et al. 2005; Ghosh et al. 1998; Souza-Neto et al. 2009). Additionally, it

was found that the mechanisms involved in the antimicrobial immunity of insects are similar to those required for the activation of NF- κ B in mammals (Silverman and Maniatis 2001).

It has been shown that nucleic acid mimics as poly(I:C) and CpG DNA could induce Toll-pathway activation in shrimp, suggesting the existence of mechanisms equivalent to those of vertebrates (Wang et al. 2013; Deepika et al. 2014). In addition, haemocytes from prawn are able to bind CpG DNA, as ODN2006 and os-ODN13, probably using different cellular surface receptors, since they differently affected immune system parameters (Sung et al. 2009).

Thus, the discovery of immune-related molecules has assumed increasing importance in unveil the fine mechanisms regulating such system.

Recently, a large transcriptome dataset (>55,000 non-redundant transcripts) of *P. clarkii* has been deposited (Jiang et al. 2014; Shen et al. 2014), thus allowing to perform computational analysis.

In order to create new insights and identify factors which may exert a role in immune mechanisms of the red swamp crayfish, an effort was made to reconstruct the mRNA sequence for the previously reported partial zinc finger containing protein in *P. clarkii*. The complete open-reading frame (ORF) for a protein containing seven tandem CCHC-type zinc fingers, arginine/glycine-rich domain (RGG), nuclear localization sequence and PKA phosphorylation site was recovered and herein designated as *PcCNBP*.

We further investigate the gene expression pattern of *PcCNBP*. Analyses of transcriptional expression profile showed that *PcCNBP* was constitutively expressed among different tissues of the adult crayfish, under normal physiological conditions. Moreover, qRT-PCR assays indicate that the transcriptional expression of *PcCNBP* responds to bacterial and viral stimulations, suggesting its involvement in immune response of *P. clarkii*.

Materials and methods

GenBank accession numbers

The *PcCNBP* partial sequence was obtained from the *P. clarkii* EST and transcriptome database (Jiang et al. 2014) available at the National Centre for Biotechnology Information (NCBI) under the following accession numbers: CD644773, SRR1144631.110557.2 and SRR1144631.289100.2.

Animals and tissue sampling

Healthy, intermolt *P. clarkii* (15–20 g) were collected from Gorghetti Tondi in the South of Sicily and maintained in Millipore Filtered Water (MFW) at ~18 °C for 2 weeks before treatments and artificially fed twice a day.

Haemocytes were collected from the dorsal sinus between the first and second abdominal segments using a 2-ml syringe fitted with a 23-gauge needle and mixed with equal volume of anticoagulant solution (10 % sodium citrate, pH 7). Haemocytes were isolated by centrifugation at 800g for 10 min at 4 °C. Crayfish tissues used in this study were dissected out at the same time and preserved in RNAlater (Life Technologies, San Diego, CA) until RNA extraction.

RNA isolation and cDNA synthesis

Total RNA was extracted from eyestalk, muscle, cuticular epidermis, gills, haemocytes, stomach, hepatopancreas and intestine tissues using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's recommendations. RNA concentrations and quality were verified by spectrophotometry (optical density (OD) at 260 nm), whereas the RNA integrity was checked using a 1.5 % agarose gel. The RNA was stored at -80 °C for future use. The extracted RNA (2 µg) was treated with RNA qualified 1 (RQ1) RNase-Free DNase (Promega, Madison, WI, USA) to remove any residual genomic DNA contamination, and the DNase was inactivated by adding 25 mM EDTA. First-strand cDNA was synthesised from 2 µg DNase-treated total RNA samples using oligo(dT)₁₈ and Superscript III (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's instructions. The cDNA mixture was stored at -20 °C.

Profiling the *PcCNBP* Tissue-Specific Expression Pattern

Based on the partial sequence of *PcCNBP* cDNA, the 3' end were obtained by PCR-RACE using the SMART RACE cDNA application kit (Clontech, USA) and the 3'*PcCNBP* primer (Table 2) as described in the user manual. The products were cloned into the pGEM-T Easy vector (Promega, USA) and transformed into DH10B *E. coli* cells (Promega, USA). Plasmid DNAs were recovered using the QIAprep Spin Miniprep Kit (QIAGEN, Japan) and sequenced using T7 and SP6 primers.

A pair of primers, *PcCNBP*-F and *PcCNBP*-R (Table 1), was used to profile the tissue specific mRNA expression of *PcCNBP*. PCR amplifications were performed using Platinum Taq DNA Polymerase (Invitrogen Corporation, Carlsbad, CA, USA) under the following conditions: pre-incubation at 95 °C for 2 min; 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s; and a final extension at 72 °C for 2 min. The amplified products were analysed on a 1.5 % agarose gel and subcloned into the *pGEM-T Easy vector* (Promega, USA), and the nucleotide sequences were verified using T7 primers.

Table 1 Specific primers used in this study and GenBank accession numbers

| | | |
|-----------------|------------------------|----------|
| <i>18S_F</i> | GCAATAACAGGTCTGTGATGCC | KR135172 |
| <i>18S_R</i> | AGGGACGTAATCAGCGCAA | |
| <i>EIF_F</i> | GGAATAAGGGGACGAAGACC | KR135170 |
| <i>EIF_R</i> | GCAAACACACGCTGGGAT | |
| <i>PcPO_F</i> | GCACAAGTTTGTGGACGACGTC | EF595973 |
| <i>PcPO_R</i> | GTCCATCTCAGCCCAGAGGAG | |
| <i>PcCNBP_F</i> | CCATCTGCTCAACCTCTGTTTG | CD644773 |
| <i>PcCNBP_R</i> | GGCACATTCTCGTGATTGTC | |
| <i>3'PcCNBP</i> | TCATCGAAAGGAAGAGACATG | CD644773 |

Sequence and phylogenetic analyses

Functional sites and domains in the predicted amino acid sequences were predicted using the Simple Modular Architecture Research Tool (SMART) program, the InterPro database, the Pfam database, the PROSITE program and the Eukaryotic Linear Motif resource (ELM) for Functional Sites in Proteins.

To reconstruct the evolutionary diversification and the molecular evolution of the multifunctional gene family of CNBP, we explored the variety of CNBP proteins in the GenBank protein database for crustaceans and for different Eumetazoan groups (Table 2) deriving a phylogenetic tree representing their relationships.

Basic Local Alignment Search Tool (TBLASTN, BLASTP) analyses were performed to recover proteins from GenBank on the basis of the presence of the consensus CCHC type zinc finger domain.

Phylogenetic and molecular evolutionary analyses were conducted using a neighbour joining (NJ) method as implemented in MEGA vers 6.0 (Tamura et al. 2013); interior branch test was used to assess confidence probability. The evolutionary distances were computed using the Equal Input method and are in the units of the number of amino acid substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons. All ambiguous positions were removed for each sequence pair.

LPS, Poly I:C and CpG ODN stimulations

Polyinosinic-polycytidylic acid (Poly I:C), unmethylated phosphorothioate CpG ODN-2006 (5'-TCGTCGTTTTGTCGTTTTGTGCGTT-3') and os-ODN13 (5'-TCGTCGTCGTGTCGTCGTCGTT-3') were synthesised by Invitrogen (San Diego, CA). LPS from *E. coli* 0111:B4 (SantaCruz Biotechnology), Poly I:C and CpG ODNs were dissolved in sterile Hank's Balanced Salt Solution (HBSS, pH 7.4) at concentrations of 1 µg/µl and 100 µl of each immunogens were injected via a 27G needle into the last abdominal segment of crayfishes. Crayfishes injected with 100 µl of HBSS were used as the control. Experimental and control groups were maintained at

Table 2 CNBPs from Eumetazoans used for phylogenetic Analysis

| Species | Accession number | Taxonomic group |
|--------------------------------------|---------------------------|-----------------|
| <i>Homo sapiens</i> | 187608726 | Mammalia |
| <i>Pongo abelii</i> | 197099834 | Mammalia |
| <i>Pan troglodytes</i> | 525344633 | Mammalia |
| <i>Mus musculus</i> | 24658883 | Mammalia |
| <i>Rattus norvegicus</i> | 12018264 | Mammalia |
| <i>Gallus gallus</i> | 45382487 | Aves |
| <i>Columba livia</i> | 449266322 | Aves |
| <i>Danio rerio</i> | 347921635 | Osteichtyes |
| <i>Salmo salar</i> | 221220712 | Osteichtyes |
| <i>Larimichthys crocea</i> | 808868651 | Osteichtyes |
| <i>Poecilia reticulata</i> | 658859226 | Osteichtyes |
| <i>Oreochromis niloticus</i> | 542253513 | Osteichtyes |
| <i>Crotalus adamanteus</i> | 818939259 | Reptilia |
| <i>Xenopus laevis</i> | 147899284 | Amphibia |
| <i>Rhinella arenarum</i> | 4929293 | Amphibia |
| <i>Xenopus laevis</i> | 147899970 | Amphibia s |
| <i>Xenopus Silurana</i> | 56118324 | Amphibia |
| <i>Callorhynchus milii</i> | 392881264 | Chondrichthyes |
| <i>Strongylocentrotus purpuratus</i> | 780103311 | Echinodermata |
| <i>Drosophila melanogaster</i> | 24658883 | Insecta |
| <i>Harpegnathos saltator</i> | 307198051 | Insecta |
| <i>Camponotus floridanus</i> | 307183595 | Insecta |
| <i>Lasius niger</i> | 861635689 | Insecta |
| <i>Lygus hesperus</i> | 732572523 | Insecta |
| <i>Vollenhovia emeryi</i> | 795010946 | Insecta |
| <i>Ixodes scapularis</i> | 215495320 | Insecta |
| <i>Daphnia magna</i> | 942318345 | Crustacea |
| <i>Limulus polyphemus CNBP X2</i> | 926627088 | Crustacea |
| <i>Limulus polyphemus CNBP X1</i> | 926627086 | Crustacea |
| <i>Macrobrachium rosenbergii</i> | SRR2082769.14458.2 | Crustacea |
| <i>Macrobrachium nipponense</i> | SRR496753.3277.2 | Crustacea |
| <i>Litopenaeus vannamei</i> | 171613412 | Crustacea |
| <i>Portunus trituberculatus</i> | SRR479039.228076.2 | Crustacea |
| <i>Crassostrea gigas</i> | 762126464 | Bivalvia |
| <i>Hydra vulgaris</i> | 221114884 | Cnidaria |
| <i>Nematostella vectensis</i> | 156369956 | Cnidaria |
| <i>Amphimedon queenslandica</i> | 340378247 | Porifera |
| <i>Schistosoma japonicum</i> | 226475522 | Trematoda |

18 °C for 24 h in MFW. Three experimental crayfishes for each treatment and three control crayfishes were collected at 0, 0.5, 1, 2, 4, 6 and 24 h post injection (hpi).

Bacterial infection

E. coli K12 MG1665 cells were harvested from logarithmic phase cultures, washed twice with HBSS pH7.4, heat inactivated and resuspended in the same buffer at

concentrations of 10^3 CFU/mL. Crayfishes were injected with 10 μ L of the inactivated bacterial suspension via a 27G needle into the last abdominal segment. Crayfishes injected with 10 μ L of HBSS served as the controls. Three experimental crayfishes and three control unchallenged crayfishes were collected at 0, 0.5, 1, 2, 4, 6 and 24 h post-injection (hpi).

Relative quantification using real-time quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed using the ABIPRISM 7500 System (Applied Biosystems, Forster City, USA) with Power Sybr Green as detection chemistry (Applied Biosystems, Forster City, USA). Genes specific primers used to determine target gene expression levels are listed in Table 1.

18S ribosomal RNA and *eif* were selected as control genes based on their expression stability in all tested conditions, and the normalisation factor was calculated using the GeNorm software (Vandesompele et al. 2002).

Serial dilutions of pooled cDNAs from both control and treated samples were prepared to determine the PCR efficiency of the target and reference genes (data not shown) and amplification efficiency ranged from 1.9 to 2.2. The PcCNBP-F and PcCNBP-R were also used for quantitative RT-PCR. All the primer sequences used in this study are listed in Table 2. A normalisation factor was calculated by GeNorm based on the expression level of these reference genes and used to quantify *PcCNBP* mRNA. Quantitative real-time PCR was conducted according to the manufacturer's recommended procedures, and every reaction was repeated in triplicate. The amplification conditions were the following: initial denaturation at 95 °C for 10 min and 40 cycles of 95 °C for 30 s and 60 °C for 50 s, followed by a melting curve from 60 to 95 °C. Amplicons were detected by agarose gel analysis after each PCR to confirm the amplification of the specific gene.

The expression of prophenoloxidase mRNA was used as a proxy for the engagement of the proPO system during the immune stimulations.

All data represented relative mRNA expressed as the mean \pm SD ($n = 3$). Significant differences between values of different treated groups and the reference control groups were determined by one-way ANOVA with Tukey's post-test.

Results

PcCNBP cDNA characterisation

Starting from three overlapped sequences (CD644773, SRR1144631.110557.2 and SRR1144631.289100.2) coding a putative ZNF protein found in *P. clarkii* EST and

SRA databases respectively, a specific primer targeting the position between 763 and 783 nt (numbering refers to the full length cDNA) was designed and used to isolate the 3'-end of the cDNA. The putative full-length cDNA was obtained by assembling the 3'RACE product with the original sequences.

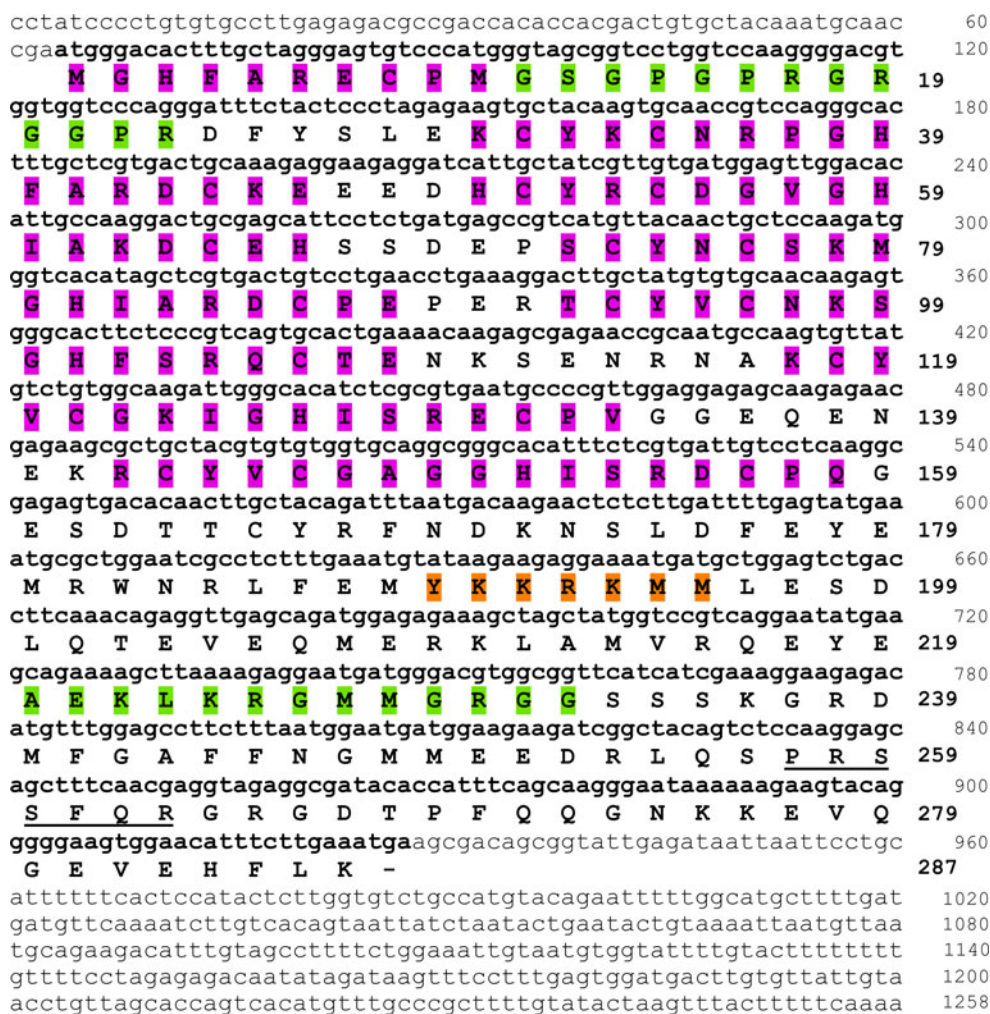
The full-length cDNA was 1257 bp with a 5'-untranslated region (UTR) of 63 bp and a 3'-UTR of 331 bp. An in-frame stop codon (TGA) and a Kozak consensus (CGA) are contained in the 5'-UTR upstream of the ATG start codon, whereas a stop codon (TAG), a classical polyadenylation signal sequence (AATATA) and a poly(A) tail were present in the 3'-UTR (Fig. 1). The cDNA contains an ORF of 864 bp corresponding to 287 amino acid residues. The predicted protein has an estimated molecular mass of 32997.99 Da, a theoretical pI of 7.78, and contains 7 tandem repeats of 14 amino acids containing the CCHC zinc finger consensus sequence (C ϕ XCGX₃HX₄C, where ϕ is an aromatic amino acid and X is variable) which represents a hallmark of the CNBP family (Fig. 2).

Analysis of the main structural features showed the presence of a glycine/arginine-rich region (RGG box) of 20 amino acids which separates the first two CCHC repeats each other and the occurrence of a second RGG box located nearby the C-terminal end (aa 230–232). These boxes are known to act by increasing the overall RNA affinity of proteins containing additional RNA-binding domain (Görlach et al. 1994).

Vertebrate CNBPs are modulated by mechanisms of phosphorylation by cAMP-dependent protein kinases. Computational searches identified several phosphorylation sites, including CK1(aa 161–167), CK2 (aa 64–70 and 199–205) and MAP Kinase such as P38 (aa 265–271), in addition to a conserved cAMP-dependent protein kinase (PKA) phosphorylation site located between residues 257 and 263 (Fig. 2).

Finally, a cluster of several basic amino acids residues (aa 189–195), representing a signal required for nuclear import (Dingwall and Laskey 1991; Kosugi et al. 2009) was also identified (Fig. 2). It may function

Fig. 1 Full-length cDNA and protein sequences of red swamp crayfish *PcCNBP*. The nucleotide and deduced amino acid sequence of the ORF, 5'- and 3'-UTRs were numbered on the right. The amino acids that constitute the zinc finger repeats are shaded in *violet*. The amino acids that constitute the RGG boxes are shaded in *green*, whereas the NLS is shaded in *orange*. The conserved PKA phosphorylation site is *underlined*



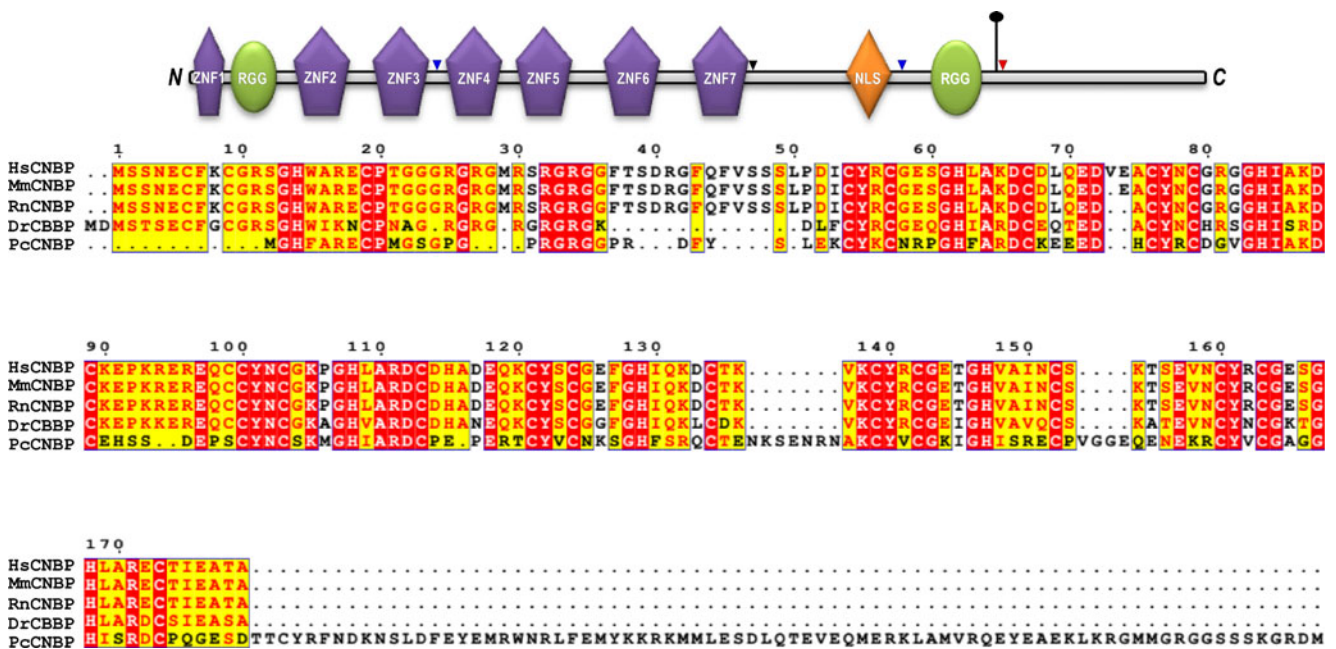


Fig. 2 Schematic diagram of PcCNBP and identification by protein alignment of seven tandem zinc finger repeats CCHC type. (On top) PcCNBP possesses seven zinc finger repeats in purple, two RGGs in green, a nuclear localization signal in orange and a conserved PKA phosphorylation site marked by lollipop. Additionally, red arrow indicates MAPK phosphorylation site, black arrow indicates CK1 ones, while CK2 phosphorylation site is marked by blue arrows. (At the

bottom) Sequence alignment of PcCNBP and homologues from human (HsCNBP), mouse (MmCNBP), rat (RnCNBP) and zebrafish (DrCNBP). Similar residues are written in black bold characters and boxed in yellow, whereas conserved residues are in white bold characters and boxed in red. The alignment was performed with T-coffe. The sequence numbering on top refers to the alignment

as monopartite nuclear targeting sequence in the predicted protein.

Phylogenetic analysis with other Eumetazoa

A Basic Local Alignment Search Tool (BLASTp) analysis indicated that PcCNBP shares moderate to high similarity with other single-stranded DNA-binding proteins isolated from other eumetazoans. Therefore, in order to gain further insights into protein conservation, multiple sequence alignment analyses were performed.

PcCNBP displayed the highest sequence similarity with single-stranded DNA-binding proteins from the crustacean group of arthropods including giant freshwater prawn *Macrobrachium rosenbergii*, oriental River Prawn *Macrobrachium nipponense* and whiteleg shrimp *Litopenaeus vannamei* (>90 % identity). PcCNBP also showed identity up to 70 % with protein from the crab *Portunus trituberculatus*.

Additionally, significant similarity (ranging from 42 to 54 % identity) was also shared with CNBP proteins from the remaining arthropods, cnidarian *Hydra*, nematode *Schistosoma japonicum*, echinoderms and from fishes, birds, reptiles, mammals.

The lowest similarities (31 to 40 % identity) were achieved comparing PcCNBP with DNA-binding proteins from porifera, starlet sea anemone *Nematostella*, mollusc

Crassostrea gigas, nematode *Necator americanus* and insects *Vollenhovia emeryi* and *Lygus hesperus*

To investigate the evolutionary relationships among the CNBP proteins, a phylogenetic tree was constructed using the PcCNBP and 39 protein sequences from different organisms (Fig. 3). Phylogenetic analysis displayed the presence of two well-supported branches of CNBP protein in which the main taxonomic group is distributed in a scattered manner showing a paraphyletic status.

The basal group includes lowest metazoans as porifera and molluscs in addition to nematodes and insects.

The second branch consists of a more derived group with vertebrates and out of this a not well-supported group including PcCNBP and the other proteins from crustaceans, cnidarians, the insect *Lasius niger*, echinoderms and the second copy of CNBP from *Xenopus laevis*.

Tissue-specific gene expression pattern

To analyse the tissue distribution and expression profiles of *PcCNBP* mRNA, total RNA was isolated from eight different tissues in unchallenged crayfishes and RT-PCR analysis were carried out. The *18S* transcript was used as an internal control. The *PcCNBP* transcript was detected in gills, cuticle, eyestalk, circulating haemocytes and in different parts of the digestive system (stomach, hepatopancreas and intestine) (Fig. 4).

Fig. 3 Neighbour-joining (NJ) bootstrap consensus tree based on *PcCNBP* and homologues from other Eumetazoa. The tree was generated using MEGA 6.0 including CNBP from different species ranging from mammals to porifera. All the sequences used were obtained from GenBank at NCBI and listed in Table 1. The evolutionary history was inferred using the neighbour-joining method. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (500 replicates is shown next to the branches). The evolutionary distances were computed using the Equal Input method and are in the units of the number of amino acid substitutions per site



To further confirm the RT-PCR expression patterns and evaluate the *PcCNBP* mRNA level in such tissues, qRT-PCR analysis were performed. The Ct values, obtained from qRT-PCR, spanned in a range from 24 to 31 (data not shown), suggesting a moderate to high level of constitutive expression when crayfishes were under normal physiological condition. Results were consistent with the expression patterns previously determined; in addition, *PcCNBP* mRNA was detected at highest level in circulating haemocytes, gills, hepatopancreas and intestine. Moderate level was detected in muscles and eyestalk, while the expression level cuticle and stomach was relatively low.

This result suggests that *PcCNBP* was ubiquitously and constitutively expressed in adult crayfishes under normal physiological conditions.

Expression profiles in response to biotic stresses

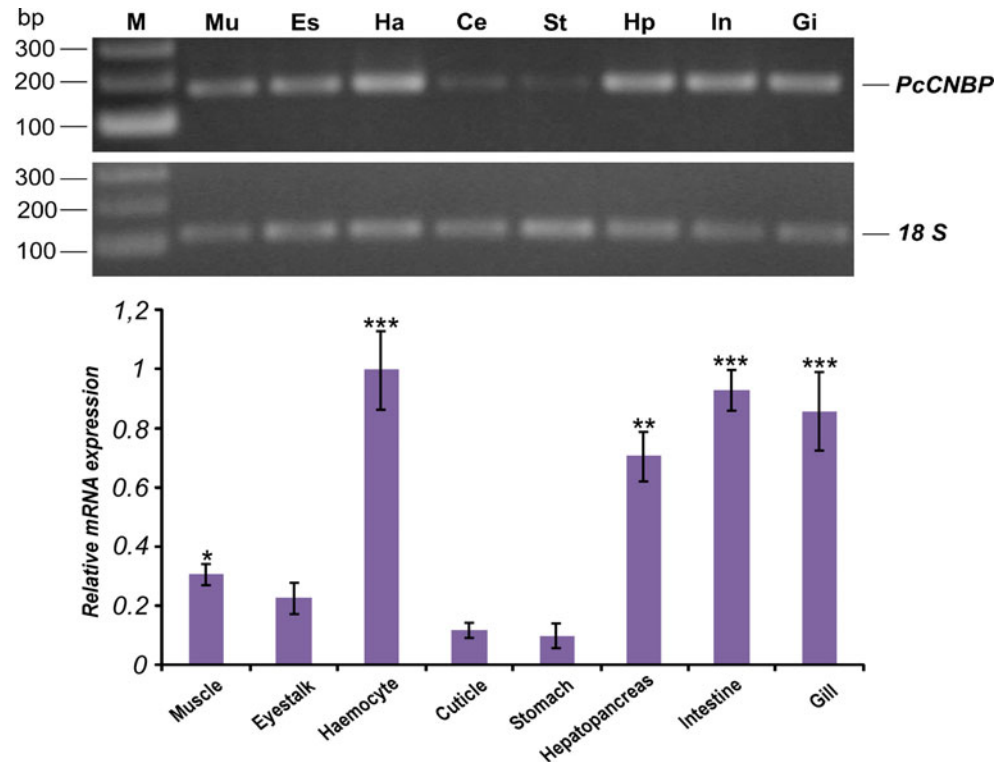
To validate the involvement in the immune response, the mRNA levels of *PcCNBP* were profiled in response to bacteria and virus challenge.

Therefore, Poly I:C and CpG ODN were used as viral and bacterial mimic agents respectively in addition to *E. coli* infection. Crayfishes were separately injected with defined immunogens, whereas HBSS injection was used as a control.

The haemocytes were selected as the target tissue because of their pivotal role in immune system (Soderhall et al. 2003; Hauton et al. 2005; Somboonwiwat et al. 2006; Ko et al. 2007) and the high expression level of *PcCNBP* herein measured.

Because the stimulation by bacterial or viral components up-regulates the expression of prophenoloxidase (*proPO*)

Fig. 4 Expression patterns of *PcCNBP* in various tissues and Relative mRNA expression. (*On top*) Total RNA was extracted, reverse-transcribed and amplified from different tissue of the crayfish: muscle (*Mu*), eyestalk (*Es*), haemocyte (*Ha*), cuticular epidermis (*Ce*), stomach (*St*), hepatopancreas (*Hp*), intestine (*In*) and gill (*Gi*) with 18S rRNA as control. (*At the bottom*) qRT-PCR-derived expression of *PcCNBP* transcript in muscle, eyestalk, cuticular epidermis, stomach, hepatopancreas, intestine and gill were normalised to that of haemocytes. The results are represented as means \pm SD ($n=3$). Statistical analysis by one-way ANOVA with Tukey's post-test. Data with different letters are statistically different: *** $p < 0.0001$; ** $p < 0.001$; * $p < 0.01$



mRNA in *P. clarkii* (Li and Xiang 2013), the *proPO* mRNA expression was herein used as a marker for monitoring the immune system activation.

The expression of *proPO* in haemocytes after immune stimulations is shown in Fig. 5. Despite a few differences in fold change amplitude, a comparison of *proPO* transcription in response to the different immune challenges reveals that the *proPO* expression displayed similar profiles in response to

Poly I:C, ODN-2006, *E. coli* and LPS challenges it increases during the experiments and remains higher than that of the control even at 24 h. This is consistent to those reported in challenged crayfishes (Li and Xiang 2013) and confirmed the status of immune system activation.

In our study, the exposure of crayfish to Poly I:C resulted in a rapid induction of *PcCNBP* transcript in *P. clarkii* haemocytes (Fig. 6), indeed it was 1.8-fold greater than the

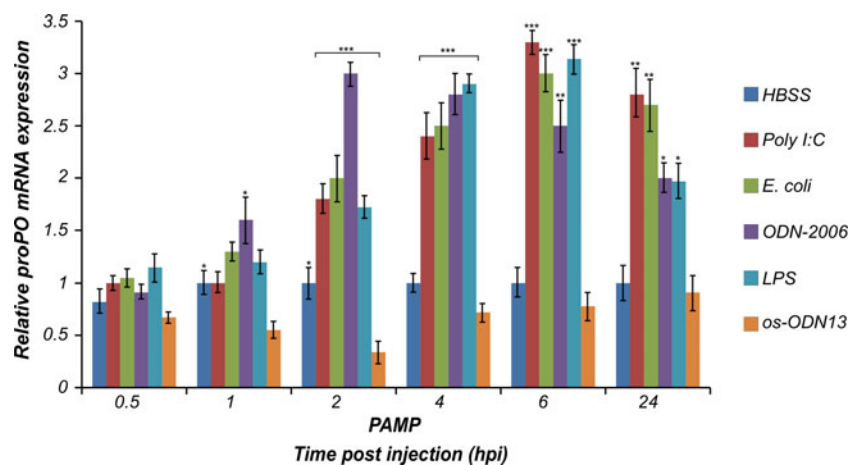


Fig. 5 Expression profiles of *proPO* in response to immune system challenges. Crayfish were challenged with Poly I:C, CpG-ODNs (ODN-2006 or os-ODN13), LPS and *E. coli* while HBSS injected crayfishes were used as control. The haemocytes were sampled at different time points post-challenge (0.5, 1, 2, 4, 6 and 24 h). The

expression level of *proPO* was analyzed by qRT-PCR with *18S* rRNA and *eif* as references. The results are represented as means \pm SD ($n=3$). Statistical analysis by one-way ANOVA with Tukey's post-test. Data with different letters are statistically different: *** $p < 0.0001$; ** $p < 0.001$; * $p < 0.01$

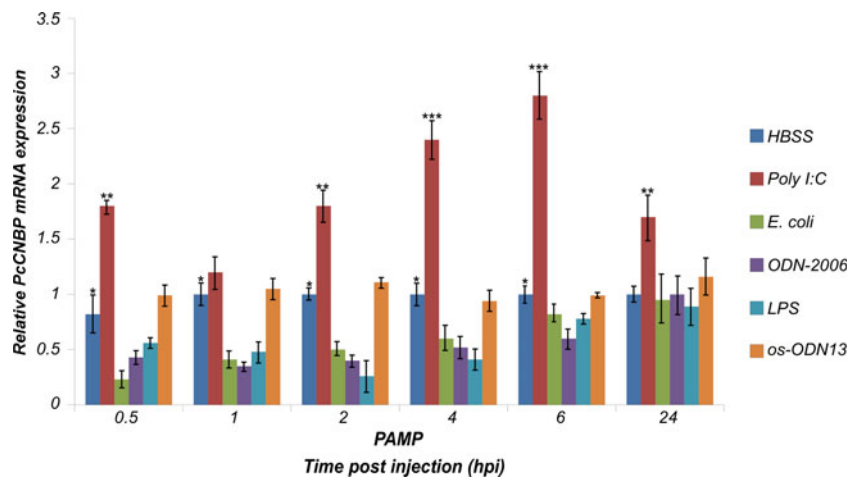


Fig. 6 Expression profiles of *PcCNBP* in response to immune system challenges. Crayfish were challenged with Poly I:C, CpG-ODNs (ODN-2006 or os-ODN13), LPS and *E. coli* while HBSS injected crayfishes were used as control. The haemocytes were sampled at different time points post-challenge (0.5, 1, 2, 4, 6 and 24 h). The expression level of

PcCNBP was analyzed by qRT-PCR with *18S* rRNA and *eif* as references. The results are represented as means \pm SD ($n = 3$). Statistical analysis by one-way ANOVA with Tukey's post-test. Data with different letters are statistically different: *** $p < 0.0001$; ** $p < 0.001$; * $p < 0.01$

control level within 30 min. The *PcCNBP* mRNA accumulated to a maximum level at 6 h (5.8-fold) and then declined. However, after 24 h, the expression was still higher than control crayfishes.

Being an aquatic animal, crayfishes are also exposed to a variety of bacteria that impair viability.

This prompted us to examine the amount of *PcCNBP* mRNA in crayfishes challenged with *E. coli*, LPS or ODN-2006 which was used as an additional bacterial elicitor of the innate immune response.

All the stimulations negatively affected the mRNA expression levels of *PcCNBP*.

After crayfishes were stimulated by *E. coli*, the relative expression of *PcCNBP* was quickly down-regulated to 2.8-fold lower than the control within 30 min (Fig. 5). Subsequently, it increased gradually and recovered to the regular level at 24 hpi. Similarly, after stimulation with LPS or CpG ODN-2006, *PcCNBP* mRNA expression was rapidly down-regulated at 30 min (1.8- and 2.4-fold lower than control, respectively) and negatively peaked 2.6-fold lower than the control group within 1 hpi in crayfishes challenged with CpG ODN-2006. Likewise, the *PcCNBP* transcript reduced to a minimum level at 2 h (3.8-fold lower than control) in LPS challenged crayfishes.

Later, transcript levels increased gradually and the physiological amount was restored at 24 hpi.

Moreover, the transcriptional changes of *PcCNBP* mRNA were better characterised using a different ODN (os-ODN 13) which has been reported to act via the NF- κ B signalling pathway and negatively affects the proPO and peroxinectin expression in the giant freshwater prawn *Macrobrachium rosenbergii* (Sung et al. 2009).

Herein, the *proPO* expression was down-regulated after os-ODN13 injection and recovered to the regular level at 24 hpi, whereas the *PcCNBP* mRNA level resulted unchanged throughout the experimental points.

Discussions

In the present study, a homolog of the transcriptional and translational regulator CNBP was identified in red swamp crayfishes *P. clarkii*, representative of the Crustacea subphylum in which the presence of this protein has not been investigated yet. The domain organisation of CNBPs, including *PcCNBP*, shows a high degree of conservation. *PcCNBP* shares moderate sequence identity also with vertebrate homologues. Homology relationships were also investigated, and the NJ tree structure is consistent with the presence of one CNBP copy per haploid genome in most vertebrates (Ruble and Foster 1998; Armas et al. 2004). Additionally, the two CNBP copies reported in *X. laevis* (De Dominicis et al. 2000) appear to be herein separated in the constructed branches (Fig. 3).

Noteworthy, no other CNBP proteins from invertebrates have been yet described, with the exception of *D. melanogaster*. Thus, data herein reported represent a first effort to comparatively analyse the evolutionary status of CNBP proteins among lower eumatazoans.

Analysis of *PcCNBP* protein sequences revealed a conserved modular structure characterised by the presence of 7 tandem repeats of the CCHC zinc finger motif, putative nuclear localization signal and conserved cAMP-dependent protein kinase (PKA) phosphorylation site. This suggests that the

protein retains the same molecular functions of the other proteins in different organisms.

These features indicated that PcCNBP represents both a novel member of CNBP homolog in *P. clarkii* and the first crustacean CNBP to have been characterised.

CNBP was found to bind single-stranded nucleic acid which promotes rearrangements of nucleic acid secondary structure in an ATP-independent manner (Armas et al. 2008; Borgognone et al. 2010). Zinc fingers and RGG motifs are responsible for the binding activity and contribute to achieving maximal activities (Borgognone et al. 2010).

In PcCNBP, two glycine/arginine-rich regions, highly similar to the RGG box of RNA-binding proteins, were identified. Thus, it is plausible that CNBP-dependent mechanisms of transcriptional and/or translational gene regulation may also occur in crustaceans.

It has been reported that *CNBP* mRNA is expressed at varying levels in different animal tissues. Thus, herein, tissue distribution analysis was carried out to unveil the *PcCNBP* expression pattern in adult crayfish. The *PcCNBP* transcripts widely express at different levels in all analysed tissues. This is consistent with previous works reporting an ubiquitous expression of *CNBP*-like transcripts. In adult chicken, *CNPB* mRNA was detected in heart, lung, liver, brain, cochlea and kidney (van Heumen et al. 1997). Similarly, *CNPB* transcripts are expressed at different levels in various normal rat tissues (Yasuda et al. 1995), while *CNPB* is expressed in ectodermal, endodermal and mesodermal germ layers of *X. laevis* (Flink et al. 1998).

It is plausible to suppose that PcCNBP may perform various function in crayfishes. In particular, *PcCNBP* exhibited high transcription levels in haemocytes, gills and hepatopancreas which are usually involved in immune system response.

In decapoda, mouth and intestine represent natural routes of infection for viruses and bacteria (Saulnier et al. 2000; Vodovar et al. 2004). Herein, we showed that *PcCNBP* transcript level in the intestine closely resembles the expression in the above-mentioned tissues. Therefore, the ubiquitous and constitutive transcriptional expression of *PcCNBP* under normal physiological conditions could serve as a general protective factor.

Crustacean stomach is lined by cuticle which acts as a physical defense barrier. Interestingly, *PcCNBP* mRNA was found to be expressed both in stomach and cuticular epidermis, although at lower levels. Therefore, it could be hypothesised that the observed mRNA expression is contributed by cells of both constituents present in the stomach.

Data focused on the involvement of CNBP as a part of immunity response are relatively limited. However, it has been reported that the transcript levels of a putative zinc finger protein, herein identified and named as *PcCNBP*, in *P. clarkii* was increased after the immune system was challenged via WSSV (Zeng and Lu 2009).

Since CNBP gene expression was found to be modulated by STAT 6 which plays a pivotal role in the immune system (Tuomela et al. 2009), fluctuations of *CNBP* mRNA level may correspond to immune system triggering.

This prompt us to evaluate the *PcCNBP* transcriptional profile in haemocytes with immune system engaged by bacterial and virus stimulations.

Interestingly, the expression profile of *PcCNBP* mRNA in haemocytes is strikingly different after *E. coli* or CpG-ODNs and Poly I:C stimulation. The mRNA expression of *PcCNBP* was significantly up-regulated within a few minutes from crayfishes Poly I:C stimulation; thus, it is reasonable to suppose that *PcCNBP* over-expression is dependent on viruses components or signalling pathways activated by virus challenge.

It has been shown that Epstein–Barr virus nuclear antigen 1 (EBNA-1) is responsible for the over-expression of CNBP mRNA, probably due to EBNA-1's ability to engage cellular promoters (Canaan et al. 2009).

Recently, it has been also reported that the CNBP homolog in *Drosophila* modulates the transcription of Wnt signalling pathway components which are involved in the regulation of virus phagocytosis (Margarit et al. 2014). Therefore, evidences for a PcCNBP involvement in defence mechanisms against viral infection are strongly suggested.

No study currently exists regarding the effects of bacterial infection on the CNBP system in arthropods. However, a previous study reported that *CNBP* mRNA levels increased in cultured chicken spleen and bursal cells after LPS stimulation (Ruble and Foster 1998).

The mRNA expression of *PcCNBP* was significantly down-regulated after both *E. coli* and CpG-ODN-2006 and LPS challenges within 1 h after stimulation. This might indicate that bacterial challenge could inhibit its expression. Because of the dual role of CNBP as transcriptional and translational regulator, a distinct role in different pathways may also be hypothesised for PcCNBP.

Interestingly, the os-ODN13 stimulation did not result in any detectable variation of *PcCNBP* mRNA level, while according to Sung et al. (2009) the *proPO* mRNA levels were significantly reduced.

It has been hypothesised that os-ODN13 and ODN-2006 may exert opposite effects on immune defense and os-ODN13 may be able to act via the NF- κ B signalling pathway (Sung et al. 2009). Thus, it is possible that other pathways, rather than NF- κ B signalling (e.g. STAT), regulate *PcCNBP* expression in *P. clarkii*.

Additionally, the return at physiological level of *PcCNBP* at 24 h post-stimulation could be due to binding of serum proteins to CpG-ODNs backbone (Meng et al. 2011) which may impair activation of TLR9-dependent signal transduction.

Taken together, our data indicate that PcCNBP is a homolog of the well-conserved family of nucleic acid binding

protein. It is modulated at transcriptional level by different immune challenges, suggesting its involvement in the immune response of *P. clarkii* during both bacteria and viruses infection. However, further studies need to be performed in order to assess the precise activity of PcCNBP in the immune system.

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