Fish and Shellfish Immunology 87 (2019) 178–183

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Characterization of small GTPase Rac1 and its interaction with PAK1 in crayfish *Procambarus clarkii*

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ARTICLE INFO

Keywords:
Procambarus clarkii
Rac1
Immune response
PAK1

ABSTRACT

Ras-related C3 botulinum toxin substrate 1 (Rac1) participates in many biological processes. In this study, a *Rac1* gene was identified in the crayfish *Procambarus clarkii* with an open reading frame of 579 bp that encoded 192 amino acids. This predicted 21.4 kDa protein was highly homologous to those in other invertebrates. Real-time PCR analysis revealed that *Pc-Rac1* was expressed in all examined tissues with the highest expression level in hemocytes. The transcriptional expression level of *Pc-Rac1* was significantly upregulated in hemocytes and hepatopancreas after lipopolysaccharide (LPS) or polyinosinic: polycytidylic acid (poly I: C) induction. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis suggested that a recombinant Pc-Rac1 protein was successfully expressed in *E. coli*. Far-western blot analysis demonstrated that Rac1 can interact with the PBD domain of p21-activated kinase 1 (PAK1). RNA interference of *Pc-Rac1* affected the mRNA expression levels of immune-related genes *lectin, Toll, crustin, TNF, ALF* and *cactus*. These results suggest that *Pc-Rac1* is involved in the innate immune responses in *P. clarkii*.

1. Introduction

The ras-related C3 botulinum toxin substrate 1 protein (Rac1) is a Rho family small GTPase and highly conserved structurally and functionally in almost all eukaryotes. As a key mediator, Rac1 regulates transport process, membrane trafficking [1,2], cell-cell adhesion and cell migration [3–7]. In vertebrates, Rac1 plays a critical role in many immune-related processes, including phagocytosis [8], inflammation [9,10] and host immune responses to extracellular stimuli [11–13]. Latest research reported that Rac1 participated in liver-mediated basal immune homeostasis and LPS-induced endotoxemia in mice [14]. Up to now, Rac1 has been reported in a few invertebrates such as fly [15,16], beet armyworm [17], nematode [18], shrimp [19,20] and sea cucumber [21]. In *Drosophila melanogaster,* Rac1 was required for integrin localization forward pathogens on hemocyte membranes [15] and participated in axonal outgrowth and myoblast fusion [16]. Moreover, Rac1 mediated natural memory decay and forgetting [22]. For *Caenorhabditis elegans*, Rac1 regulated cell-to-cell heterogeneity during epidermal morphogenesis [18]. It was also found that Rac1 triggered cytokine-stimulated hemocyte spreading in *Spodoptera exigua* [17]. In addition, the expression of Rac1 was upregulated by microorganism challenges [19–21] and Rac1 overexpression enhanced Egr-induced cell death [23]. These clues indicated that Rac1 had multiple functions in animals. However, little is known about its roles in crustaceans, especially in crayfish and crabs.

The crayfish *Procambarus clarkii* is an economically important cultured species in China. In the present study, the identification of *Pc-Rac1* and its expression patterns under various immune inductions were analyzed. In addition, Pc-Rac1 protein expression, interaction between Pc-Rac1 and PAK1, and the effects of *Pc-Rac1* RNAi on the expression of immune-related genes were also investigated. All data observed here will provide more clues for understanding the functions of Rac1 in innate immunity.

2. Materials and methods

2.1. Experimental animals

Healthy crayfish with body length of 5–6 cm and body weight 8–10 g were collected from the Hupo market in Hefei. These crayfish were kept in a 12 h-light and 12 h-dark photoperiod at room temperature and fed small invertebrates. Carbon dioxide was used to euthanize the crayfish following procedures in agreement with the principles for the care and use of animals [24].

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https://doi.org/10.1016/j.fsi.2019.01.013
Received 19 November 2018; Received in revised form 4 January 2019; Accepted 8 January 2019
Available online 09 January 2019

Table 1
Summary of primers used in this study.

Name	Primer sequences (5′-3′)
Rac1-1	CGGGATCCATGCAAGCCATTAAG
Rac1-2	CCCTCGAGTTAAAGGAGGGTACA
PBD-1	CGCGGATCCTGCATGGAAGAAGGACATATCG
PBD-2	CCGCTCGAGGAAGTCCTTGAAGGGCGGCGAC
Rac1-F	CCTAACACACCCATCATCTTG
Rac1-R	TAATAGGACACAGCACAGCAC
Lectin-F	TGCTCCTGGTGGTAGTGGT
Lectin-R	GTAGAGGTGTTTGGGTGTGG
Toll-F	GGGTTGAAGTTGGATGTGGG
Toll-R	TCTTGAGACACTGGACCTGC
Crustin-F	GGTATGGAGGTCGAGACAGG
Crustin-R	GCCGGGCTGTAACTACTACT
TNF-F	CACCTTTCATCCCCTTTCCAT
TNF-R	AATGCAGATGATAAAGCCCG
ALF-F	TTTGGCCATAGTGCGCATAC
ALF-R	TGCAGGGAGACACTACAAGG
Cactus-F	GAGATTCGAGCCGGAGAGAA
Cactus-R	GTGCCCCGGTGTTATTGATC
18S-F	CTGTGATGCCCTTAGATGTT
18S-R	GCGAGGGGTAGAACATCCAA
dsRNA-Rac1-F	**GGATCCTAATACGACTCACTATAGG**
	TGGAGCCGTGGGTAAGAC
dsRNA-Rac1-R	**GGATCCTAATACGACTCACTATAGG**
	ACTTCGTGGCACCAACCT
dsRNA-EGFP-F	**GGATCCTAATACGACTCACTATAGG**
	CAGTGCTTCAGCCGCTACCC
dsRNA-EGFP-F	**GGATCCTAATACGACTCACTATAGG**
	ACTCCAGCAGGACCATGTGAT

Note: Restriction sites are underlined, T7 promoters are in bold and underlined.

2.2. Cloning and sequencing of Pc-Rac1

Total RNA was extracted from hemocytes of *P. clarkii* using Trizol reagent (Takara, China). RNA quality was assessed by 1% agarose gel electrophoresis. The cDNA was synthesized using PrimeScript™ RT Master Mix (Takara, China) according to the instruction. Specific primers (Table 1) were designed to clone the open reading frame (ORF) of the *Pc-Rac1*. The PCR reaction was performed as 1 cycle of denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, extended at 72 °C for 10 min. PCR products were cloned into the PMD-19T vector (Takara) and transformed into competent cell *Escherichia coli* (DH5α) for sequencing.

2.3. Sequence analysis of Pc-Rac1

Sequences of *Pc-Rac1* were analyzed by NCBI BLAST (https://blast.ncbi.nlm.nih.gov). The molecular weight of the protein was calculated using the Compute PI/Mw tool (https://web.expasy.org/compute_pi/). Multiple sequence alignments were performed using the Clustal X program [25] with its default parameters. A neighbor-joining phylogenetic tree was constructed by MEGA software (version 5.0) [26] based on the Rac1 sequences from different species.

2.4. Protein expression, purification and antibody preparation

The PMD-19T vector plasmid containing the *Pc-Rac1* ORF was digested with restriction enzymes *Bam* H I and *Xho* I, then ligated into PET-32a (+) vector (Novagen, USA), finally transformed into *E. coli* Rosetta (DE3). The expression of the recombinant Pc-Rac1 protein was induced by Isopropyl-β-D-thiogalactopyranoside (IPTG) for 20 h at 16 °C. The recombinant proteins were purified using the Express Ni-NTA Fast Start Kit (Qiagen, Germany) according to the instructions. The recombinant fusion protein was analyzed by 12% SDS-PAGE. Quantification of the recombinant protein was performed using the bicinchoninic acid (BCA) method [27]. The antiserum was prepared according to previously described standard procedures [28]. Briefly, purified fusion proteins were homogenized in Freund's complete adjuvant and used to immunize male New Zealand rabbits twice at two-week intervals. The antiserum was collected after the third immunization boost.

2.5. Real-time PCR

Hemocytes, gill, heart, stomach, intestine, muscle and hepatopancreas were dissected for the analysis of tissue distribution. To investigate the expression of *Pc-Rac1* in response to lipopolysaccharide (LPS) or polyinosinic: polycytidylic acid (poly I:C) challenge, crayfish were randomly divided into three groups of 21 individuals per group. LPS (10 μL, 1 μg/μL) or poly I:C (10 μL, 1 μg/μL) was respectively injected into each crayfish. The PBS injection was used as the negative control group. Hemocytes, hepatopancreas and intestine were collected from crayfish 3, 6, 9, 12, 24, 48 and 72 h after injection. All specific primers (Table 1) were designed for real-time PCR based on known sequences. *P. clarkii* 18S rRNA gene (GenBank accession no. AF436001) was used as internal control. The real-time PCR reactions were performed in a total volume 20 μL comprising 10 μL of 2 × TransStrart®Tip qPCR SuperMix (Trans), 1 μL of cDNA template, 1 μL each of Rac1-F and Rac1-R, and 7 μL of ultrapure water. The mixtures were carried out the following procedure: 94 °C for 30 s; 40 cycles of 94 °C for 5 s, 55 °C for 15 s and 72 °C for 10 s, followed by a melting curve analysis at 65 °C-95 °C. The relative expression of *Pc-Rac1* was calculated by the $2^{-\triangle\triangle CT}$ method [29]. Before using the comparative C_T method, we had verified that the PCR efficiency of reference and target genes was approximately equal. All the experiments were repeated three times. All data were assessed using Student's *t*-test or one-way analysis of variance (SPSS software version 18.0) and represented as the means ± standard error (SE). Differences were considered significant when the P-value was less than 0.05.

2.6. dsRNA synthesis and Pc-Rac1 RNAi analysis

The dsRNA sequences for *Pc-Rac1* and EGFP (enhanced green fluorescent protein) were synthesized with T7 RioMAX Express RNAi System (Promega, USA) according to the manufacturer's protocol. Briefly, the DNA templates from in vitro single-strand RNA transcription were amplified by PCR with specific primers (Table 1) and then the single-stranded RNA was annealed to generate dsRNA. After purification, the dsRNA concentration was measured by absorbance at 260 nm and dsRNA quality was checked by 1% agarose gel electrophoresis. The experimental crayfish were injected with Pc-Rac1 dsRNA (10 μg, 1 μg/μL) while the control groups were injected with EGFP dsRNA. Hemocytes were collected from per treatment group at 24 h and 48 h after injection. The total RNA was isolated and used for the expression analysis of *Rac1*, *lectin*, *Toll*, *crustin*, *TNF*, *ALF* (anti-lipopolysaccharide factor) and *cactus* using real-time PCR.

2.7. Far-western blotting

To investigate the activity of recombinant Rac1, the interaction between Rac1 and PAK1 protein was determined. The PBD domain of PAK1 gene was cloned using specific primers PBD-1 and PBD-2 (Table 1). The DNA fragment was ligated into vector PGEX 4T-1 after digestion with restriction enzymes (*Bam* H I and *Xho* I), and the resulting plasmid was transformed into *E. coli* Rosetta (DE3) for protein expression. PBD was purified using the GST Fusion Protein Purification Kit (GenScript, China). Purified PBD was used as the prey protein, and Rac1 as the bait protein. Purified Rac1 and PBD were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. One of the membranes was incubated with purified Rac1 protein and then incubated with the anti-Rac1 antibody after PBST washing. The membrane was then washed with PBST, and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG

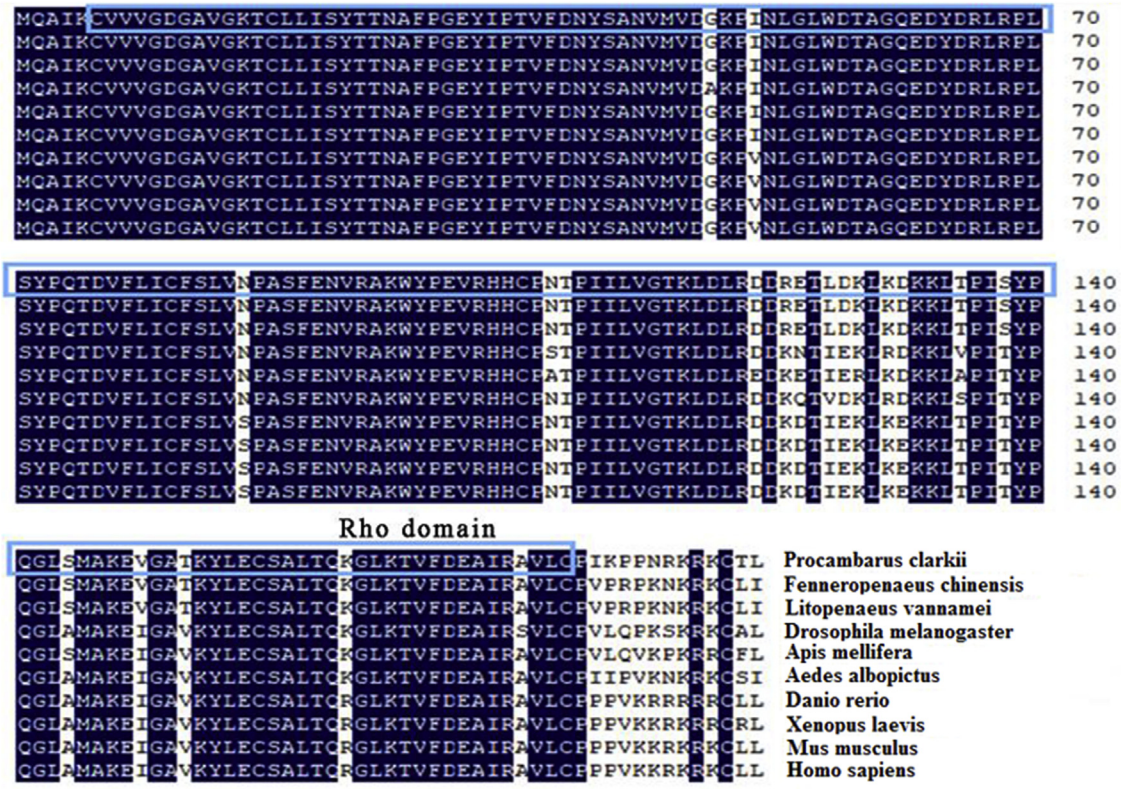

Fig. 1. Alignment of Pc-Rac1 with Rac proteins from other animals. Similar and identical amino acids are marked in gray and black, respectively. The conserved Rho domain (6-178aa) is boxed.

(TransGen, Beijing, China) diluted 1:5000 with 5% non-fat milk in PBST (PBS containing 0.1% Tween-20) for 1 h at room temperature. The protein bands were detected using an HRP-DAB Detection Kit (Sangon, Shanghai, China). Bovine serum albumin (BSA) was used as the negative control [30]. The other membrane was incubated with an anti-Rac1 antibody, without the incubation with purified Rac1 protein, as a standard western blot.

3. Results

3.1. Cloning and sequence analysis of Pc-Rac1

An open reading frame of 579 bp encoding Pc-Rac1 was obtained by PCR and it encodes 192 amino acid residues containing a conserved Rho domain (6-179aa) (Fig. 1). The molecular weight and theoretical isoelectric point of the protein are 21.4 kDa and 8.62, respectively. The homology analysis showed that the known Rac1 proteins can be divided into invertebrates and vertebrates (Fig. S1). The deduced amino acid sequence of Pc-Rac1 showed 96% similarity to that from *Fenneropenus chinensis,* 92% similarity to that of *Litopenaeus vannamei* and about 90% similarity to other Rac1 proteins from invertebrates, suggesting that Rac1 is conservative in evolutionary relationships.

3.2. Tissue distribution of Pc-Rac1

The expression profile of the *Pc-Rac1* gene in different tissues was analyzed at the transcriptional level by real-time PCR. Pc-Rac1 expression was detected in all the examined tissues including hepatopancreas, gill, intestine, heart, stomach, muscle and hemocytes. The highest expression level of *Pc-Rac1* was found in hemocytes and lowest in hepatopancreas (Fig. 2).

3.3. Induced expression pattern of Pc-Rac1 in various tissues

To understand the function of *Rac1* in the immune process of *P. clarkii*, the induced expression profiles of *Pc-Rac1* in hemocytes, hepatopancreas and intestine under LPS and poly I: C challenges were explored. In hemocytes, the mRNA expression level of *Pc-Rac1* was significantly upregulated from 3 to 72 h after LPS and poly I: C induction (Fig. 3 A and B). In hepatopancreas, *Pc-Rac1* was upregulated from 3 to 72 h after LPS challenge. The expression level of *Pc-Rac1* did not change remarkably after poly I:C injection compared with PBS-injection from 3 to 24 h, but it was strongly upregulated from 48 to 72 h (Fig. 3C and D). In intestine, the *Pc-Rac1* transcription level was upregulated from 3 to 72 h following LPS stimulation. At 12 h post-injection of poly I: C, the expression level of *Pc-Rac1* in intestine was significantly upregulated; after which it decreased, but was still significantly higher than the level in the control group (Fig. 3E and F). These results indicated that *Pc-Rac1* expression was significantly induced by different pathogen-associated molecular patterns, suggesting it has a role in immune response.

3.4. Protein expression and western blot

SDS-PAGE revealed that recombinant Pc-Rac1 protein was successfully expressed in *E. coli* and its expression was not influenced by IPTG concentration (Fig. 4A). The Pc-Rac1 protein was visualized as a single band after purified using the Express Ni-NTA Fast Start Kit (Fig. 4B). SDS-PAGE and western blot analysis showed the band with a size at 26 kDa (Fig. 4C). These results showed that recombinant Pc-Rac1 protein was obtained successfully through the prokaryotic expression system.

3.5. Protein-protein interaction between Rac1 and PAK1

Far western blot was carried out to investigate the interaction

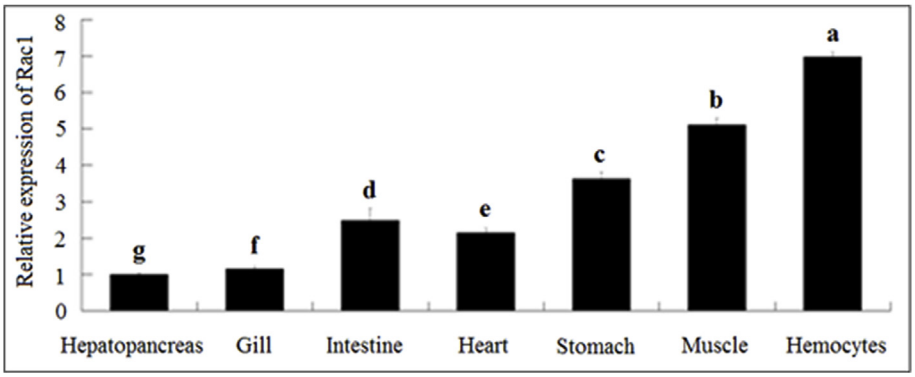

Fig. 2. Expression level of *Pc-Rac1* in various tissues. The expression of *Pc-Rac1* was determined by real-time PCR. The data were analyzed by one-way ANOVA and were presented as the mean ± SE of independent experiments performed in triplicate. Bars labeled with different letters are statistically significantly different ($p < 0.05$).

between Rac1 and PAK1, the vital downstream mediator of Rac1. PBD domain of PAK1 protein was expressed in *E. coli* and Glutathione Sepharose 4B chromatography was used to purify the recombinant protein (Fig. S2). GST-PBD was used as the prey protein, and His-Rac1 as the bait protein. Only the bait protein Rac1 could be observed in the standard control western blot (Fig. 5A). Both the bait and prey proteins were detected after incubation with the bait protein Rac1 in the far-western blot (Fig. 5B). These results confirmed that Rac1 can interact with the PBD domain of PAK1 directly in vitro.

3.6. Effect of Pc-Rac1 RNAi on immune-related genes

To determine the effects of *Rac1* RNAi on the mRNA expression level of several immune-related genes in hemocytes, dsPc-Rac1 was injected into healthy crayfish. The group injected with dsEGFP was used as a control. As shown in Fig. 6A, the mRNA expression level of *Pc-Rac1* in hemocytes significantly declined 24 and 48 h after dsRNA injection, which was consistent with the protein level determined by western blotting. Real-time PCR was performed to detect the expression of immune related genes *lectin* (GenBank accession number MK284235), *Toll* (GenBank accession number MF185747), *crustin*

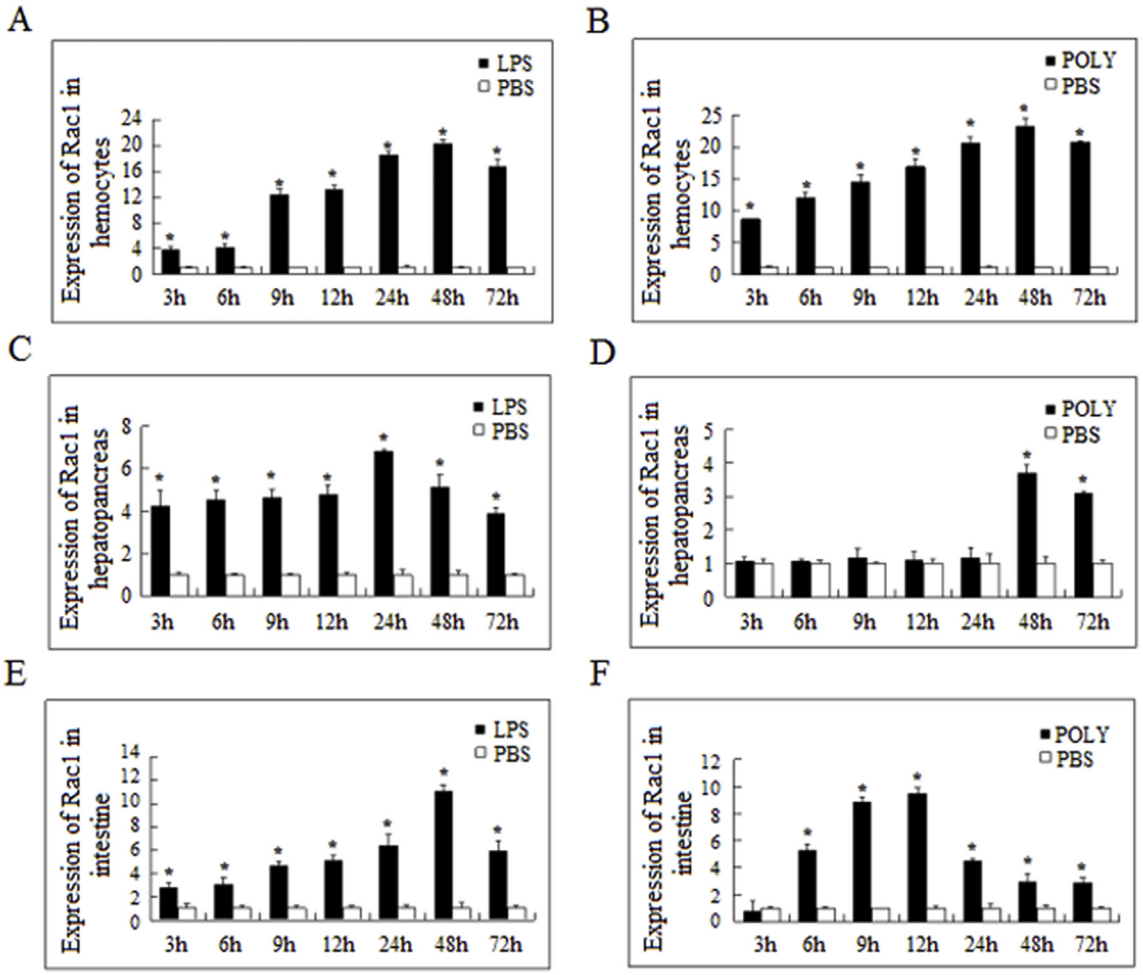

Fig. 3. Expression profiles of Pc-Rac1 in hemocytes (A and B), hepatopancreas (C and D) and intestine (E and F) after LPS and poly I:C challenges. The tissues were collected 3, 6, 9, 12, 24, 48 and 72 h post-injection. The *Pc-Rac1* mRNA level in the PBS-injected group was designated as the calibrator. Bars represent mean ± S.E. ($n = 3$) and asterisks represent significant difference ($P < 0.05$) between the control and experimental groups.

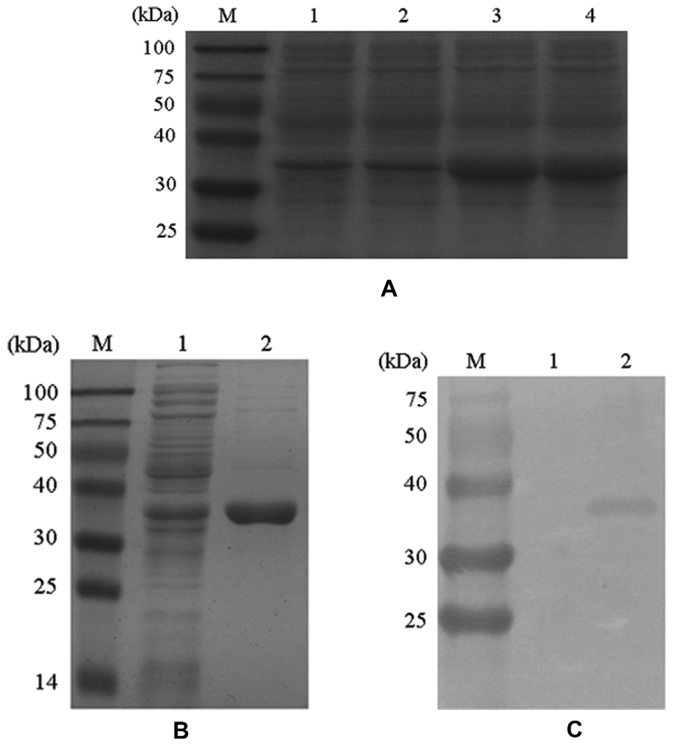

Fig. 4. Pc-Rac1 protein expression in *E coli* cells induced by IPTG and protein purification. (A) Lane 1: crude extracts of *E. coli* transformed with pET-32(a+) vector; Lane 2: no IPTG induction; Lane 3: induction by 0.5 mM IPTG; Lane 4: induction by 1 mM IPTG. (B) Lane 1: induction by 0.5 mM IPTG; Lane 2: purified recombinant Pc-Rac1 protein. **(C)** Western blotting of recombinant protein using anti His-tag antibody. Lane 1, untransformed *E. coli* BL21 (DE3); Lane 2, after induction by 1.0 mM IPTG. M: protein marker.

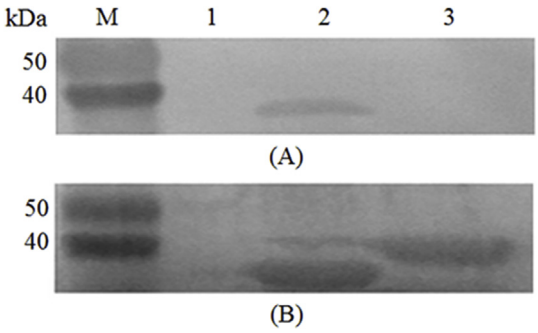

Fig. 5. The protein-protein interaction between Pc-Rac1 and PAK1. (A) Standard Western blot. (B) Far-western blotting of Rac1 and PAK1. Lane 1: BSA (bovine serum albumin) control; Lane 2: purified Pc-Rac1 protein; Lane 3: purified PAK1 protein.

(GenBank accession number MF185748), *ALF* (GenBank accession number MF185749) and *cactus* (GenBank accession number MF185745). Compared with the control, the expression level of *Toll* was significantly upregulated, while the expression levels of *TNF, ALF, lectin* and *cactus* were obviously downregulated. However, no significant changes in the expression level of *crustin* were found (Fig. 6B).

4. Discussion

In mammals, Rac1 GTPase makes impact on a variety of cellular processes such as phagocytosis, apoptosis and inflammation [31,32]. Here, a Rac1 gene was identified in crayfish *P. clarkii* and it encoded a 21.4 kDa protein with a conserved Rho domain. The 3D structure diagram of Pc-Rac1 was made up of six β-strands, six α-helixs and an Mg^{2+}

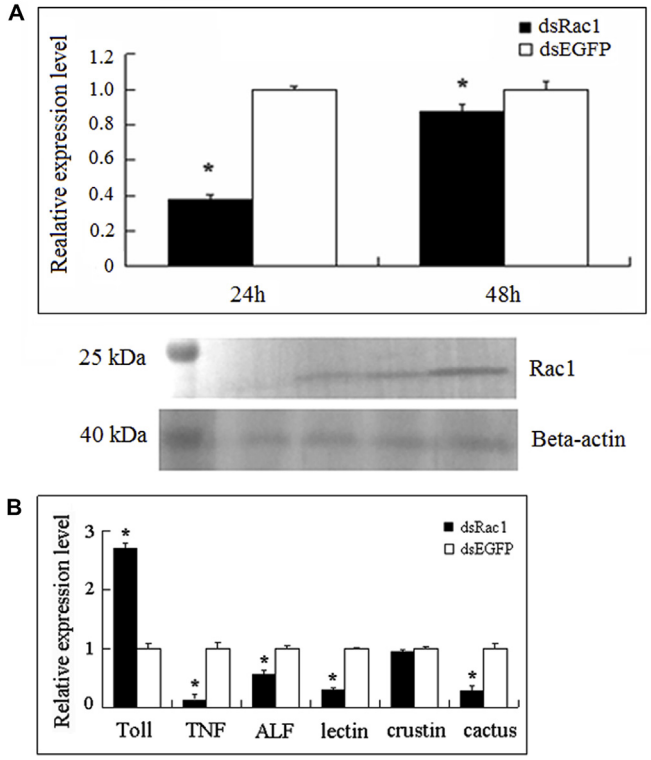

Fig. 6. Effects of Pc-Rac1 RNAi on immune-related genes expression. (A) RNA interference of the Pc-Rac1 gene in hemocytes. The relative expression of Pc-Rac1 was detected 24 and 48 h after injection of dsRNA. The dsEGFP-injected group was used as a control. (B) Expression of immune-related genes in hemocytes after Pc-Rac1 RNAi. Bars represent mean ± S.E. (n = 3) and asterisks indicate significant differences ($P < 0.05$).

ligand (Fig. S3). The amino acid sequence of Pc-Rac1 has higher identity with Rac1 proteins invertebrates than those in vertebrates, suggesting that Pc-Rac1 may have the similar biological functions to the former.

Real-time PCR analysis indicated that *Pc-Rac1* was expressed in all tissues examined, suggesting *Pc-Rac1* may be involved in diverse biological processes. Compared with the other tissues tested, the mRNA level of *Pc-Rac1* was higher in hemocytes, which was similar to that in *L. vannamei* and *F. chinensis* [19,20]. It was known that hemocytes removed invading pathogens by phagocytosis in crustaceans [33]. The high expression level of *Pc-Rac1* in hemocytes was likely related to its function in immunity.

In order to investigate the role of Rac1 in the immune response of *P. clarkii*, the expression patterns of *Pc-Rac1* in hemocytes, hepatopancreas and intestine were detected after immune challenges. Generally, *Pc-Rac1* expression in these three tissues was significantly upregulated following LPS and poly I: C challenges. The expression patterns varied in different tissues and *Pc-Rac1* was more sensitive to LPS challenge than to poly I:C. Compared with the other examined tissues, the relative expression of *Pc-Rac1* changed remarkably in hemocytes.

The interaction between PAK1 and Rac1 was confirmed by far-western blotting in this experiment. PAK1 was a well-known effector of Rho GTPase and its kinase activity was increased after binding with active Rac [34,35]. It was reported that PAK1 promoted NF-κB activity through suppression of PPARγ in intestinal inflammation [36] and played a role in p53-mediated apoptosis [37]. Meanwhile, Rac1 controlled an IκB-independent pathway to NF-κB activation via Toll-like receptor 2 (TLR2) [38,39]. Our results indicated that RNA interference of the Rac1 gene affected the mRNA expression of immune-related genes like *Toll*, *TNF* and *Cactus*. These clues showed the role of *Pc-Rac1* in innate immunity.

In conclusion, Pc-Rac1 shares conserved domains of Rac proteins in other animals and is involved in immune processes of *P. clarkii*. However, further studies are needed to investigate the Rac1-mediated signaling pathways and the alternative functions of *Pc-Rac1*.

Conflicts of interest

The authors have no conflict of interest to declare.

Acknowledgements

This research was supported by the Natural Science Foundations of Anhui Province of China [1508085MC52] and the Natural Science Foundations of Anhui Higher Education Institutions of China [KJ2017A145].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.01.013.

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