Fish and Shellfish Immunology 95 (2019) 259–267

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Rab7 controls innate immunity by regulating phagocytosis and antimicrobial peptide expression in Chinese mitten crab

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ARTICLE INFO

Keywords:
Antimicrobial peptides
Eriocheir sinensis
Phagocytosis
Rab7
Toll signaling

ABSTRACT

The Rab family is the most significant subfamily of small GTP-binding proteins. These proteins have widespread intracellular localization and play an important role in many biological processes. *Rab7* plays a crucial role in the innate immune system of crustaceans. In the present study, we cloned and characterized *Rab7* from Chinese mitten crab (*Eriocheir sinensis*), designated *EsRab7*. The full-length of the *EsRab7* cDNA sequence is 1,257 bp and contains a 618-bp open reading frame encoding a 205-amino acid polypeptide. Bioinformatics analysis showed that the *Rab7* protein was highly conserved during evolution. Quantitative real-time PCR showed the highest tissue expression in muscle, followed by hepatopancreas. *EsRab7* was significantly upregulated in hemocytes after stimulation by Gram-positive *Staphylococcus aureus* or Gram-negative *Vibrio parahaemolyticus*. Further studies showed that *EsRab7* knockdown during bacterial stimulation resulted in decreased bacterial phagocytosis. In addition, *EsRab7* regulated the expression of antimicrobial peptides via the Toll signaling pathway. Collectively, these results demonstrate that *EsRab7* plays critical roles in antimicrobial function in the Chinese mitten crab.

1. Introduction

Chinese mitten crab (*Eriocheir sinensis*) is an important economic aquatic species found widely in Southeast Asia and cultivated in China [1]. Due to its widespread distribution, the environment to which these crabs are exposed is quite complex. Especially in large-scale farming environments, it is threatened by a large number of diseases, resulting in low yield and serious economic losses [2,3]. Therefore, research on *E. sinensis* immunity is particularly important.

The Ras superfamily of small GTP-binding proteins comprises the Rab, Rho, Ras, Ran and Arf subfamilies [4]. The most important of these is the Rab subfamily, which has broad intracellular localization and plays an important role in vesicle formation, transport, adhesion, anchoring and fusion [5]. The Rab family has a number of members which have been divided into *Rab1, Rab3, Rab4, Rab5, Rab6, Rab8, Rab11, Rab22, Rab27* and *Rab40* according to distinct subfamily-specific sequence motifs; however, many Rab family members remain to be classified [5]. As GTPases, Rab proteins are characterized by a cyclical mechanism of activation and inactivation depending on GTP-binding and hydrolysis [6]. Rab GTPase has a wide range of functions, and numerous genetic and biochemical studies have shown that Rab GTPases act as modulators of specific intracellular trafficking pathways [7].

The *Rab7* protein plays critical roles in the endocytic processes and regulates phagocytosis by interacting with upstream regulatory factors and downstream effectors [8]. In addition, *Rab7* also participates in multiple regulatory mechanisms in endosomal sorting and lysosomal biogenesis [9]. For instance, Rab7 is essential for the phagosomes present in *Leishmania* and *Salmonella* to fuse with late endosomes and lysosomes in macrophages [10,11]. Moreover, *Rab7* plays an momentous role in antiviral actions by regulate phagocytosis, inhibition of *PmRab7* could be a novel approach to prevent both DNA and RNA virus infection of shrimp [12]. Collective data demonstrated the significant roles of *Rab7* in the phagosome formation and maturation, as well as pathogens clearance in innate immunity. Nevertheless, the research of *Rab7* in crustacean immunity were mainly focused on antiviral immunity, the antibacterial activity of *Rab7* remains largely unclear.

Recently, an increasing number of studies have confirmed that Rab proteins play a significant role in the immune system. For instance, the expression of *Rab11* and *Rab7*, but not *Rab5*, is downregulated during *T. cruzi* infection in cardiomyocytes [13]. Stimulation of macrophages with *M. smegmatis* and *M. avium* resulted in upregulated expression of *Rab20*, *Rab34* and *Rab10*, which are small GTPases regulated at the

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https://doi.org/10.1016/j.fsi.2019.10.037
Received 25 June 2019; Received in revised form 3 September 2019; Accepted 20 October 2019
Available online 23 October 2019

transcriptional level in an NF-κB dependent manner. These findings indicate that Rab proteins play a role in innate immunity [14]. *LcRab5* is involved in the immune response of the large yellow croaker and improves the inflammatory response through activation of *TNF-α* and *IL-6* expression [15]. Furthermore, *Rab14* shown to mediate phagocytosis in the immune response to *S. aureus* in *Drosophila melanogaster* [16].

In addition to its role in vertebrate and insect immunity, immune functions of Rab family proteins in crustaceans are under investigation. For example, expression of *EsRab3, EsRab5* was significantly upregulated when the crab were challenged with *V. anguillarum* [17]. In *Penaeus monodon*, suppression of *PmRab11* at both the mRNA and protein levels either before or after YHV-challenge resulted in significant inhibition of YHV levels in the hemocytes and viral release in the supernatant, suggesting that *PmRab11* is required for YHV infection in shrimp [18]. Nevertheless, the specific function of *Rab7* protein of *E. sinensis* in immunity is still unclear.

In this study, we cloned and characterized *EsRab7* and investigated its role in antibacterial immunity in Chinese mitten crabs. Results of tissue distribution revealed that *EsRab7* mRNA expression were widely observed in all the tested tissues, especially high expression levels were found in muscle and hepatopancreas. Additionally, we investigated the expression patterns of *EsRab7* following pathogen attack, RNA interference of *EsRab7* reduced the phagocytosis rate of crab hemocytes and inhibited the expression level of AMP genes via the Toll pathway. Therefore, the *Rab7* gene is closely related to antibacterial immune responses in *E. sinensis*.

2. Material and methods

2.1. Experimental animals and sample preparation

Healthy adult Chinese mitten crabs (*E. sinensis*; n = 160; wet weight = 100 ± 15 g) were purchased from a local agricultural market in Shanghai, China. Crabs were maintained at a constant temperature (20–25 °C) and acclimated in filtered, aerated freshwater for 1 week before experiments. For RNA extraction, hemocytes were collected from hemocoel in the arthrodial membrane of the last two pairs of walking legs of each crabs using a 10 ml sterile syringe containing 5 ml of anticoagulant solution (0.1 M glucose, 30 mM citrate, 26 mM citric acid, 0.14 M NaCl, and 10 mM ethylenediaminetetraacetic acid) [19]. The supernatant was discarded after centrifugation (800 ×*g* at 4 °C for 10 min), and the hemocytes were stored at −80 °C. Other tissues including stomach, gill, intestine, brain, heart, hepatopancreas and muscle were collected, frozen immediately in liquid nitrogen, and stored at −80 °C.

2.2. Bacterial challenge and sample collection

The Gram-positive bacterium *Staphylococcus aureus* (BYK0113) and the Gram-negative bacterium *Vibrio parahaemolyticus* (BYK00036) were obtained from the National Pathogen Collection Center for Aquatic Animals (Shanghai Ocean University, Shanghai, China). Before the immune challenge, bacterial stains were cultured in Luria–Bertani medium (120 rpm, 37 °C) and collected by centrifugation at 5,000 ×*g* for 5 min. Bacteria were then washed (3×), resuspended in phosphate-buffered saline (PBS) and adjusted to 1×10^8 colony forming units (CFU)/ml. The CFU counts of bacteria were determined by plating the diluted suspension onto agar plates. Crabs (n = 150) were then randomly divided into three groups: Gram-positive bacterium (*S. aureus*), Gram-negative bacterium (*V. parahaemolyticus*) and control group and 200 μL of the corresponding bacterial suspension was injected into the hemolymph from the non-sclerotized membrane of the posterior walking leg; the control group received 200 μL of PBS. More than five crabs in each group were selected randomly at specific time-points [0 (blank control), 2, 4, 8, 12, 24 and 48 h] after bacterial challenge and hemocytes samples were collected as described in section 2.1. Primary hemocytes were cultured using previously described methods [20].

2.3. Total RNA extraction and full-length cDNA cloning of EsRab7

Total RNA was extracted from *E. sinensis* using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The concentration and quality of total RNA were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and 1% agarose gel electrophoresis. For quantitative real-time RT-PCR (qRT-PCR), total RNA (4 μg) was reverse transcribed using the PrimeScript™ Real-time PCR Kit (TaKaRa, Shiga, Japan). For full-length cloning, total RNA (5 μg) was reverse transcribed using the SMARTer rapid amplification of cDNA ends (RACE) cDNA Amplification kit (Clontech, Shiga, Japan).

The partial cDNA sequence of *EsRab7* in the form of expressed sequence tags (EST) was obtained from a hemocyte cDNA library of Chinese mitten crab (unpublished). Full-length *EsRab7* cDNA was cloned using RACE technology (SMARTer™ RACE cDNA Amplification Kit, Clontech) with gene specific primers for 3′- and 5′- RACE designed based on the *EsRab7* EST and synthesized by RuiMian (Shanghai, China) (Table 1). The PCR product was constructed according to the manufacturer's protocol, then purified and sequenced by Invitrogen (Shanghai, China) and submitted to NCBI under accession number MK285363.

2.4. Bioinformatic analysis

The ExPASy Translate tool (http://web.expasy.org/translate/) was used to translate the nucleotide sequence, and the obtained protein sequences were compared against other sequences in the NCBI database using the online search tool BLASTX (http://www.ncbi.nlm.nih.gov/). ORF finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) was used to identify the open reading frames (ORF) of *EsRab7*. CD-search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was used to predict the GTP/Mg^{2+} site. The isoelectric point and molecular weight were calculated by ExPASy Compute pI/Mw tool (http://web.expasy.org/compute_pi/). Signal P 4.1 Server (http://www.cbs.dtu.dk/services/

Table 1
Sequences of primers used for *EsRab7* analysis.

Primer name and purpose	Sequence (5′-3′)
cDNA cloning	
EsRab7-5GSP1	TGCTTTGCCTTGTTGTCGTTGG
EsRab7-5GSP2	GGGCGACGCTTGGATTAGGAAC
EsRab7-3GSP1	TCATCATCCTTGGTGACTCGGGTGT
EsRab7-3GSP2	GACCCTGACCACTTCCCATTCG
Quantitative real-time PCR	
β-actin qF	GCATCCACGAGACCACTTACA
β-actin qR	CTCCTGCTTGCTGATCCACATC
EsRab7 qF	CAGTCACTCGGTGTTGCCTTCT
EsRab7 qR	TGGGCGACGCTTGGATTAG
ALF1 qF	GACGCAGGAGGATGCTAAC
ALF1 qR	TGATGGCAGATGAAGGACAC
ALF2 qF	GACCCTTTGCTGAATGCTTGA
ALF2 qR	CTGCTCTACAATGTCGCCTGA
ALF3 qF	ACGAGGAGCAAGGAAAGAAAG
ALF3 qR	TTGTGCCATAGACCAGAGACTT
Crus-1 qF	GCTCTATGGCGGAGGATGTCA
Crus-1 qR	CGGGCTTCAGACCCACTTTAC
Crus-2 qF	GCCCACCTCCCAAACCTAT
Crus-2 qR	GCAAGCGTCACAGCAGCACT
DWD1 qF	CAGTGTGTCCTGACCCGAAC
DWD1 qR	AAGCAGCACTTTTGGGTC
LYS qF	CTGGGATGATGTGGAGAAGTGC
LYS qR	TTATTCGGTGTGTTATGAGGGGT
RNA interference	
si*EsRab7* F	GACCCUGACCACUUCCCAUTT
si*EsRab7* R	AUGGGAAGUGGUCAGGGUCTT

SignalP/) was used to predict the presence of signal peptides, and SMART (http://smart.emblheidelberg.de/) was used to predict the structure and function of domains. TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict transmembrane regions. SWISS-MODEL (http://swissmodel.expasy.org/) was used to construct the three-dimensional models of *EsRab7*. Multiple sequence alignment of *EsRab7* was performed using the ClustalX 2.0 program and DNAMAN software. Phylogenetic trees were constructed using the neighbor-joining (NJ) method in MEGA 6 software. The amino acid sequences of *Rab7* from different species were searched using the BLASTp program in the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/) for multiple sequence alignment and phylogenetic analysis.

2.5. Quantitative real-time PCR (qRT-PCR) analysis of EsRab7 expression

Total RNA from various tissues and hemocytes was extracted using the TRIzol® reagent (RNA Extraction Kit, Invitrogen, Carlsbad, CA, USA) and first-strand cDNA was synthesized. Specific primers for *EsRab7* (*EsRab7* qF and *EsRab7* qR; Table 1) and β-actin [21] (β-actin qF and β-actin qR; Table 1) were used for qRT-PCR. The mRNA expression of *EsRab7* was analyzed by qRT-PCR using a QuantStudio 5 Real-Time PCR System (96-well, 0.2 mL block)* (Applied Biosystems). TB Green™ Premix Ex Taq™ (TaKaRa, Japan) was used to perform real-time PCR according to the following protocol: 95 °C for 30 s; and 39 cycles at 95 °C for 5 s and 58.5 °C for 30 s; followed by 95 °C for 10 s and a 0.5 °C/5s incremental increase from 65 °C to 95 °C. The results were analyzed using QuantStudio Design & Analysis Software, and quantified using the comparative CT method ($2^{-\Delta\Delta Ct}$ method) based on Ct values for *EsRab7* and β-actin [22]. All data used in the analysis were obtained from three independent experiments.

2.6. EsRab7 RNA interference (RNAi)

The *EsRab7*-specific si RNA and targeting green fluorescent protein (GFP; control) used in RNAi experiments were synthesized by GenePharma (Shanghai, China). The primer sequences used are listed in Table 1. *E. sinensis* primary hemocytes isolated from healthy crabs as described in section 2.1 were transfected with siRNA using the siRNA-Mate reagent (GenePharma, China) at a final concentration of 10 nM according to the manufacturer's instructions. At 24 h post-siRNA transfection, total RNA was extracted to evaluate the RNAi efficiency by qRT-PCR and semi-quantitative RT-PCR.

2.7. Antimicrobial peptide (AMP) gene expression after RNAi-mediated silencing of EsRab7

For analysis of AMP gene expression, RNAi-mediated silencing of *EsRab7* was performed as described in section 2.6. *E. sinensis* hemocytes were challenged by *S. aureus* or V. *parahaemolyticus* for 24 h before extraction and reverse transcription of total RNA as described in section 2.5. Hemocytes were transfected with siGFP and prior to bacterial stimulation as a negative control (NC), and an equal volume of medium was added to the blank control group. Using the primers listed in Table 1, expression of the following AMP genes was analyzed by qRT-PCR: *Es*ALF1 [23] (ALF1 qF and ALF1 qR), *Es*ALF2 [24] (ALF2 qF and ALF2 qR), *Es*ALF3 [25] (ALF3 qF and ALF3 qR), Crus1 [26] (Crus-1 qF and Crus-1 qR) and CrusEs2 [27] (Crus-2 qF and Crus-2 qR), Lys [28] (Lys qF and Lys qR), EsDWD1 [29] (DWD1 qF and DWD1 qR). All experiments were performed in triplicate.

2.8. Fluorescent labeling of bacteria and phagocytosis assay

Phagocytosis assays were performed according to previously reported methods [30,31]. Overnight-cultured *S. aureus* or *V. parahaemolyticus* were heat-killed (70 °C, 30 min), washed with PBS and then resuspended in 0.1 M $NaHCO_3$ containing 0.5 mg/ml FITC (Sigma, St. Louis, MO, USA). After incubation for 2 h at 37 °C, the bacteria were pelleted and washed three times with PBS by centrifugation until no dissociated FITC was visible and the suspension was adjusted to 1×10^9 microbes/ml.

Hemocytes were isolated from healthy crabs and cultured according to previously described methods [20]. The hemocytes were counted using an automated cell counter (Invitrogen Countess), seeded at 6×10^5 onto a 24 × 24 mM cell climbing slice (WHB, Shanghai, China) and cultured for 12 h in 6-well plates in vitro. Subsequently, interference and/or bacterial stimulation of the cultured cells was performed according to our experimental requirements with the addition of 10 μL FITC-labeled bacteria (1×10^9 CFU/ml) per well. The phagocytosis assay was performed 1 h after bacterial challenge. The cells were washed (2×) with PBS (4 °C) and stained with trypan blue (2 mg/ml) for 20 min to quench the non-phagocytosed bacteria. The hemocytes were then washed (3×) with PBS, and stained with DAPI for 1 min before observation under a fluorescence microscope (Echo Revolve FL, USA). Five hundred hemocytes were counted for each sample, and samples were obtained from three crab. The phagocytosis rate was calculated as the ratio of the number of phagocytic cells containing fluorescent bacteria to the total number of cells.

The phagocytic rate was also analyzed by flow cytometry according to the following method: 1×10^6 hemocytes were mixed with 1×10^7 FITC-labeled microbes in a total volume of 200 μL of mPBS and incubated for 40 min in the dark at 25 °C. The hemocytes were then isolated by centrifugation at 600 ×*g* for 8 min, washed, and resuspended in 200 μL of mPBS before the addition of 4 μL of 0.5% trypan blue to quench extracellular fluorescence. A total of 10,000 cells were counted for each sample. All experiments were performed in triplicate.

2.9. Immunofluorescence detection of nuclear factor translocation

The primary culture of crab hemocytes and preparation of cell climbing slices was performed as described in section 2.7. The treated cells were washed (3×) with PBS (Solarbio, Beijing) for 3 min, fixed with 4% paraformaldehyde for 15 min, and washed again (3×) with PBS. The cells were then permeabilized with 0.5% Triton X-100 in PBS for 10 min, washed with PBS and blocked with 3% BSA for 2 h before incubation with primary detection antibodies (antibodies produced in our laboratory using recombinant protein) at a dilution of 1:200 in 3% BSA overnight at 4 °C. The next day, the cells were washed (3×) with PBST (0.2% Tween 20 in PBS), before incubation with the FITC-conjugated secondary detection antibodies (at a dilution of 1:200 in PBST; CWBIO, Beijing) for 2 h at 37 °C. The cells were then washed (3×) with PBST and stained with DAPI (Beyotime, China) for 5 min. After a final wash (3×) in PBST, fluorescent signals in the cell climbing slices were observed under the fluorescence microscope (Echo Revolve).

2.10. Statistical analysis

SPSS software (ver. 11.0) and GraphPad Prism 5 were used for statistical analysis, and statistical significance was calculated by one-way ANOVA and post-hoc Duncan's multiple range tests. The error bars represented standard deviation (SD). Significance was set at $p < 0.05$.

3. Results

3.1. Gene cloning and bioinformatics analysis of EsRab7

The cloned full-length *EsRab7* cDNA contains 1,257 bp, with a 618-bp ORF encoding a 205-amino acid protein, a 72-bp 5′ untranslated region (UTR), a 567-bp 3′ UTR and a typical poly(A) tail. The GTP/Mg^{2+} binding sites were highly conserved in the amino acid sequences of *EsRab7* (Fig. 1). The protein was predicted to have a molecular weight of 23.25 kDa and an isoelectric point of 5.78. No signal peptides

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                                                        GCGCCAGAC 9
GCCATATTGATACCACCGAGGGGAGACCTCCTGTAGTTTTCGTCGTGTTTTCCGCCGCCCAGA 72
ATGGCATCCAGAAAGAAGATACTGCTGAAGGTCATCATCCTTGGTGACTCGGGTGTGGGCAAG 135
 M  A  S  R  K  K  I  L  L  K  V  I  I  L  G  D  S  G  V  G  K  21
ACGTCGCTCATGAACCAGTTTGTCAACAAGAAGTTCAGCAACCAGTACAAGGCTACCATCGGT 198
 T  S  L  M  N  Q  F  V  N  K  K  F  S  N  Q  Y  K  A  T  I  G  42
GCTGACTTTCTCACCAAAGAAGTGATGGTGGATGACAGACTGGTCACAATGCAGATATGGGAC 261
 A  D  F  L  T  K  E  V  M  V  D  D  R  L  V  T  M  Q  I  W  D  63
ACTGCTGGCCAGGAGAGATTCCAGTCACTCGGTGTTGCCTTCTACCGCGGGGCAGACTGCTGT 324
 T  A  G  Q  E  R  F  Q  S  L  G  V  A  F  Y  R  G  A  D  C  C  84
GTCTTGTGCTATGATGTCACTTCTCCCAACTCCTTCAAGTCACTAGACTCCTGGAGGGACGAG 387
 V  L  C  Y  D  V  T  S  P  N  S  F  K  S  L  D  S  W  R  D  E  105
TTCCTAATCCAAGCGTCGCCCAGAGACCCTGACCACTTCCCATTCGTTGTCCTCGGAAACAAG 450
 F  L  I  Q  A  S  P  R  D  P  D  H  F  P  F  V  V  L  G  N  K  126
ATTGACCTGGAAAATAGAGCGGTGTCTACAAAGCGAGCACAGCAGTGGTGTCACAGTAAAAAT 513
 I  D  L  E  N  R  A  V  S  T  K  R  A  Q  Q  W  C  H  S  K  N  147
GAGGTTCCCTACTTTGAGACCAGTGCAAAGGAGGCCATAAACGTGGAGTTGGCCTTCCAGACG 576
 E  V  P  Y  F  E  T  S  A  K  E  A  I  N  V  E  L  A  F  Q  T  168
ATCGCCCGCAACGCCTTGGCCCAGGAGAATGAGGTAGAGCTGTACAATGAGTTCCCCGACCAG 639
 I  A  R  N  A  L  A  Q  E  N  E  V  E  L  Y  N  E  F  P  D  Q  189
ATCAAGCTGACCAACGACAACAAGGCAAAGCAAGAGGCCTGTTCCTGCTAATGATCACTGTAA 702
 I  K  L  T  N  D  N  K  A  K  Q  E  A  C  S  C  *                205
TGATTTTCTTAGTTGATAAGACAGAATCCCTCTGTCCTACTTGTGATATTCGAGAAGCGAGCG 765
TGGGAGAAGAGTTGCAGTCCAAGGCCTGGCCCAAGAGCAACGTTTGAGTGGTCAAAGTCTTGT 828
ACATTTCAGTTTTTTTATATATCTATATATTTGTAGTTCCCCACCTGAAAATTTGCAAACATA 891
AATTCCTATCATATCAAGGGTGGCATAAATAGATTTAGGGAAGGACTTTACAGATTTATACAT 954
ACAGATTATTAAGGCTTAAATTTTTAAGGAAGAACGCTCTTATTGATCCCTCAAATGGTGGGT 1017
TAGGTGGGTTGACCAAGCTCTTGCAGAATCATAGCCTGACTGGCAACCTTGGTAGACCCAGCG 1080
TGTGTATAGCATTCGGGTGTTATCACTTCTTGGTTTCACAATGTGCAGATCAAAGAATTATTG 1143
GTATTCATGTTTGTTAGCATGTTATAATATCGAGGAAAAGTGTTGATTTTGTTAGTAAAAAAC 1206
AAACAATTTATAAATTTACTCATAATGCTAAAAAAAAAAAAAAAAAAAAAA              1257
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Fig. 1. Nucleotide and deduced amino acid sequences of *EsRab7*. The amino acid sequence is shown in blue under the cDNA sequence, with the RAB domain shaded in dark gray. The conserved GTP/Mg^{2+} binding sites are marked in bold italic, while letters representing start codons (ATG) and stop codons (TAG) are shown in bold non-italic. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

were predicted by the Signal P software. The amino acid residues 9 to 176 of the *EsRab7* protein were predicted as the RAB domain by the SMART software. No transmembrane regions were predicted by the TMHMM server (Fig. 2A and B). A three-dimensional model of *EsRab7* was constructed using the online SWISS-MODEL software (Fig. 2C).

To investigate the evolution of *EsRab7*, we compared the sequence with those of *Rab7* from other species available in GenBank. The DNAMAN multi-sequence alignment revealed that the protein sequence of *EsRab7* was more than 98% similar to the *Rab7* sequences of *Penaeus monodon*, *F. chinensis* and *L. vannamei*, showing high conservation of the *Rab7* family across species (Fig. 3). The phylogenetic NJ tree revealed a relatively high level of evolutionary conservation of *Rab7*. The relationships shown in the phylogenetic tree are consistent with the taxonomy of *Rab7*, with a closer relationship with invertebrates than vertebrates (Fig. 4).

3.2. Tissue expression pattern of EsRab7

Relative *EsRab7* mRNA expression levels in different tissues were measured by qRT-PCR using β-actin as an internal control. *EsRab7* expression was observed in all the tissues analyzed, with high abundance in muscle and hepatopancreas, but low expression in hemocytes and stomach (Fig. 5).

3.3. EsRab7 expression profiles in hemocytes after bacterial challenge

Following stimulation of hemocytes with *S. aureus* and *V. parahaemolyticus* as representatives of Gram-positive and Gram-negative bacteria, respectively, the temporal expression profiles of *EsRab7* were detected by qRT-PCR. *EsRab7* expression was upregulated significantly ($p < 0.05$) compared with that in controls at 24 h after *S. aureus* challenge (Fig. 6A). In response to *V. parahaemolyticus* challenge, *EsRab7* expression was upregulated ($p < 0.01$) at 8, 24 and, especially at 48 h, compared with the levels detected in the control (Fig. 6B). No significant changes in *EsRab7* expression levels were observed in the control (PBS).

3.4. Efficiency of siRNA-mediated silencing of EsRab7 expression

To investigate the function of *EsRab7*, we used siRNA to interfere with *EsRab7*, expression in crab hemocytes. qRT-PCR evaluation of the silencing efficiency showed an 85% decrease ($p < 0.001$) in *EsRab7*, mRNA expression following transfection with si*EsRab7*, for 24 h compared with that in the siGFP group (Fig. 7A). These results confirmed the effective silencing of *EsRab7*, expression.

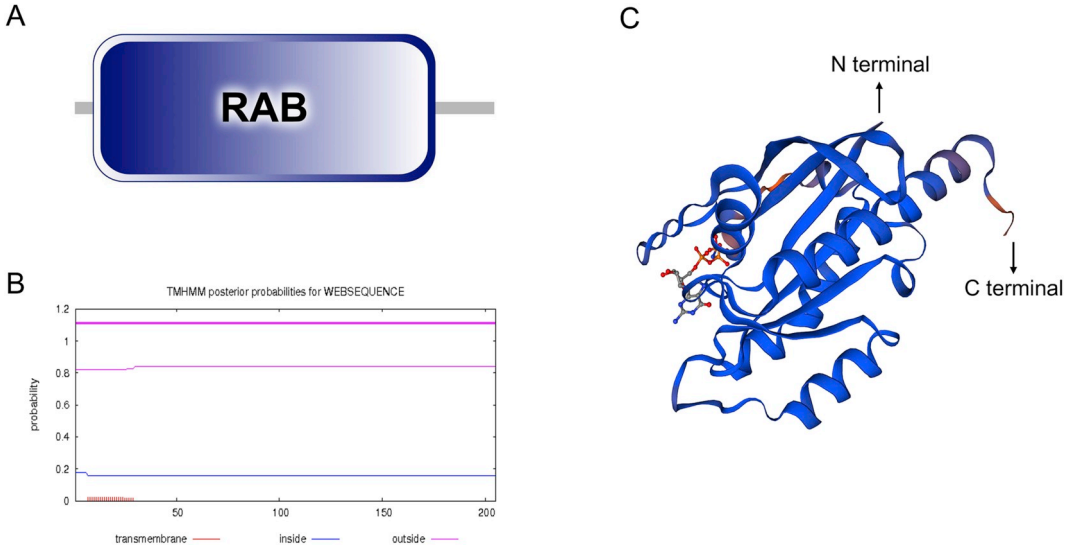

Fig. 2. A schematic view of the structure of the *Es*Rab7 protein and its three-dimensional model. (A) Domain analysis of the putative *Es*Rab7 protein by the SMART tool showing that it contains a RAB domain. (B) No transmembrane regions were predicted in the Rab7 protein by the TMHMM server. (C) A three-dimensional model of the *Es*Rab7 built by the online software SWISS-MODEL with PDB ID 1vg8.1. A as a template.

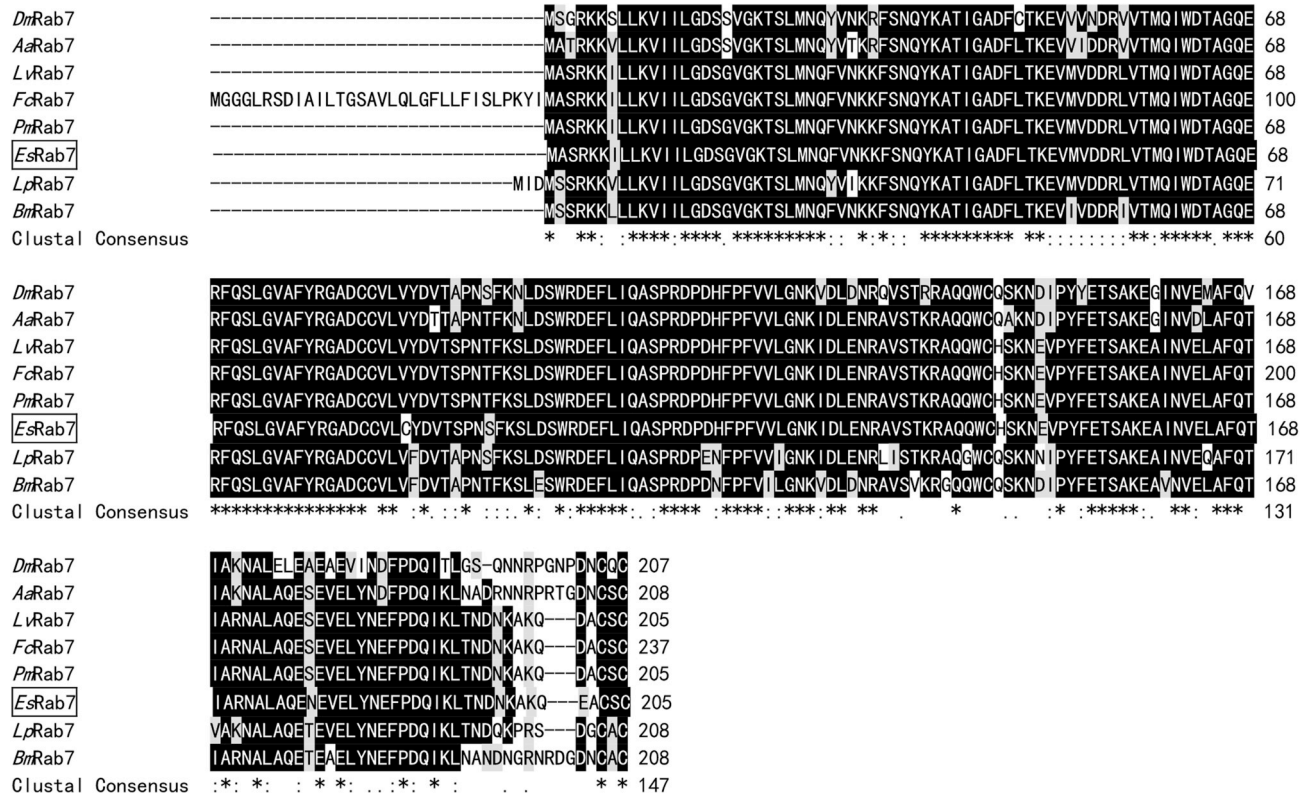

Fig. 3. Multiple sequence alignment of *Es*Rab7 with other reported Rab7 proteins from invertebrates. The sequences and accession numbers are as follows: *Dm*Rab7, *Drosophila melanogaster*, NP_001247277.1; *Aa*Rab7, *Aedes albopictus*, ABL74414.1; *Lv*Rab7, *Litopenaeus vannamei*, ACT65737.1; *Fc*Rab7, *Fenneropenaeus chinensis*, AEF33797.1; *Pm*Rab7, *Penaeus monodon*, ADM66002.1; *Lp*Rab7, *Limulus Polyphemus*, XP_013771808.1; *Bm*Rab7, *Bombyx mori*, NP_001040368.1.

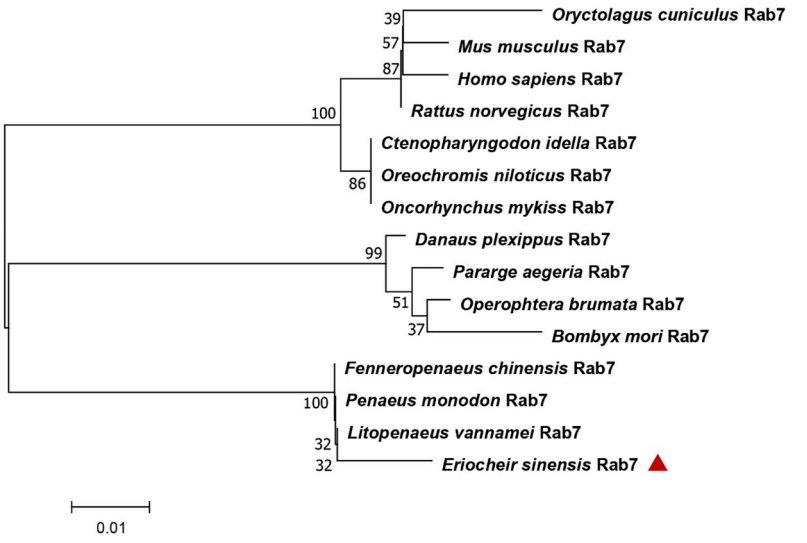

Fig. 4. Neighbor-joining (NJ) phylogenetic comparison of *Es*Rab7 sequences with those from vertebrates and invertebrates. The NJ phylogenetic tree was constructed with the MEGA 6 sequence analysis tool. The red triangle indicates *Es*Rab7. The sequences and their accession numbers are as follows: *Oryctolagus cuniculus*, AAD02564.1; *Mus musculus*, CAA61797.1; *Homo sapiens*, AAD02565.1; *Rattus norvegicus*, BAE17000.1; *Ctenopharyngodon idella*, AUG68890.1; *Oreochromis niloticus*, XP_003442718.1; *Oncorhynchus mykiss*, XP_021425323.1; *Danaus plexippus*, OWR48446.1; *Pararge aegeria,* JAA85499.1; *Operophtera brumata,* KOB74263.1; *Bombyx mori*, NP_001040368.1; *Fenneropenaeus chinensis*, AEF33797.1; *Penaeus monodon*, ADM66002.1; *Litopenaeus vannamei*, ACT65737.1.

3.5. Effects of *EsRab7* silencing on the expression of AMPs

The mRNA expression levels of *Es*ALF-1, *Es*ALF-2, *Es*ALF-3, *Es*Crus-1, *Es*Crus-2, *Es*Lys, and *Es*DWD1 were measured in *EsRab7*-silenced hemocytes after *S. aureus* or *V. parahemolyticus* challenge for 24 h. The expression levels of *Es*ALF-1, *Es*ALF-2, *Es*Crus-2, and *Es*DWD1 were significantly downregulated after *S. aureus* stimulation compared with the levels detected in the NC group (Fig. 7B). In addition, *Es*ALF-1, *Es*ALF-2, *Es*ALF-3, *Es*Crus-2 and *Es*DWD1 were significantly downregulated following *V. parahemolyticus* challenge, whereas the expression of *Es*Crus-1 was upregulated (Fig. 7C). These results showed that *EsRab7* regulates the expression of antibacterial peptides stimulated by Gram-negative and Gram-positive bacteria, indicating that *EsRab7* is involved in humoral innate immunity.

3.6. Regulation of phagocytosis by EsRab7

To investigate the effect of *EsRab7* on bacterial phagocytosis in vitro, we compared the bacterial phagocytosis rate in hemocytes expressing normal levels of *EsRab7* with that of *EsRab7*-silenced hemocytes. Immunofluorescence assay showed that the phagocytic rates for Gram-positive and Gram-negative bacteria were approximately 15% and 20%, respectively, in the normal and siGFP-transfected groups (Fig. 8). However, following siRNA-mediated silencing of *EsRab7*

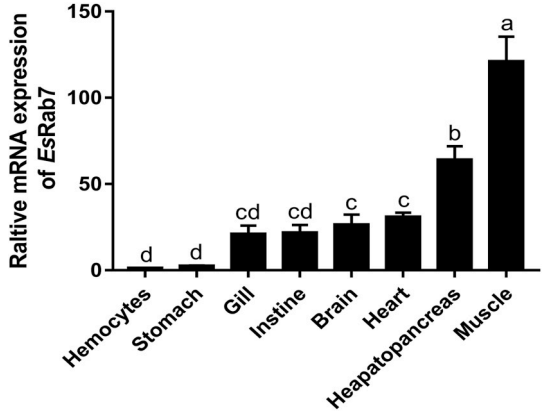

Fig. 5. Tissue distribution analysis of *EsRab7* at the mRNA level. Total RNA was extracted from eight different tissues of unchallenged crab. First-strand cDNAs were generated by reverse transcription and diluted 20-fold for use as templates for qRT-PCR analysis; *β-actin* was used as the control. Assays were performed three times, and data are presented as means ± SD. Statistical significance was determined by one-way ANOVA and post-hoc Duncan's multiple range tests. The same letters above bars indicate that expression levels were not significantly different, while different letters indicate significant differences ($P < 0.05$).

expression, the phagocytosis rates for Gram-positive and Gram-negative bacteria decreased by approximately 25% ($p < 0.05$)and 33% ($p < 0.01$), respectively, compared with the rates in the NC group (Fig. 8). These trends were confirmed by flow cytometric analysis (Fig. 9). These observations demonstrated that *EsRab7* promotes phagocytosis of both Gram-positive and Gram-negative bacteria in *E. sinensis*.

3.7. EsRab7 induces dorsal translocation into the nucleus

Recent studies have shown that AMP expression in crustaceans is regulated mainly by the Toll and Imd pathways. Therefore, we analyzed nuclear translocation of the transcription factors Dorsal and Relish to explore the role of *Es*Rab7 in the Toll and Imd signaling pathways, respectively. Previous studies have confirmed that the Toll pathway is activated mainly by Gram-positive bacteria and fungi in *Drosophila melanogaster*, and the Imd pathway is mainly resistant to Gram-negative bacteria; therefore, we selected *S. aureus* and *V. parahemolyticus* to detect the involvement of the Toll and Imd pathways, respectively. Immunocytochemical analysis showed that the distribution of *Es*Dorsal and *Es*Relish protein in the untreated group was mainly in the cytoplasm (Fig. 10A and B). Nuclear translocation of the transcription factor *Es*Dorsal was observed in siGFP-transfected hemocytes stimulated with Gram-positive or Gram-negative bacteria (Fig. 10A and B). In *EsRab7*-knockdown hemocytes challenged with Gram-positive *S. aureus*, *Es*Dorsal protein was predominantly detected in the cytoplasm (Fig. 10A). In contrast, *Es*Relish protein was predominantly observed in the nucleus in *EsRab7*-knockdown hemocytes after challenge with Gram-negative *V. parahemolyticus* (Fig. 10B). These findings indicated that *Es*Rab7 promotes the entry of *Es*Dorsal transcription factors into the nucleus following simulation with *S. aureus*. Taken together, our results suggested that *EsRab7* regulates AMP expression via the Toll pathway, but not the Imd pathway.

4. Discussion

Rab plays an significant role in many biological processes in eukaryotic cells, such as cell division and proliferation, cell differentiation [4]. A growing number of studies have shown that the Rab family performs immune functions, which are characterized by phagocytic clearance under bacterial stimulation. For instance, *Rab6* regulates shrimp hemocyte phagocytosis to protect against white spot syndrome virus (WSSV) infection. *Rab6* performs the same function in *Drosophila* melanism, which illustrates the conservation of the function of *Rab6* in phagocytosis in invertebrates [32]. Moreover, the intracellular transport function of Rab is essential for the invasive reproduction of certain viruses. For example, in *P. monodon*, *PmRab7* silencing prevents successfully the viral trafficking necessary for replication, leading to a reduction in WSSV and YHV replication [33]. Therefore, the potential involvement of *Rab7* in the immune response of crustaceans and the underlying mechanism warrants investigation.

In this study, we cloned the full-length *Rab7* cDNA from *E. sinensis*. Analysis of the sequence in the NCBI database revealed that the *EsRab7* gene-encoded protein has all the conserved domains and active sites associated with the Rab gene family and the conserved sequence of the Rab subfamilies (RabSF). With the CSC amino acids at the C-terminus, *EsRab7* is a CXC type protein, and the potential for isoprene-modification of the two cysteines in this location suggest that the protein may have strong hydrophobicity and membrane affinity [34,35]. The conservation analysis of protein sequences showed a high degree of similarity at the amino acid level between *Rab7* in crab and other species. The N-terminus is highly conserved, while the C-terminal sequence is highly variable and is very similar to the amino acid sequences of *PmRab7* and *LvRab7* [36,37]. The phylogenetic tree demonstrated the *EsRab7* gene clustered with different groups of homologous genes of other species, and the distance from the vertebrate genes was also consistent with the evolutionary relationships among the species. These findings indicate that the influence of selection pressure on the evolution of the gene and its crucial role in various species.

qRT-PCR analysis of different *E. sinensis* tissues showed that *EsRab7* is expressed at high levels in muscle and hepatopancreas (Fig. 5). *Rab7*, which is an active GTPase with GDP/GTP-binding function, regulates vesicle trafficking by catalyzing the transformation of *Rab7*-GDP and *Rab7*-GTP and the translocation of *Rab7* between the cytoplasm and membrane. *EsRab7* is most abundant in muscle, probably due to its involvement in membrane transport in muscle cells, a phenomenon that has also been found for *EsRab-1* [17]. Hepatopancreas tissue is important for the production of immune response factors in crustaceans,

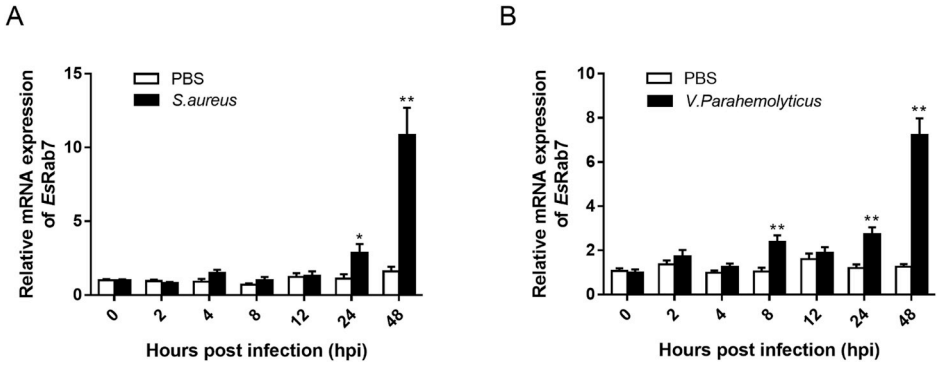

Fig. 6. Expression profile of *EsRab7* mRNA after bacterial stimulation in the hemocytes. Total RNAs were extracted from hemocytes at 0, 2, 4, 8, 12, 24 and 48 h after challenge with *S. aureus*, or *V. parahaemolyticus*; PBS was used as a control. *EsRab7* expression in each sample was analyzed by qRT-PCR with *β-actin* as the reference gene. Three independent repeats were performed with at least three crabs for each sample. Data represent the mean ± SD and analyzed by Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

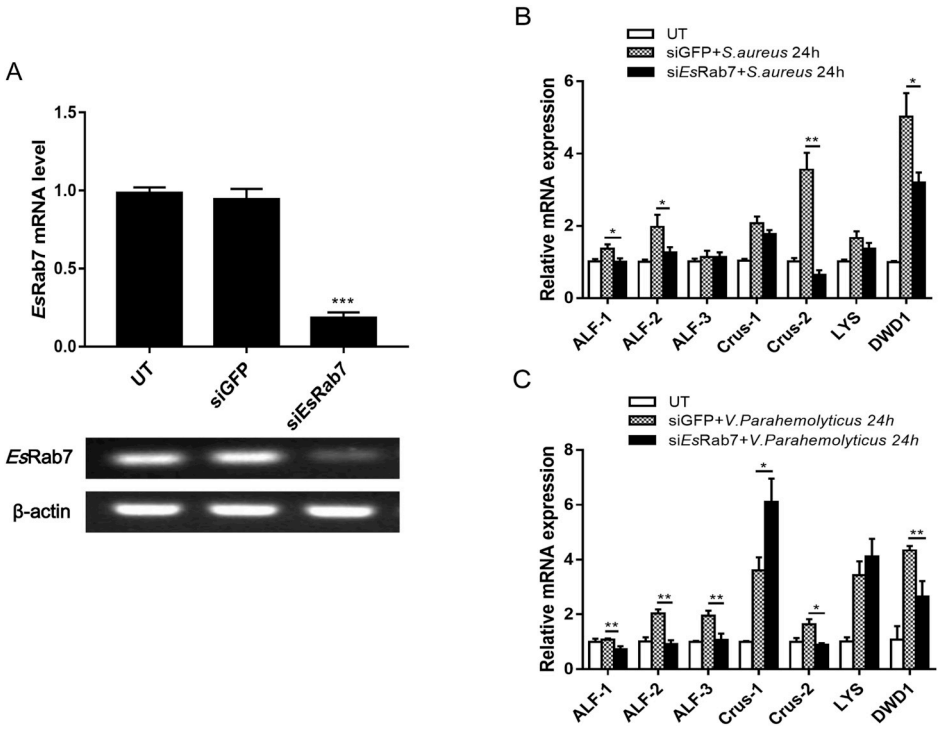

Fig. 7. Effects of *EsRab7* RNAi on expression of AMPs in hemocytes following bacterial stimulation. (A) The *EsRab7* mRNA expression level in hemocytes after transfection with siGFP or *EsRab7* siRNA; *EsRab7* mRNA expression in normal hemocytes was used as a control (UT). For qRT-PCR analyses, data represent the mean ± SD of triplicate experiments analyzed by one-way ANOVA and post-hoc Duncan's multiple range tests. ***$P < 0.001$. (B) Effects of *EsRab7* RNAi on expression of antimicrobial peptides (AMPs) in hemocytes challenged with *S. aureus*. After 24 h of bacterial stimulation, the mRNA expression levels of seven antibacterial peptides (ALF1, 2 and 3, Crus 1 and 2, DWD1 and Lys) were determined by qRT-PCR with *β-actin* as the reference gene; AMP mRNA expression levels in normal hemocytes were used as controls (UT). Data represent the mean ± SD of three independent analyzed by Student's *t*-test. *$P < 0.05$, **$P < 0.01$. (C) Effects of *EsRab7* RNAi on expression of AMPs in hemocytes challenged with *V. parahaemolyticus*.

not only initiating humoral immune responses, but also containing highly specialized cells and phagocytic cells, which play an important role in cellular immune responses [38,39]. It has been reported in Chinese mitten crab that both *EsRab1* and *EsRab-3* are expressed at relatively high levels in hepatopancreas [17]. Although *EsRab7* is expressed at low levels in unstimulated blood cells, it is significantly increased after bacterial stimulation.

To verify the immune function of *EsRab7*, we analyzed the expression of *EsRab7* after bacterial stimulation. *EsRab7* expression was significantly increased in the late stages of stimulation with both Gram-positive and Gram-negative bacteria (Fig. 6); therefore, we speculate that *EsRab7* represent a supplemental immune molecule, that functions in the late stage of bacterial stimulation, such as a C-type lectin [40].

It is well known that invertebrates fight pathogens through innate immunity consisting of humoral and cellular components. The cellular immune system of crustaceans consists mainly of blood cell-based innate immunity and is a very important in the defense against pathogenic microorganisms. Furthermore, cellular and humoral immunity in

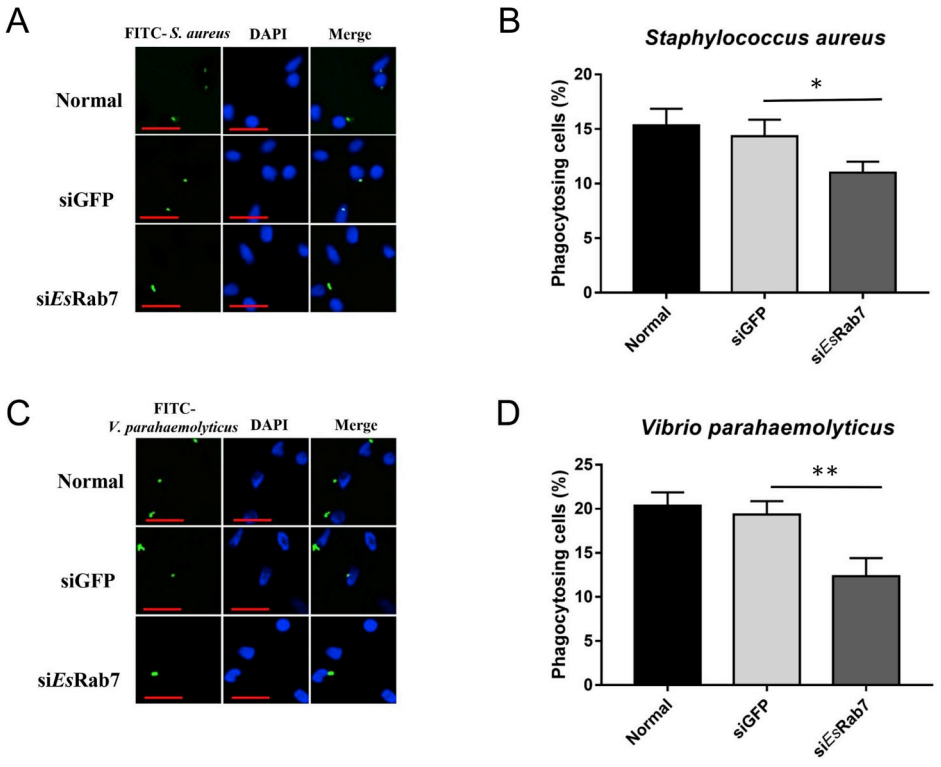

Fig. 8. *EsRab7* regulated phagocytosis of bacteria in vitro. (A and C) Cultured hemocytes were challenged with FITC-labeled *S. aureus* and *V. parahaemolyticus*. After 1 h, cells were collected and washed with PBS. After staining with DAPI and quenching with trypan blue, hemocytes were observed under a fluorescence microscope. Scale bar = 15 μm. (B and D) Phagocytosis was detected by fluorescence microscopy, and a total of 500 cells were counted. Data are representative of three independent repeats and analyzed by post-hoc Duncan's multiple range tests. *$P < 0.05$, **$P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

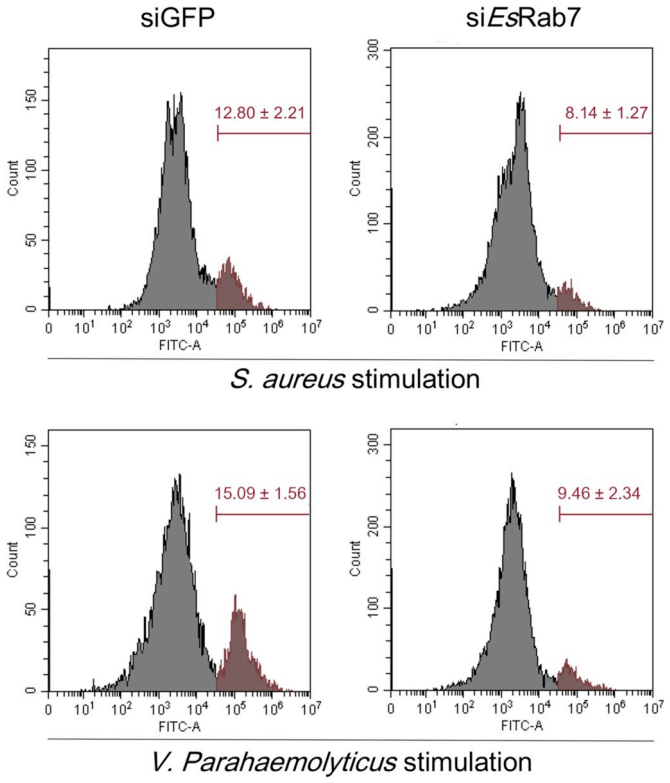

Fig. 9. Detection of phagocytosis by flow cytometry. Bacteria (1×10^7 CFU) were incubated with 1×10^6 hemocytes for 40 min at 25 °C. Cells were obtained by centrifugation at 600 $\times g$ for 8 min. After washing, the hemocytes were analyzed by flow cytometry. A total 10,000 hemocytes were counted for each sample. Data represent the mean ± SD of three independent repeats.

crustaceans are closely related and complementary systems [41]. Humoral immunity involves mainly the melanization of hemolymph and the production of AMPs [42]. However, the immune responses that are regulated by *Rab7* in crustacean immunity remain to clarified.

Phagocytosis is an important cellular immune response process, especially for invertebrates. Small pathogenic microbe particles can be recognized by phagocytic receptors on the surface of the immune cells, and then the pathogens are directly phagocytized by the specific immune cells [43]. Rab GTPases are involved in phagosome maturation and trafficking, and studies have demonstrated an important role in actin-dependent phagocytosis by transporting vesicles to their target compartments [44]. We hypothesized that *EsRab7* also plays a role in the immune phagocytosis of *E. sinensis*. Our phagocytosis experiment confirmed that the phagocytosis of Gram-positive and Gram-negative bacteria by hemocytes decreased significantly after silencing of *EsRab7* expression, with a greater effect observed for the Gram-negative bacteria (Figs. 8 and 9). Thus, it can be speculated that the formation of phagocytic vesicles and phagocytosis of bacteria is inhibited in the absence of *EsRab7*.

As an intracellular receptor, the C-terminal of Rab7 is variable, we suspected that Rab7 is closely related to intracellular signal transduction [36]. Meanwhile, previous research also has reported that Rab7 is a signaling-related factor [8,9]. Crustacean AMPs are immunological effectors that directly kill or eliminate infectious pathogenic microorganisms. They are widely found in organisms and are an important component of the innate immune system of crustaceans [25,26]. And their production is also regulated by signaling pathways such as the Toll and Imd etc. The two pathway signaling pathways play key roles in regulating the antibacterial and antiviral responses of invertebrates [45,46]. Few studies have focused on the intracellular signaling cascade triggered by *Rab7* or its role in regulating the expression of AMPs. Therefore, we analyzed the expression of AMPs in *E. sinensis* following bacterial stimulation after silencing *EsRab7*. A significant change in the expression of some AMPs in response to bacterial stimulation was observed after *EsRab7* silencing, indicating that *EsRab7* regulates AMP expression via an unknown signaling pathway (Fig. 7). Immunofluorescence experiments demonstrated that nuclear translocation of *Es*Dorsal, which is a nuclear factor in the Toll pathway, was inhibited following *EsRab7* silencing Fig. 10. Therefore, we hypothesize that *Rab7* regulates the expression of AMPs in the crustacean through the Toll signaling pathway. Previous studies have shown that the small GTPase *Rab7b* is important for the correct transport of several receptors, such as Toll-like receptors (TLRs) and sorting receptors [47]. In addition, the Calpain-Myosin 9-*Rab7b* pathway regulates the expression of Toll-like receptor 4 in platelets [48]. Although the specific mechanism by which *EsRab7* regulates the Toll pathway is still not clear, our findings provide a foundation for further investigation of the signaling pathways and immune responses regulated by Rab family proteins.

In summary, we cloned and characterized the *EsRab7* gene of *E. sinensis* through bioinformatics and transcriptional analysis. *EsRab7* is distributed in a variety of tissues including gill, hepatopancreas and blood cells. Furthermore, after exposure to Gram-positive and Gram-negative bacteria, *EsRab7* expression was significantly upregulated in hemocytes. In addition, *EsRab7* promotes the phagocytosis of pathogens via cellular immunity. In humoral immunity, *EsRab7* may regulate AMP

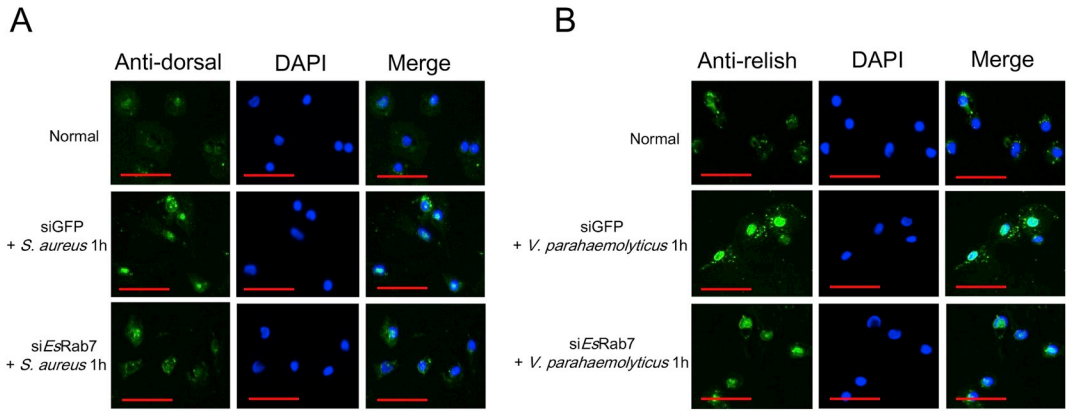

Fig. 10. Knockdown of *EsRab7* expression suppressed nuclear translocation of Dorsal (but not Relish). (A) In the normal, siGFP + *S. aureus* and si*EsRab7* + *S. aureus* groups, *Es*Dorsal translocation in the hemocytes was evaluated by immunocytochemical staining with a Dorsal-specific antibody; the green signal indicates the distribution of *Es*Dorsal, and nuclei were stained with DAPI. (B) In the normal, siGFP + *V. parahaemolyticus* and si*EsRab7* + *V. parahaemolyticus* groups, *Es*Relish translocation in the hemocytes was evaluated by immunocytochemical staining with a Relish-specific antibody. Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

gene expression via the Toll signaling pathway. Therefore, our findings indicate that the antibacterial function of *EsRab7* is mediated phagocytosis and regulation of AMPs.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (31602189 and 31672639). We want to thank Mr. Puxu Mao from Shanghai Nanyang Model High School for his participation and constructive suggestions in this study.

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