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RESEARCH REPORT

Molecular cloning and characterization of *Rheb* from white shrimp (*Litopenaeus vannamei*)

X Liu[1,2,3], M Wang[1,2], J Shao[1,2,3], B Wang[1,2], K Jiang[1,2], M Liu[1,2]*, L Wang[1,2]*

[1]Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
[2]Laboratory of Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237
[3]University of Chinese Academy of Sciences, Beijing 100049, China

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Abstract

The Ras family GTPase Rheb, a GTP-binding protein, binds specifically to the mTOR catalytic domain and induces activation of the mTOR catalytic function. Furthermore, Rheb is related to a stable modification of the configuration of mTORC1 that increases access of substrates to their binding site on the raptor polypeptide. In the present research, a cDNA of 898 bp for the *Litopenaeus vannamei Rheb* was cloned via rapid amplification of cDNA ends (RACE) technique. The complete cDNA sequence of *Rheb* contained an open reading frame (ORF) of 549 bp, which encoded a protein of 182 amino acids. The amino acid sequence of Rheb shared more than 60% similarity with other identified Rheb proteins. A Ras domain (from P3 to A169) was found in the amino acid sequence of Rheb that can react selectively and non-covalently with GTP. The mRNA transcripts of *Rheb* were consistently expressed in all the tested tissues, including muscle, gill, hepatopancreas, eyestalk, intestine and stomach. The mRNA expression profiles of *Rheb* in muscle after the stimulation with rapamycin were promoted, which further proved that Rheb protein could be a feedback regulator to mTOR signaling pathway. Furthermore, the results of the present study indicated that Rheb played an important role in the regulation of mTOR signaling pathway during the stimulation of dietary restriction, amino acid supplementation and rapamycin stimulation in shrimp.

Key Words: *Litopenaeus vannamei*; molecular cloning; mTOR; Rheb

Introduction

Mechanistic target of rapamycin (mTOR) is a fundamental regulator of cell growth and proliferation in all eukaryotes (Wullschleger *et al*., 2006). mTOR can sense stress, oxygen, amino acids, energy levels and growth factors to perform cell function (Laplante *et al*., 2012). The functions of mTOR were performed by two independent complexes. The mTOR complex 1 (mTORC1) is comprised of mTOR in association with raptor, LST8, Deptor, Ttl1/Tel2 and PPRAS40, which could be inhibited by rapamycin (RAPA) (Kunz et al., 1993; Hara et al., 2002; Kim *et al*., 2002; Loewith *et al*., 2002). The mTOR complex 2 (mTORC2) shares include mTOR, mLST8, Deptor, and Ttl1/Tel2 with mTORC1. In addition, Rictor, mSIN1, and PPR5/Protor are specific for mTORC2 (Loewith *et al*., 2002; Jacinto *et al*., 2004; Sarbassov *et al*., 2004). Its output is insensitive to RAPA.

In mammalian cells, mTORC1 output is sensitive to amino acid (especially leucine and arginine) sufficiency (Long *et al*., 2005a). Amino acid withdrawal can lead to the inhibition of mTORC1 signaling (Demetriades *et al*., 2014). The overexpression of Rheb can rescue mTOR from inactivation in *vivo* caused by amino-acid withdrawal (Long *et al*., 2005a). What's more, genetic evidence from *Drosophila* indicated that the Rheb is an indispensable activator of mTORC1 in the insulin/IGF-I receptor pathway. The studies indicate that Rheb is a key regulator of the output of mTORC1.

Corresponding authors:
Mei Liu
Key Laboratory of Experimental Marine Biology
Institute of Oceanology
Chinese Academy of Sciences
Qingdao 266071, China
E-mail: liumei@qdio.ac.cn

Lei Wang
Key Laboratory of Experimental Marine Biology
Institute of Oceanology
Chinese Academy of Sciences
Qingdao 266071, China
E-mail: wanglei@qdio.ac.cn

RAPA is a specific inhibitor of mTORC1 and the effects of RAPA on mTOR signaling are complicated. Many mTORC1 functions are highly sensitive to RAPA, for example, mTOR-mediated protein synthesis, lipogenesis, energy metabolism, and lysosome biogenesis (Thomas *et al*., 1997; Schmelzle *et al*., 2000; Brugarolas *et al*., 2003; Laplante *et al*., 2009; Settembre *et al*., 2012). RAPA forms a complex with the intracellular 12-kDa FK506-binding protein (FKBP12) (Brown *et al*., 1994; Sabatini *et al*., 1994). This complex directly combines with and inhibits mTORC1. Rheb can relieve the inhibition of the mTORC1 (Ma *et al*., 2008; Sun *et al*., 2008). The influence and mechanism of Rheb to control the mTORC1 are as yet unknown.

Rheb can bind specifically to the mTOR catalytic domain in *vivo* and in *vitro* (Avruch *et al*., 2014). The mTOR–Rheb interaction can promote the activation of mTOR kinase (Long *et al*., 2005a). Amino acids appear to control the efficacy of Rheb-GTP towards mTORC1 (Avruch *et al*., 2009). Besides, Rheb promotes a reconfiguration of the mTORC1 complex that enhances the accession and binding of substrates to raptor. A previous research showed that the mTORC1 substrates S6K1 and 4E-BP bound directly to raptor (Hara *et al*., 2002). The process can promote the phosphorylation of translational regulators eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1), which, in turn, promote protein synthesis (Ma *et al*., 2009). However, further work is needed to elucidate the mechanisms of Rheb–mTOR interaction that promotes mTOR kinase activity.

The researches about Rheb functions concentrates upon mammals and some insects. Su *et al*. found that *Rheb* mRNA expression was increased in WSSV-infected shrimp (Su *et al.,* 2014). Beyond that, very few studies have been done on its functions in crustaceans. What's more, a relatively small number of studies explore the interaction between Rheb and mTORC1. So, the mechanism of activation is not fully understood. Therefore, studies to elucidate the Rheb-mTOR signaling pathway are of great importance. *Litopenaeus vannamei* has proven to be a useful decapod crustacean model system for the study of evolution, in addition to its importance as a food source (Sakthivel, 2014). The research about mTOR signaling pathway in *L. vannamei* can deepen our understanding of the important signaling pathway that regulate growth and metabolism in eukaryotes and reveal the evolutionary traces of mTOR signaling pathway. Meanwhile, the studies about growth mechanism in shrimp can lead to more reliable way to the selection of the new breed and optimizing the feed formula.

Materials and methods

Experimental animals

Litopenaeus vannamei (5 ± 0.5 g) from the Qingdao Ruizi aquaculture base (Shandong, China) were used in this study and were stocked in two tanks at a density of 100 shrimps per tank (1000 L) at 30‰ salinity. Shrimp were maintained for 1 week and fed commercial pellets (42.3% protein, 7.2% fat, 11.6% water, and 15.5% ash, supplied by Da Le Co., Ltd, Yantai, China). The water quality parameters were evaluated 2−3 times per week and maintained at pH 7.5−8.2, temperature 25−29 °C, dissolved oxygen 5.0−6.5 mgL^{-1} during the trial.

Rapamycin injection

In the first tank, we injected shrimps on the sixth uromere with 100 µL RAPA (500 mM), and shrimps in the other tank were injected with 100 µL DMSO diluted by phosphate-buffered saline (PBS) (0.01 M, pH 7.4) on the ratio of 1:1000 (the solvent of RAPA). Shrimps were dissected at different time-points (0, 0.5, 1, 2, 4 and 6 h) after injection with RAPA (experimental group) or DMSO diluent (control group) to obtain muscle tissue. The control and experimental groups (n = 9 shrimps/group, conducted in triplicate) obtained at each time-point were used to obtain muscular tissue for real-time (RT)-PCR analysis. The muscle tissue was preserved in RNA store solution (Beijing ComWin Biotech Co., Ltd., Beijing, China).

Amino acid injection

The shrimps were divided into six groups. The shrimps in the first group injected with 100 µL PBS (0.01 M, pH 7.4) were the control group. The last group srhimps were injected with 100 µL RAPA (500 mM). The remaining 4 groups were starved for 3 days. After the dietary restriction (DR), shrimps in the second group were injected with 100 µL PBS (0.01 M, pH 7.4). The shrimps in the third and fourth groups were injected with 100 µL 0.1 M leucine or 100 µL 0.1 M arginine, respectively. The shrimps in the fifth group were injected with 100 µL leucine (0.1 M) and 100 µL RAPA (500 mM). The control and experimental groups (n = 9 shrimps/group, conducted in triplicate) were used to obtain muscular tissue for RT-PCR analysis 30 min after injection. The muscle tissue was preserved in RNA store solution (Beijing ComWin Biotech Co., Ltd., Beijing, China).

RNA preparation and cDNA synthesis

Total RNA was extracted using a MiniBEST Universal RNA Extraction kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. RNA degradation and contamination was monitored on 1% agarose gels. The cDNA synthesis was carried out by TransScript II One-Step gDNA Removal and cDNA Synthesis SuperMix (AH311-02, TransGen Biotech, China). The reactions were performed at 42 °C for 30 min, terminated by heating at 85 °C for 5 min and then stored at -80 °C.

Cloning the cDNA of Rheb

The partial sequence of *Rheb* cDNA was obtained from the transcriptome database of *L. vannamei*. Standard procedures were used for cDNA cloning. Two pairs of gene-specific primers, 3R1/2 and 5R1/2, were designed based on this partial sequence to clone the 3' end and 5' end of *Rheb* cDNA by rapid-amplification of cDNA ends (RACE) technique. The full-length cDNA of the *Rheb* from *L. vannamei* was amplified by PCR using the primers Rheb3'-F and Rheb5'-R. The primers were designed by NCBI primer blast and were given in Table 1. All PCR amplification was performed in a MJ

Table 1 Oligonucleotide primers used in the current experiments.

Name	Sequence (5'-3')	Brief information
3R1	AGCGTGGGCAAATCCTCCTT	Gene specific primer for RACE
3R2	ATGCCCCGACCATCGAGAAC	Gene specific primer for RACE
5R1	CCCTCGGGACTGATGTTGCC	Gene specific primer for RACE
5R2	GTTGCCCACCAAGACAATGGG	Gene specific primer for RACE
QRHE-F	AGGAAAGTGGCCGTTATGGG	Gene specific primer for real-time PCR
QRHEB-R	TACCAGCTCCAGGCCATACT	Gene specific primer for real-time PCR
Qβ-actin-F	GCCCATCTACGAGGGATA	Internal control for real-time PCR
Qβ-actin-R	GGTGGTCGTGAAGGTGTAA	Internal control for real-time PCR
Rheb3'-F	TGTCTCTCCCTTCCCTTCGG	Gene specific primers used to amplify full-length Rheb
Rheb5'-R	AAGGTCCATCCTATAACCCAGG	Gene specific primers used to amplify full-length Rheb
NUP	AAGCAGTGGTATCAACGCAGAGT	Universal primers for RACE
UPML	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	Universal primers for RACE
UPMS	CTAATACGACTCACTATAGGGC	Universal primers for RACE
3'CDS	AAGCAGTGGTATCAACGCAGAGTAC(T)30V N	Oligo (dT) for cDNA synthesizing
5'CDS	AAGCAGTGGTATCAACGCAGAGTGGGGGGGGGGHN	Anchor primer for 5' RACE
M13F	TGTAAAACGACGGCCAGT	Vector primer for sequencing
M13R	CAGGAAACAGCTATGACC	Vector primer for sequencing

Mini Personal Thermal Cycler (Bio-Rad, USA), and the PCR products were purified using DNA Gel Extraction Kit (DP210, Tiangen, China) and cloned into the pEASY-T1 Cloning Vector (CT101, TransGen Biotech, China). After being transformed into the Trans5α Chemically Competent Cell (CD201, TransGen Biotech, China), the positive recombinants were identified via anti-ampicillin selection.

Sequence characterization and multiple sequence alignment

The protein sequence similarities were discovered by Protein BLAST at the National Center for Biotechnology Information (NCBI). The physicochemical property of protein Rheb were analyzed by ProtParam tool (https://www.expasy.ch/tools/protparam.html). SignalP 4.1 program was utilized to predict the presence and location of signal peptide (http://www.cbs.dtu.dk/services/SignalP/). The protein domain features of Rheb were predicted by Simple Modular Architecture Research Tool (SMART) 7.0 (http://smart.emblheidelberg.de/). Multiple sequence alignment of Rheb and other Rhebs was performed with ClustalW multiple alignment program 2.1 (http://www.ch.embnet.org/software/ClustalW.html) and multiple alignment show program 2.0 (http://www.bioinformatics.org/sms2/color_align_cons.html). A Neighbor-Joining (NJ) phylogenic tree of Rheb was constructed with MEGA 6.0 software package. To derive confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times.

Real-time PCR analysis of Rheb mRNA expression

The mRNA transcripts of *Rheb* in muscle, gill, hepatopancreas, eyestalk, intestine and stomach were quantified by RT-PCR, and its temporal expression profiles in muscle of *L. vannamei* stimulated with RAPA were determined by RT-PCR. PCR amplification was performed using the following cycling conditions: denaturation for 30 s at 94 °C, followed by 40 cycles of 5 s at 94 °C, and 30 s at 60 °C. To confirm that only one PCR product was amplified and measured, dissociation curve analysis was performed at the end of each PCR. All RT-PCR was performed with the SYBR Premix Ex Taq kit (TaKaRa Biotechnology Co., Dalian, China). The information of all primers used in this assay was shown in Table 1. The primers were designed by NCBI primer blast. The expression of *Rheb* was normalized to the expression of *β-actin* gene for each sample. The comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to analyse the expression level of *Rheb* (Schmittgen *et al*., 2008).

Statistical analysis

Results are expressed as means ± SD. The statistical analysis were performed by one-way analysis of variance (one-way ANOVA) using SPSS software to detect significant intergroup differences. The *p* values less than 0.05 were considered statistically significant.

Results

The molecular features, sequence alignment and phylogeny relationship of Rheb

A nucleotide sequence from the *L. vannamei* transcriptome is homologous to *Rheb* identified previously confirmed by sequencing and blast analysis. Based on this fragment, a fragment was amplified with nested primers UPM/3R1 and NUP/3R2 to obtain the 3' end of the sequence. A fragment was amplified with nested primers UPM/5R1 and NUP/5R2 to obtain the 5' end of the sequence. A 898 bp nucleotide sequence representing the complete cDNA sequence of *Rheb*

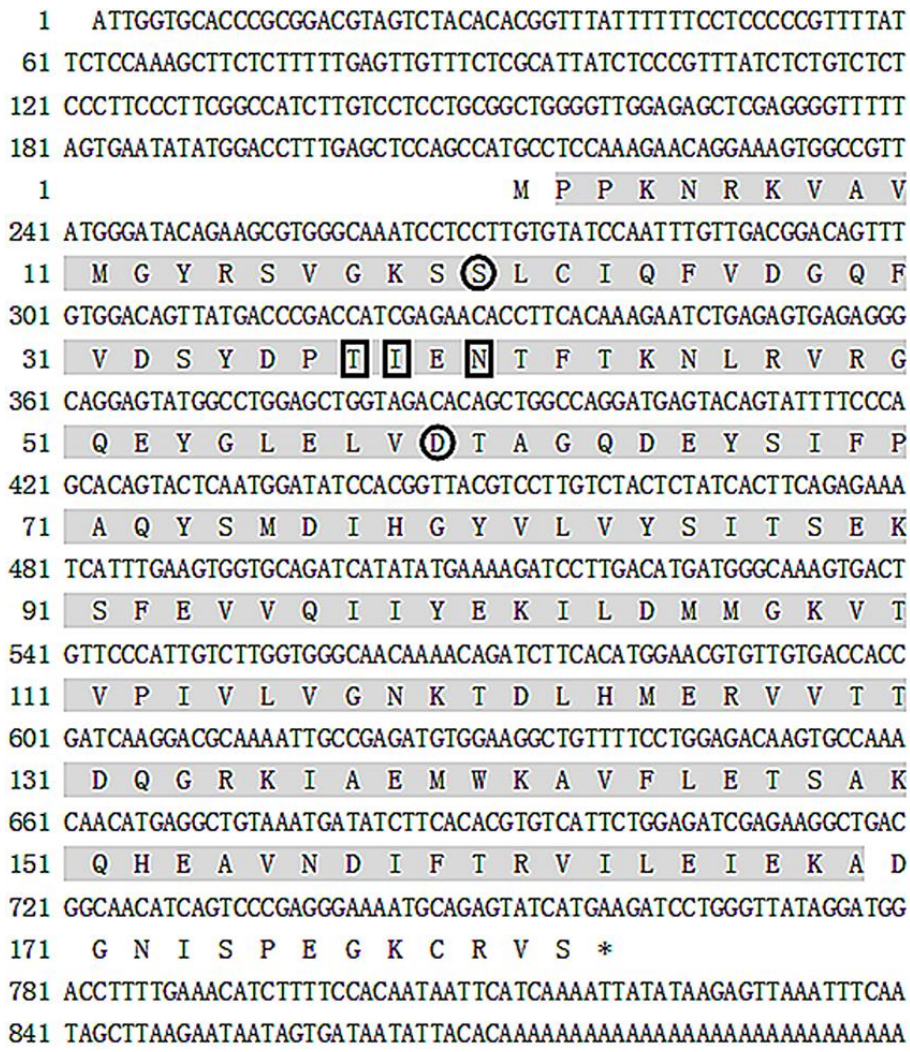

Fig. 1 Nucleotide and deduced amino acid sequences of Rheb. The nucleotides and deduced amino acids are numbered along the left margin. The Ras domain was in shade. Conserved amino acids involved in mTOR binding activity are boxed. Conserved amino acids involved in guanyl nucleotides binding activity are circled. The asterisk indicated the stop codon. The amino acid sequences of Rheb has been submitted to GenBank and the accession number is MG696863.

of *L. vannamei* was assembled. The complete cDNA sequence of *Rheb* contains a 208 bp 5' untranslated region, a 141 bp 3' untranslated region with a poly (A) tail and the complete sequence of an open reading frame (ORF) of 549 bp (Fig. 1). The ORF encoded a polypeptide of 182 amino acid residues with a calculated molecular mass of approximately 20.55 kDa. The theoretical isoelectric point is 5.67. No signal peptide was predicted in the deduced amino acid sequence of Rheb by SignalP program. A Ras domain (from P3 to A169) was found in the amino acid sequence of Rheb. The deduced amino acid sequences of the six crustacean Rheb proteins were highly conserved, showing high identity and similarity to each other (Fig. 2). Sequence identity was particularly high within the 'G box' motifs (G1–G5) in all the Rheb proteins, particularly in the G2 motifs (Fig. 2). The effect domain (switch I) is highly conservative region too. The deduced amino acid sequence of Rheb exhibited high similarity with other reported Rhebs, such as 91% with that from *Homarus americanus* (ADV76255), 86% with that from *Carcinus maenas* (ADV76253) and 66% with that from *Homo sapiens* (NP_005605) (Fig. 3). The nucleotide sequence of *Rheb* has been submitted to GenBank and the accession number is MG696863.

The tissue distribution of Rheb mRNA

The RT-PCR analysis was employed to detect the tissue distribution of the Rheb mRNA in different tissues and the *β-actin* gene as internal control. The lowest expression level of *Rheb* transcripts was present in intestine. In other detected tissues, the Rheb mRNA transcripts were significantly higher

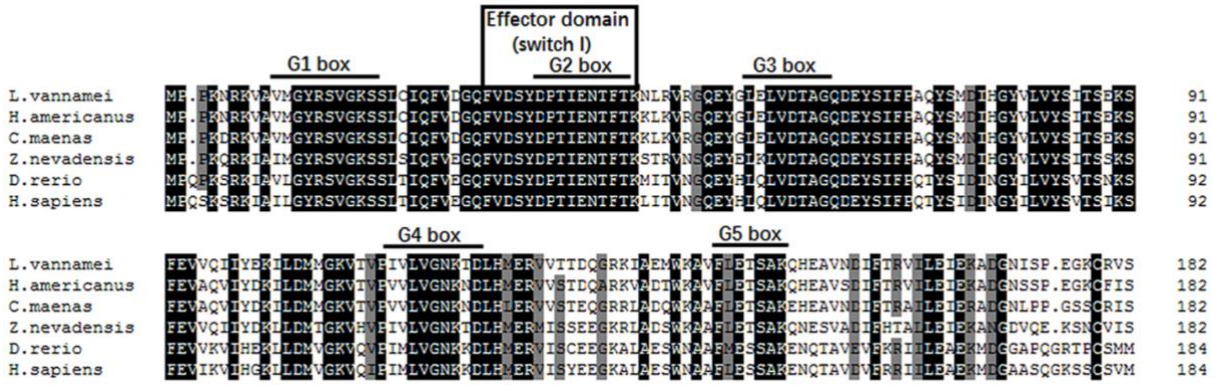

Fig. 2 Multiple alignments of deduced amino acid sequences for Rheb proteins. The black shadow region indicated positions where all sequences share the same amino acid residue. Similar amino acids are shaded in grey. G boxes and effector domain (switch I region) has been marked in the figure. The effector switch I region is responsible for interactions with the mTOR protein, FKBP38 and other proteins (Aspuria and Tamanoi, 2004; Ma *et al*., 2008). Species and gene accession numbers are as follows: *Litopenaeus vannamei* (MG696863), *Homarus americanus* (ADV76255), *Carcinus maenas* (ADV76253), *Zootermopsis nevadensis* (XP_021934312), *Danio rerio* (NP_957023) and *Homo sapiens* (NP_005605).

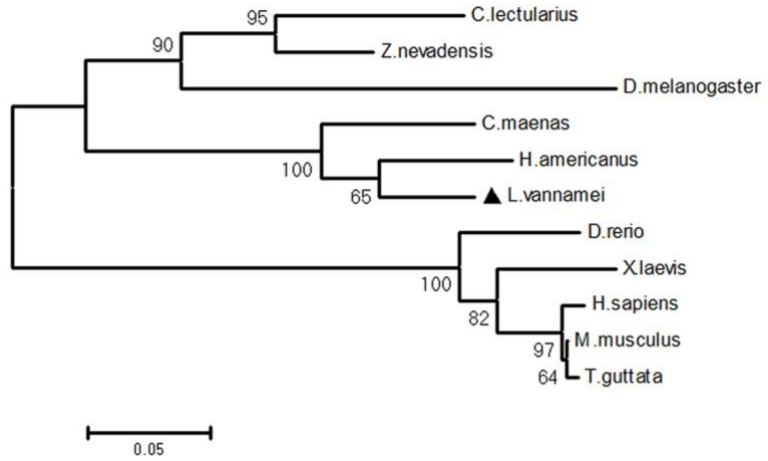

Fig. 3 Neighbor-Joining (NJ) phylogenic tree of Rheb constructed using MEGA 6.0 software package based on the amino acid sequences of Rhebs from different organisms. To derive confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times. Species and protein sequences ID are as follows: *Litopenaeus vannamei* (MG696863), *Drosophila melanogaster* (NP_730950), *Danio rerio* (NP_957023), *Homo sapiens* (NP_005605), *Mus musculus* (NP_444305), *Homarus americanus* (ADV76255), *Carcinus maenas* (ADV76253), *Cimex lectularius* (XP_014260514), *Zootermopsis nevadensis* (XP_021934312), *Xenopus laevis* (NP_001080494), *Taeniopygia guttata* (NP_001232539), The Rheb protein of *L. vannamei* is indicated with a black triangle. The numbers at the forks indicated the bootstrap value. The scale bar represents the proportion of amino acid differences between sequences based on nucleotide substitutions per site.

than those in intestine. What's more, the expression level of *Rheb* among muscle, gill, hepatopancreas, eyestalk, intestine and stomach showed no significant difference (Fig. 4).

The temporal expression profile of Rheb mRNA post RAPA stimulation

The temporal mRNA expression profile of *Rheb* in muscles after RAPA stimulation was examined via RT-PCR. The mRNA expression of *Rheb* in muscles increased significantly during 0.5 h-6 h ($p < 0.05$) after the stimulation of RAPA. The mRNA transcripts increased to the peak level at 1 h post stimulation (19.53-fold, $p < 0.05$). In the control group, no significant change of *Rheb* mRNA expression was observed after DMSO injection during the whole experiment (Fig. 5).

The temporal expression profile of Rheb mRNA post dietary restriction, amino acid and RAPA stimulation

RT-PCR analysis was employed to examine the mRNA expression profile of *Rheb* in muscles after dietary restriction and at 30 min after injection of amino acid and RAPA. As shown in Fig. 6, the mRNA

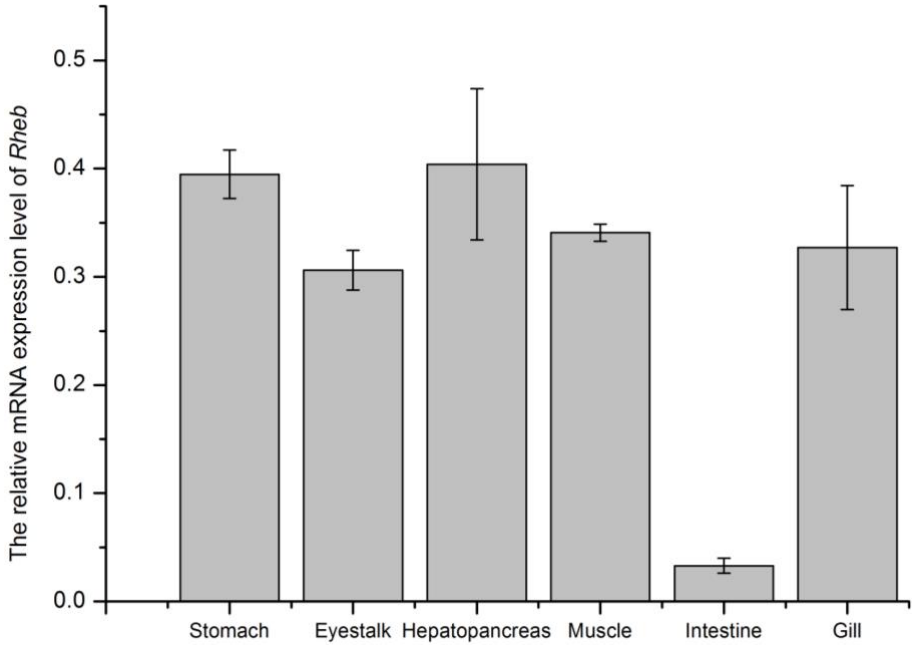

Fig. 4 Tissue distribution of *Rheb* mRNA transcripts detected by RT-PCR technology. *β-actin* gene was used as an internal control. *Rheb* mRNA transcripts come from stomach, eyestalk, hepatopancreas, muscle, intestine and gill of five adult shrimp. Vertical bars represented mean ± S.D. (n = 5), and bars with different characters indicated significantly different ($p < 0.05$).

transcripts of *Rheb* in muscles increased after the shrimps deprived of feed for 3 days (3.2-fold compared with the origin level, $p < 0.05$). The leucine and arginine injections can relieve the influence caused by hunger and make the expression of *Rheb* decrease to the original expression level. The injection of RAPA can hinder the ability of leucine injection to relieve the increase expression level of *Rheb* due to dietary restriction. There was still a significant 3.0-fold increase in *Rheb* mRNA in the group injected with leucine and RAPA compared to control group ($p < 0.05$) (Fig. 6).

Discussion

The Small GTPase Rheb protein that can bind directly to mTORC1 and play a positive role in the regulation of mTOR signaling pathway is involved in the activation of protein synthesis and growth (Sato *et al*., 2008). In the present study, the full-length cDNA of *Rheb* was cloned from *L. vannamei*. The deduced polypeptide of Rheb consisted of 182 amino acids, and its calculated molecular weight was 20.55 kDa, which was close to those from vertebrate and invertebrate (Fig. 1). The amino acid sequence of Rheb shared over 60% similarities with other identified Rhebs (Fig. 2). Moreover, a typical Ras domain (Fig. 1) was found which can react selectively and non-covalently with GTP (Akashi *et al*., 2007). The switch I domain (switch I) is a highly conservative region that is crucial for the direct bond between mTOR and Rheb (Long *et al.*, 2007). Our work also verified the viewpoint that the decapod crustacean Rheb contained the five highly conserved G boxes which are related to GTP binding and GTPase activity (Maclea *et al*., 2012). As shown in Fig. 3, the Rheb is a very conservative protein. The amino acid sequence of Rheb in *L. vannamei* shared more than 60% similarity with other identified Rheb proteins.

To investigate the function of Rheb in controlling cell function of shrimp, the distribution of its mRNA in different tissues was detected by RT-PCR technique. The Rheb mRNA transcripts were observed consistently expressed in all the detected tissues. The analogous expression of *Rheb* in the tissues indicated that it played vital role in the regulation of cell function. The reason for lower expression of *Rheb* in intestine maybe was that intestine is the main tissue absorbing amino acids that makes the concentration of amino acids in intestine is higher than other tissues. In order to prevent the irrational regulation of mTOR signaling pathway caused by amino acids, the low expression of *Rheb* could be essential for the cell function regulation in intestine.

To further understand the regulative roles of Rheb in mTOR signaling pathway, we depressed the mTOR signaling pathway by RAPA. The temporal expression profile in muscle post RAPA stimulation was detected by RT-PCR technique. We found the expression of *Rheb* increased when the mTOR signaling pathway was depressed by RAPA. The highest expression was found when we injected RAPA into shrimps for 1 h. which was 19.53-fold ($p < 0.05$) of that in control group. Previous studies found that *Rheb* overexpression activates TORC1 signaling (Sun *et al.,* 2008). Ma *et al.* (2008) identified that Rheb binds specifically to FKBP38

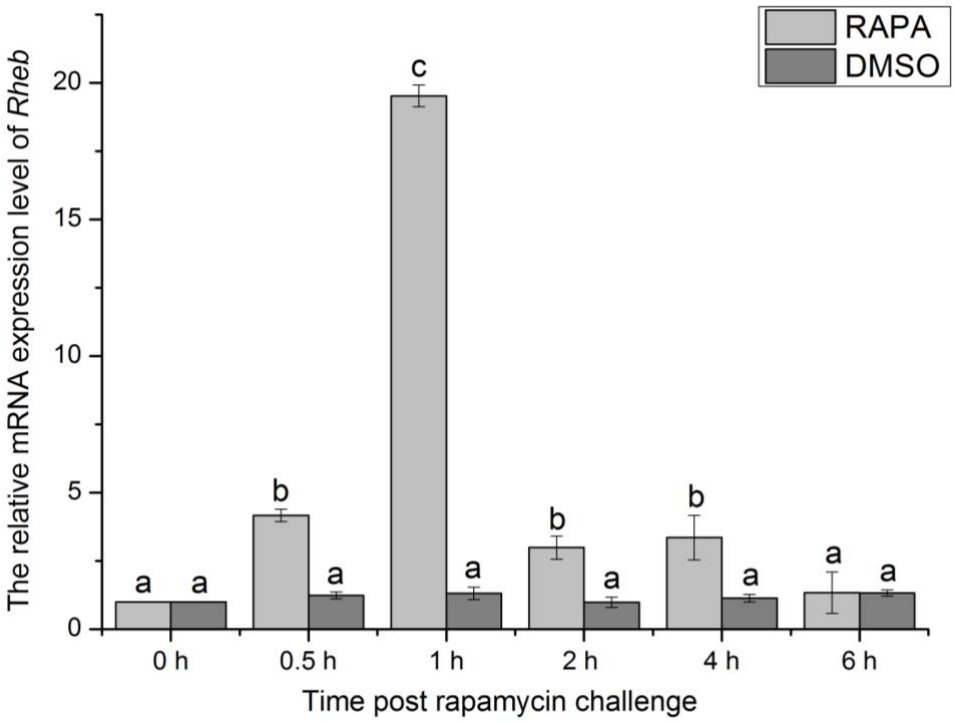

Fig. 5 Temporal mRNA expression profiles of *Rheb* detected by RT-PCR in shrimp muscle at 0, 0.5, 1, 2, 4 and 6 h post RAPA stimulation. The shrimps injected with DMSO were employed as control groups. *β-actin* gene was used as an internal control to calibrate the cDNA template for all the samples. Each value was shown as mean ± S.D. (n = 5), and bars with different characters indicated significantly different ($p < 0.05$).

(highly similar to FKBP12) in a GTP-dependent manner through its switch 1 region in HEK293 cells. The combination between Rheb and FKBP38 can displace FKBP38 (an mTOR inhibitor) from the FKBP/RAPA-binding domain and contributes to mTORC1 activation (Avruch *et al*., 2009). Our work suggested that the feedback regulations between Rheb and mTOR were existing in shrimp. The depression of mTOR signaling pathway caused by RAPA can promote the expression of *Rheb. Rheb* overexpression might be able to activate TORC1 signaling in shrimp, too. However, as yet unknown and the considerable additional work is needed to elucidate the feed-back mechanisms by which the Rheb–TOR interaction promotes TOR kinase activity.

mTOR output is sensitive to amino-acid (especially leucine and arginine) (Long *et al*., 2005a). Furthermore, Kimball *et al*. found leucine caused the most obvious stimulation of the TOR signaling pathway compared with other amino acids (Jefferson *et al*., 2001). So, we injected shrimps which were deprived of food for three days with leucine, arginine or rapamycin alone or leucine and rapamycin combination to explore the regulation of *Rheb* expression related to mTOR pathway under these circumstances. Previous works have found dietary restriction (DR) can reduces mTORC1 activity (Mejia *et al*., 2015; Garratt *et al*., 2016). In our work, The DR group expression level of *Rheb* was obviously increased. This result further proved the existence of feedback regulation between Rheb and mTOR. Previous research has proved the inhibition of mTORC1 signaling path caused by leucine withdrawal can be completely reversed by overexpression of *Rheb* (Avruch *et al*., 2009). In our work, the adding of leucine and arginine to shrimp deprived food for three days can relieve the accelerated expression of *Rheb*. The result proved that Rheb was regulated by amino acids, and the regulation play an important role in mTOR signaling path in shrimp. In “Leu + RAPA” group, we injected leucine and RAPA into shrimps. We found that RAPA can totally hinder the regulation of leucine to *Rheb* compared with leucine group in shrimp.

Long X *et al*. found that Rheb-mTOR interaction can activate mTORC1 activity in vitro and the interaction appears to be regulated by amino acid (Long *et al*., 2005a; Long *et al*., 2005b). Some work has been proved that Rheb can relieves the inhibition mTOR signaling path caused by RAPA (Ma *et al*., 2008). Our results are consistent with these findings. So, we suspect that the inhibitory effect of RAPA-FKBP12 complex and the positive impact of Rheb on mTOR signaling pathway are mutually inhibited in shrimp. The dominant RAPA hamper the activation of mTOR caused by amino acid. So, the *Rheb* expression of “Leu + RAPA” group don’t have obvious change compared with DR group. And it was shown that the DR and RAPA injection exhibited a similar effect on the expression of *Rheb* (Fig. 6).

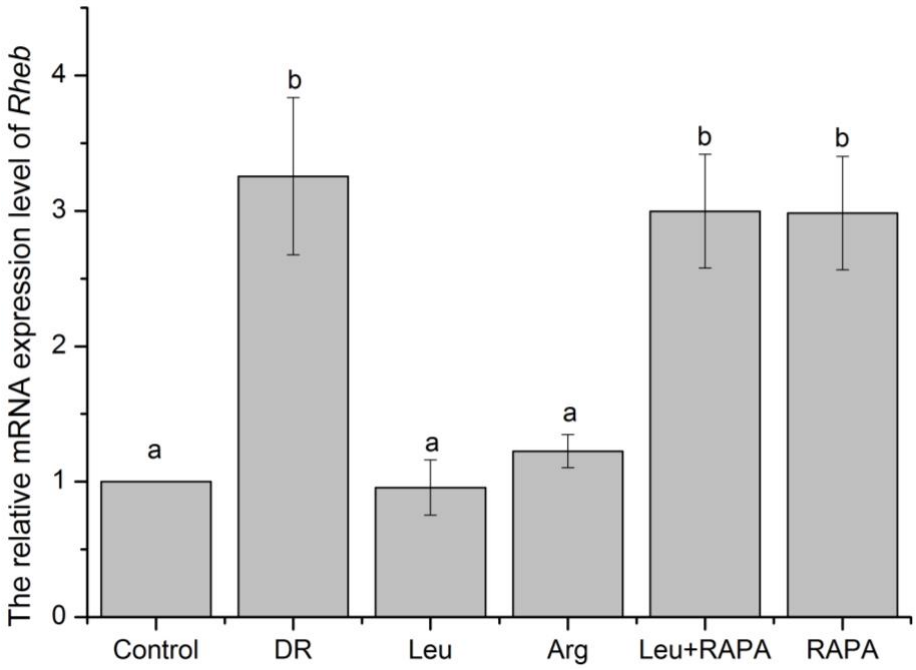

Fig. 6 The mRNA expression profiles of *Rheb* detected by RT-PCR post dietary restriction (DR), amino acid and RAPA stimulation. The shrimps injected with PBS was the control group. The expression profiling of second group was done 3 days after DR and 30 min after injection with PBS. The mRNA expression profiles of other groups were done 30 min after injection with leucine (Leu), arginine (Arg), rapamycin (RAPA) and "leucine + rapamycin" (Leu + RAPA). *β-actin* gene was used as an internal control to calibrate the cDNA template for all the samples. Each value was shown as mean ± S.D. (n = 5), and bars with different characters indicated significantly different ($p < 0.05$).

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Competing financial interests

The authors declare no competing financial interests.

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