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The small GTPase Rheb is a key component linking amino acid signaling and TOR in the nutritional pathway that controls mosquito egg development

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Abstract

Mosquitoes transmit numerous devastating human diseases because they require blood feeding for egg development. Previously, we have shown that the nutritional Target-of-Rapamycin (TOR) pathway mediates blood meal activation of mosquito reproductive cycles. Blood-derived amino acid (AA) signaling through the nutrient-sensitive TOR kinase is critical for the transcriptional activation of the major yolk protein precursor (YPP) gene, vitellogenin (*Vg*), initiation of vitellogenesis and egg development. In this study, we provide *in vitro* and *in vivo* evidence that the Rheb GTPase (Ras Homologue Enriched in Brain), which is an upstream activator of TOR, is required for AA-mediated activation of the TOR pathway in the fat body of the mosquito *Aedes aegypti*. Using RNA interference (RNAi) methods, we showed that Rheb was indispensable in AA-induced phosphorylation of S6 kinase, a key downstream substrate of TOR activation. *Rheb* RNAi depletion resulted in significant downregulation of *Vg* transcription and translation in the mosquito fat body, which was monitored *in vivo* after blood meal or *in vitro* organ culture after AA stimulation. Egg development was severely hindered in mosquitoes with a *Rheb* RNAi depletion background. This study represents a notable step in deciphering molecular pathways controlling reproduction of this important vector of human diseases.

Keywords

RNA interference; S6 kinase; vitellogenin; egg development; fat body; nutrition

1. Introduction

Mosquito-borne diseases, such as malaria and Dengue fever, are among the most threatening in modern times. Throughout the course of evolution, many species of mosquitoes have developed variations in their requirement for blood as a food resource in order to initiate and maintain egg development. Anautogenous mosquitoes use a reproductive strategy that requires the intake of vertebrate blood to obtain nutrients for each cycle of egg development. Repeated cycles of blood feeding and egg development make mosquitoes an efficient vehicle by which disease pathogens can spread from one host to another. Thus, a detailed

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understanding of the reproductive processes at the molecular level may reveal new insights for interrupting the process of disease transmission.

In mosquitoes, vertebrate blood is an important source of proteins for the development of eggs. Amino acids (AAs) derived from the blood meal are used by the mosquito fat body, a tissue analogous to vertebrate liver and white fat tissue, to synthesize yolk protein precursors (YPPs) (Raikhel et al., 2002). These YPPs are then taken up by the ovaries and deposited into developing oocytes (Raikhel & Dhadialla, 1992). In anautogenous mosquitoes, the reproductive system is held in a state of arrest during which the expression of *YPP* genes is repressed and the ovarian development halts until the mosquito acquires a blood meal. After such a meal, the *YPP* genes shift to a remarkable level of activation, a phenomenon termed vitellogenesis. In the mosquito *Aedes aegypti*, vitellogenin (*Vg*) is the most highly expressed and best characterized *YPP* gene. Transcription of this gene is regulated by the combined inputs of the steroid hormone 20-hydroxyecdysone (20E) cascade and nutritional AA/Target-of-Rapamycin (TOR) signaling (Raikhel et al., 2005; Attardo et al., 2005). *Vg* transcript expression follows the 20E titer, which reaches its peak at around 24 h post-blood meal (PBM) (Martin et al., 2001; Fallon et al., 1974; Wheelock & Hagedorn, 1985). However, 20E alone is not capable of activating vitellogenesis and subsequent egg maturation, and signaling by AAs via TOR is required (Hansen et al., 2004).

In mosquitoes, the nutritional AA/TOR signaling is regulated by increased concentration of specific AAs, particularly leucine, in the hemolymph after a blood meal (Attardo et al., 2006). The serine/threonine kinase TOR is responsible for transducing the AA signal, activating downstream events of vitellogenesis in the fat body cells (Hansen et al., 2004). Inhibition of TOR by either the drug rapamycin or RNA interference (RNAi)-mediated gene depletion has been shown to result in a severe downregulation of *Vg* gene transcription after AA stimulation in an *in vitro* fat body culture system and inhibition of egg development *in vivo*. The AA-dependent nutrient signaling mediates the phosphorylation of S6 kinase (S6K) in the fat body (Hansen et al., 2005). In turn, S6K is required for activation of translational events, including that of the GATA factor, which is a key regulatory factor of *Vg* gene transcription (Attardo et al., 2003; Park et al., 2006).

The TOR pathway integrates extracellular signals derived from growth factors, stress or nutrients such as AAs (Nave et al., 1999; Jacinto & Hall, 2003; Raught et al., 2001; Colombani et al., 2003). The small GTPase Rheb (Ras Homologue Enriched in Brain) positively activates the protein kinase activity of TOR complex 1 (TORC1) (Saucedo et al., 2003; Sarbassov et al., 2005; Stocker et al., 2003; Castro et al., 2003; Garami et al., 2003; Patel et al., 2003). The Rheb GTP-binding proteins define a unique family within the Ras superfamily of G-proteins and it is found in many species, ranging from yeast to mammals (Urano et al., 2000). Rheb has received considerable attention due to its critical role in regulating growth and cell cycle through the insulin/TORC1 signaling pathway (Li et al., 2004; Manning & Cantley, 2003). Epistasis studies in *Drosophila* placed Rheb downstream of the tuberous sclerosis tumor suppressor protein complex (TSC), a repressor of TORC1, but upstream of TORC1 (Marygold & Leever, 2002; Gao et al., 2002; Zhang et al., 2000; Zhang et al., 2003). The Rheb constitutes a major component of the insulin-mediated branch of the TOR pathway that regulates cell growth in eukaryotic organisms (Inoki et al., 2005; Yamagata et al., 1994). Overexpression of Rheb in *Drosophila* results in increased cell and tissue size, whereas reduced Rheb leads to a decrease in the same (Saucedo et al., 2003; Stocker et al., 2003; Garami et al., 2003; Patel et al., 2003). Although several studies have implicated Rheb in the AA nutritional branch of the TOR pathway, its precise role in mediating AA activation of TOR is not completely clear (Avruch et al., 2009; Zhang et al., 2003).

Considering the importance of the nutritional signaling in reproduction of mosquitoes, we sought to further characterize the TOR pathway components and determine their role in mosquito egg development. Our present study has revealed that Rheb is required for the AA-mediated TOR activation of vitellogenic events in the mosquito fat body. These results provide direct proof of the role of Rheb as a major upstream signal transducer involved in the nutritional branch of the TOR pathway.

2. Materials and Methods

2.1. Mosquito rearing and in vitro fat body culture

The *A. aegypti* mosquito strain UGAL/Rockefeller was maintained in laboratory culture as described in by Hansen et al. (2005). Other specific experimental details regarding mosquito culture and fat body *in vitro* culture were followed as described previously (Roy et al., 2007).

2.2. Reagents used for in vitro fat body culture

The dissected fat bodies were incubated in *Aedes* physiological saline (APS) and in medium either lacking AAs (containing equimolar amounts of mannitol in place of AAs) or containing AAs together with other reagents (Attardo et al., 2006). Henceforth, the former will be referred to as AA[−] medium and the latter as AA⁺ medium. The detailed composition for *A. aegypti* incubation media can be found elsewhere (Roy et al., 2007). 20E was obtained from Sigma.

2.3. Cloning and Sequencing of Rheb cDNA from *A. aegypti*

Standard procedures were used for recombinant DNA manipulations. Expressed sequence tag cDNA sequences coding for the *A. aegypti* Rheb gene were identified in the MIT BROAD (http://www.broad.mit.edu/annotation/genome/aedes_aegypti.2/Blast.html) database, using the *Drosophila* Rheb protein as the template (tBLASTn). Common PCR techniques, using gene-specific primers, were employed for the amplification of the full-length Rheb cDNA from the fat body cDNA pool of *A. aegypti*. All PCR products were cloned in pCRII-TOPO vector (Invitrogen).

The full-length cDNA and deduced AA sequences of *AaRheb* were compared using the BLAST tool at the National Center for Biotechnology Information (NCBI). Sequence alignments were performed using the T-COFFEE server (<http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee.cgi/index.cgi>) via the Clustal algorithm (Notredame et al., 2000). Conserved AA sequences between different species were shaded using the BOXSHADE program. The values for the fraction of sequences that must agree to shading were set for at least 60% identity.

2.3. RNA extraction, reverse-transcription and quantitative PCR

Total RNA from dissected mosquito fat bodies was extracted using a well-established methodology and reverse-transcription PCR (Supplemental Material).

Quantitative PCR (qPCR) was performed using the iCycler iQ system (Bio-Rad, Hercules, CA), and reactions were performed in 96-well plates using TaqMan primers/probes for *Vg* and SYBR green primers for *S7* ribosomal protein (internal control). We used a qPCR master mix, iQ Supermix (Bio-Rad) for the TaqMan reactions or the iQ SYBR green supermix (Bio-Rad) for the SYBR green reactions. All qPCR reactions were run in duplicate using 2 µl cDNA per reaction. Data were generated from three different cohorts of female mosquitoes for each experiment. Quantitative measurements were performed in triplicate and normalized to the internal control of *S7* ribosomal mRNA for each sample (except for

Fig.1). *In vivo* fat body Rheb transcript expression levels (Fig.1) were standardized by total RNA input, because the fat body is a dynamically developing tissue, both before and following a blood meal, thus precluding the use of a 'normalizing' transcript. Time points were chosen for the current study to represent the complete vitellogenic cycle: pre-vitellogenesis (1–4 days pre-vitellogenesis), vitellogenesis (6–30 h PBM), early post-vitellogenesis (36–48 h PBM) and late post-vitellogenesis (72 h PBM), with these progressions including, respectively, active ribosomal biogenesis, massive protein synthesis, tissue autophagy and ribosomal biogenesis again. This developmental course can be observed through the dynamic expression profile of the commonly used 'housekeeping' transcript ribosomal protein S7, as well as Actin (not shown). Such a condition is not applicable to the *in vitro* experiments, because all fat bodies used in these studies were at the same developmental stage, hence the use of ribosomal protein S7 transcripts as the 'housekeeping' normalizer.

The standard curves for qPCR experiments were generated using a serial dilution of the cDNAs/PCR products containing transcript of the genes of interest. The lowest dilution of the standard curve was given an arbitrary value of 1×10^5 ; subsequent values for five lower dilutions, which were 10-fold serially diluted, were obtained using the lowest dilution as the reference. Amounts of amplicon in the test samples were generated by comparing with the standard curve. Hence, the term "relative" means that these samples were measured relative to the standard curve of the gene concerned. The number on the Y-axis thus represents a relative value and has no units as such.

Primers and probes (all TaqMan probes used the Black Hole Quencher and were synthesized by Qiagen) are as follows: Vg forward, 5'-ATGCACCGTCTGCCATC; Vg reverse, 5'-GTTCTAGTTGG-AAAGCTCG; Texas Red labeled Vg probe, 5'-AAGCCCCGCAACCGTCCGTACT; S7 forward, 5'-TCAGTGTACAAGAAGCTGACCGGA; S7 reverse, 5'-TTCCGCGCGCGC-TCATTATTAGATT; Rheb forward, 5'-GCTCAGTATTCAGTTCGTCGAAGGGC; Rheb reverse, 5'-ACCTCGTAGTCGGTTGAGTTGACG. Primers used specifically for reverse-transcription PCR analysis: Rheb forward (for RNAi confirmation), 5'-ACTGTGCGAACGCAATTCGGAAGC; Rheb reverse (for RNAi confirmation), 5'-GGCTACTGTGATTGGGCTGGGGAGA. Reactions were carried out as described previously (Attardo et al., 2003). qPCR data were collected by the ICYCLER IQ REAL TIME DETECTION SYSTEM SOFTWARE V3.0 for WINDOWS. Raw data were exported to EXCEL (Microsoft) for analysis.

2.4. Protein extraction and Western blot analysis

Total protein extracts were prepared from dissected fat bodies and subjected to Western blot analysis using a well-established methodology (Supplemental Material). This was performed using antibodies that detect phosphorylated S6 kinase (Thr-412; number 07-018, Upstate, Lake Placid, NY). These polyclonal antibodies detect the human S6 kinase phosphorylated on Thr 412 and the human splice variant of human S6 kinase phosphorylated on Thr 389 [Thr 388 in *A. aegypti*]. The antibody detects a phosphoprotein with a molecular mass of ~62 kDa (Hansen et al., 2005). The antibodies against native S6 kinase protein were also used for loading controls (p70 S6K antibody from Cell Signaling, catalog no. 9202). Identifying a protein band as belonging to S6k has been confirmed through S6k RNAi depletion in the mosquito fat body (data not shown). A mixture of monoclonal antibodies raised against the small subunit (66 kDa) of the mature Vg protein was used to estimate the Vg protein level in immunoblots (Raikhel et al., 1986). Mouse monoclonal antibodies against β -actin were purchased from Sigma. Each experiment was performed at least three times using different cohorts of female mosquitoes and had similar outcomes. In this case, data from a typical experiment is shown.

2.5. Rheb depletion by RNA interference

Generation of Rheb dsRNA and mosquito injections were performed according to previously described techniques (Roy et al., 2007). One day after emergence, female mosquitoes were injected with 1 µg of Rheb dsRNA. After a 5-day recovery period, mosquitoes were given a blood meal and examined for the effect of Rheb depletion. dsRNA from a small portion of the bacterial gene *Mal* (*dsMal*) was used as a control. The control dsRNA *Mal*, which contains a nonfunctional part of the *E. coli MalE* gene that encodes the maltose-binding protein, was amplified from the plasmid 28iMal (NEB). After a 5-day recovery period, mosquitoes were either given a blood meal or directly dissected and incubated in vitro in various media and examined for the effect of Rheb depletion.

3. Results

3.1. Cloning and characterization of *A. aegypti* Rheb

We cloned the Rheb cDNA from the Rockefeller/UGAL strain of the mosquito *A. aegypti* (GenBank™ accession number FJ357570). The sequence data for Rheb (Liverpool strain) has also been independently reported in the GenBank™ database under accession number XM_001658963 and in the Vectorbase (Vectorbase.org) database under AAEL008179. This cDNA codes for a protein comprised of 182 AAs and with a relative molecular mass of about 20.53 kDa. The cDNA cloned from our strain (Rockefeller/UGAL) was shown to differ slightly from that of the Liverpool strain (MIT BROAD/Vectorbase/ GenBank™ data) and differences are noted as follows: in our Rockefeller/UGAL strain, (1) at nucleotide position 168, G changed to A; (2) at nucleotide position 486, A changed to G; and (3) at nucleotide position 525, A changed to G. The deduced Rheb protein sequence was 100% identical in both these strains. Of note, Rheb protein sequences have been shown to be highly identical and the domain structure conserved in all known Rheb proteins (Supplemental Fig.S1 & Supplemental Table 1). AaRheb exhibits 97% overall AA identity with *Culex*, 62% with humans, and 40% with the *C. elegans* (Supplemental Table 1).

3.2. Expression profile of Rheb mRNA in *A. aegypti* female fat body

To determine whether the *Rheb* gene identified from the *A. aegypti* genome (*AaRheb*) is expressed in adult female fat bodies, we used qPCR to obtain a detailed expression profile of the *AaRheb* gene. RNA was collected from pre-vitellogenic (PV) and blood-fed female mosquitoes PBM at several time points (Fig. 1). Rheb mRNA was found in both PV and vitellogenic mosquito fat bodies. *AaRheb* transcript was expressed at the higher levels at the beginning of previtellogenesis, with a gradual decrease in abundance toward 120 h PV; it increased again after the completion of vitellogenesis at 48 h and 72 h PBM (Fig. 1). We examined the relative levels of ribosomal S7 mRNA, a housekeeping gene commonly used as a control in expression studies. However, in the mosquito fat body, S7 mRNA levels were not constant, reflecting dramatic and rapidly changing transcriptional activity in this tissue during egg developmental cycles, making it difficult to use as a control (Supplemental Fig. S2).

3.3. Rheb is required for AA-mediated TOR activation and S6k phosphorylation

To determine whether AaRheb is involved in transducing AA-mediated signal to TOR, we used a reverse-genetic approach to disrupt the function of the *AaRheb* gene *in vivo* through the utilization of RNAi, which causes the disruption of specific mRNA in response to the presence of double-stranded RNA homologous to the mRNA of interest (Hannon, 2002). TOR signaling is necessary for AA-dependent phosphorylation of S6k in the fat body of female mosquitoes. The presence of AAs causes an increase in S6k phosphorylation, thereby

promoting protein translation (Hansen et al., 2005). Henceforth, phosphorylation of S6k can serve as a reliable readout of TOR activity.

One-day-old female mosquitoes were injected with dsRheb, ds4E-BP (eukaryotic initiation factor 4E binding protein) or the control dsMal. We wanted to confirm that depletion of Rheb via RNAi is specific to the phosphorylation of S6k and that activation of the RNAi machinery causes no aberrant effects on the TOR pathway itself. Therefore, RNAi against 4E-BP, another well-known downstream effector of TOR, served as a control in addition to dsMal. RT-PCR showed that the level of Rheb mRNA was significantly reduced in fat bodies from mosquitoes with Rheb-depleted background when compared with Mal control, but not completely eliminated (Fig. 2A). After 5–6 days of recovery, the dissected fat bodies were incubated in the AA+ culture medium for 3 h, then immediately homogenized in ice-cold protein breaking buffer. Total proteins were extracted and subjected to SDS PAGE. Western blot analysis was performed using a phospho-S6k antibody. The full-length open reading frame of this protein consists of 550 AAs and has a molecular weight of approximately 62 kDa (Hansen et al., 2005). In both the dsMal control and the ds4E-BP-injected mosquitoes, S6k was highly phosphorylated in the presence of AAs. Under the same conditions, S6k phosphorylation was not detectable in the fat bodies of dsRheb-treated mosquitoes (Fig. 2A). Thus, RNAi depletion of Rheb renders the fat body unresponsive to the AA signaling, indicating that Rheb is essential for AA-mediated TOR activation.

In the next experiment, mosquitoes were injected in the same way as described above. Fat bodies were collected 3, 6 and 24 h PBM and Western blot analysis was performed to evaluate the phosphorylation status of S6k. Previously, we reported that, in wild-type mosquitoes, S6k was highly phosphorylated within 3–6 h PBM and reduced thereafter (Hansen et al., 2005). In our present experiments, the level of S6k phosphorylation was high at 3 h and 6 h PBM and undetectable at 24 h PBM in mosquitoes with a Mal background; this concurs with our previous study. S6k phosphorylation level in the fat bodies from mosquitoes with Rheb depletion background was significantly reduced when compared with Mal controls (Fig. 2B). Residual low levels of S6k phosphorylation observed at 3 h and 6 h PBM in fat bodies from dsRheb-treated mosquitoes was likely due to incomplete depletion of Rheb mRNA by RNAi. Taken together, results of *in vitro* and *in vivo* experiments strongly suggest that Rheb is an intermediate factor in the AA/TOR pathway that mediates the AA signal transduction in regulating activation of TOR and S6k.

3.4. RNAi depletion of Rheb inhibits vitellogenin expression and egg development

To investigate the role of *AaRheb* in activation of downstream events in response to AA signaling, we first evaluated *Vg* gene expression in *AaRheb*-depleted females (*in vivo*). As seen in Fig. 3A, females with depleted *AaRheb* demonstrated an approximate 50% reduction in *Vg* gene expression level when compared with the controls (*Mal* & WT). Western blot analysis using a mixture of monoclonal antibodies, a technique which recognizes the small 66-kDa *Vg* subunit, was utilized to detect changes in the *Vg* protein level. Again, there was a significant reduction in the amount of *Vg* protein in fat bodies of females with depleted *AaRheb* at 3, 6 and 24 h PBM when compared with the Mal control (Fig. 3B). However, the *Vg* protein level did increase over time in fat bodies of females with depleted *AaRheb*, most likely due to incomplete removal of Rheb mRNA. Existence of an alternative regulatory pathway complementary to the AA/TOR pathway cannot be ruled out.

To confirm that the *Rheb* depletion effect on *Vg* expression and synthesis is due to the role of Rheb in mediating AA signaling, we conducted an *in vitro* fat body culture assay. *AaRheb* RNAi was performed as described above, and fat bodies dissected from PV females 5 days after Rheb dsRNA or Mal dsRNA injections were incubated in either AA+ or AA- culture media. qPCR assay showed a significant reduction in *Vg* gene expression in fat bodies of

Rheb-depleted mosquitoes incubated in the AA+ culture medium when compared with the *Mal* control treated similarly (Fig. 4A). Western blot analysis revealed that, under these conditions, Vg protein was not present at a detectable level in fat bodies of the *Rheb* RNAi-treated mosquitoes, although a considerable amount of Vg was present in the *Mal* control tissue (Fig. 4C). Thus, these results of *in vitro* and *in vivo* experiments indicate that *Rheb* is required for AA signaling in activation of Vg gene expression and subsequent translation.

20E is the key regulator of *YPP* gene expression in the mosquito fat body (Raikhel et al., 2005; Sun et al., 2002). However, the nutritional AA/TOR pathway is required for this gene-specific action of 20E (Hansen et al., 2004; Attardo et al., 2005). Because the experiments described above suggested that *Rheb* is an essential factor in mediating the AA signal to TOR, RNAi depletion of *Rheb* was expected to block 20E-dependent Vg gene expression in the presence of AAs. Incubation of fat bodies from mosquitoes with a *Mal* depletion background in the AA+ medium containing 20E resulted in a strong upregulation of Vg expression. In contrast, Vg transcript level was only slightly elevated in the fat bodies from *Rheb* RNAi-treated mosquitoes incubated in the same medium (Fig. 4B). The reason for this slight increase is unclear. It is possibly due to incomplete removal of *Rheb* mRNA by RNAi. Although, there was no statistically significant elevation of Vg mRNA levels in fat bodies with *Rheb* RNAi background exposed to AAs and 20E (Fig. 4B, last column) relative to those in *Rheb*+ control tissue supplied with AAs and lacking 20E (Fig. 4B, third column), Vg was produced only in the latter sample.

In order to evaluate the effect of *Rheb* depletion on ovarian development, ovaries from experimental and control mosquitoes were examined 24 h and 48 h PBM. In mosquitoes treated with *Rheb* dsRNA, there was a dramatic reduction in ovary size compared with that in the ds*Mal*-injected mosquitoes; the number and size of developing eggs were also reduced (Fig 5A). Some ds*Rheb*-treated mosquitoes had ovaries with no egg development (data not shown). A significant reduction in the number of eggs deposited was also observed in these *Rheb* dsRNA-injected mosquitoes when compared with the *Mal* control (Fig 5B).

4. Discussion

In this study, we have demonstrated the importance of *Rheb*, an activator of TOR, in egg development of the blood-feeding insect *A. aegypti*. Blood-feeding, anautogenous mosquitoes have developed a unique lifestyle in which to ensure that adequate nutrients are obtained for rapid and massive egg development; AAs signaling through the TOR pathway serve as a gateway for commencement of vitellogenesis and egg maturation. Although 20E is the major regulator of vitellogenesis, it cannot initiate this process without the involvement of AA/TOR signaling (Hansen et al., 2004; Attardo et al., 2005). One of the key mechanisms linking these two regulatory cascades is the TOR-dependent translation of GATA factor, which is required for initiation of transcription of 20E-dependent target genes, such as Vg (Park et al., 2006).

Here, we have shown that *Rheb* is an integral part of the AA branch of the TOR pathway and is required for mosquito egg development. The *Rheb* transcript level remains elevated during the PV period and again after the cessation of vitellogenesis in the mosquito fat body. This pattern of *Rheb* expression is similar to that of other genes coding for components of the AA/TOR signaling pathway—such as AA-transporters, TOR and S6k (Shiao et al., 2008)—suggesting that *Rheb*, as for those other genes, is expressed at the higher level in preparation for transduction of nutritional blood-meal-activated signaling at the onset of egg developmental cycles. Such an elevation of expression in the mosquito fat body does not appear to correlate with nutrient starvation as has been demonstrated in other organisms (Panepinto et al., 2002). In contrast, their expression is controlled by juvenile hormone

(Shiao et al., 2008). In mosquitoes, this hormone regulates PV events required for female competency for subsequent blood feeding and egg development (Zhu et al., 2006; Zhu et al., 2003).

To examine the requirement of Rheb in AA-mediated activation of TOR in the mosquito fat body, we took advantage of biochemical readout of TOR function: S6k phosphorylation (Hansen et al., 2005; Long et al., 2005b; Long et al., 2005a). Indeed, RNAi-mediated Rheb silencing strongly downregulated S6k phosphorylation, which is normally activated in mosquito fat bodies by a blood meal. Importantly, *in vitro* organ culture experiments showed that RNAi depletion of *Rheb* rendered the fat body irresponsive to AA-mediated S6k phosphorylation. Hence, our data indicate that Rheb is absolutely essential for AA-mediated TOR activation.

Our previous studies have established a key role of the nutritional AA/TOR pathway in activation of vitellogenesis (Hansen et al., 2004; Attardo et al., 2005; Hansen et al., 2005; Attardo et al., 2006; Park et al., 2006). Dependence of *Vg* gene transactivation in the mosquito fat body on the presence of Rheb, shown in the current study, serves as an important confirmation of the requirement of Rheb in the AA signaling. RNAi depletion of *Rheb* significantly downregulated *Vg* expression in blood-fed female mosquitoes and rendered *in vitro* AA-treated fat bodies incapable of activating *Vg* expression. The overall translation of the *Vg* protein was also severely affected in Rheb-depleted female mosquitoes. AAs alone have been shown to activate low levels of both transcription and translation of *Vg*, but the presence of 20E increases this effect several fold (Hansen et al., 2004; and this study). The transactivating action of 20E on genes such as *Vg* in the mosquito fat body depends on prior AA/TOR signaling (Hansen et al., 2004). Our experiments showed that RNAi Rheb depletion blocked this transactivating ability of 20E. When fat bodies from mosquitoes with a Rheb-depleted background were incubated in the presence of 20E in the AA+ medium, *Vg* gene expression was severely impaired, but its expression was greatly upregulated in Mal controls. Taken together, these experiments clearly demonstrate that Rheb is indispensable for the AA/TOR signaling in its crosstalk with the 20E regulatory pathway in controlling vitellogenic events in the mosquito female fat body.

Although, our results have clearly shown that Rheb is necessary for AA-mediated activation of TOR during mosquito egg development, the mechanism by which it positively signals TOR is yet to be clarified. Rheb has been shown to bind directly to the amino-terminal lobe of the mammalian TOR (mTOR) catalytic domain and activate mTOR in a GTP-dependent manner (Long et al., 2005a). Withdrawal of all extracellular AAs or of leucine alone results in inhibition of the binding of Rheb to mTOR (Long et al., 2005b). Another study suggested that Rheb activates the mTOR complex by antagonizing its inhibitor FK506 binding protein 38 (FKBP 38). Rheb binds directly with FKBP 38 in a GTP-dependent manner to prevent its association with the mTOR (Bai et al., 2007). FKBP38 interacted with mTOR in cells deprived of AAs, and the interaction was reduced in the presence of AAs. Moreover, overexpression of Rheb also reduced the interaction of FKBP38 with mTOR in AA-deprived cells, and the interaction was further diminished when AAs were restored (Bai et al., 2007). Recently, four Rag proteins (Ras-related GTP binding protein) have been implicated in AA sensing (Kim et al., 2008; Sancak et al., 2008). In the presence of AAs, the RagA/B-RagC/D^{GTP} heterodimer was activated and bound directly to TOR subunit raptor. However, Rag did not appear to activate mTOR kinase directly; instead, in the presence of AAs Rag^{GTP}, it was observed to promote translocation of mTOR from diffuse locations throughout the cytoplasm to vesicular compartments that contain Rheb (Kim et al., 2008; Sansak et al., 2008). This is in agreement with a previous observation showing that AAs promote the association between Rheb and mTOR (Long et al., 2005a; 2005b). Mosquito TOR, which has a diffuse cytoplasmic distribution pattern in fat body cells before a blood

meal, dramatically changes its localization, concentrating near the plasma membrane after activation by a blood meal (Hansen et al., 2005). Whether mosquito Rheb is closely associated with the TOR after activation by a blood meal remains to be established. Future study should investigate further the mechanism of AA sensing in the mosquito.

Finally, our data demonstrated that silencing of *AaRheb* blocks mosquito egg development. Consequently, RNAi-mediated Rheb knockdown was shown to result in a significant inhibition of egg development, a phenomenon similarly seen earlier in S6k-depleted female mosquitoes, causing a severely reduced number of eggs deposited at the end of the reproductive cycle (Hansen et al., 2005). Pertinent to this fact, it has previously been reported that female *Drosophila* with a viable loss-of-function mutant *Rheb* allele had rudimentary ovaries and were sterile (Stocker et al., 2003). These reports suggest that manipulation of this pathway can lead to a reproductive failure, which in turn may be useful in devising various pest control strategies. Dramatic inhibition of Vg production in the fat body as a result of Rheb depletion has undoubtedly a significant negative effect on ovarian development in these mosquitoes. However, because the RNAi approach used in this study is not tissue specific but rather systemic in nature, we cannot rule out the direct effect of Rheb depletion on ovarian development. Targets of the AA/TOR pathway in the mosquito ovary in which Rheb plays an integral role remain to be established in future research.

In conclusion, our present study has contributed to deciphering the nutritional regulation of female reproduction in anautogenous mosquitoes, which serve as major vectors of human diseases. We have shown that Rheb is indispensable for mediating the AA signaling to TOR-S6k and thereby is involved in the regulation of YPP synthesis in the fat body and is required for egg development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The nucleotide sequence reported in this paper has been submitted to the GenBank™ data bank with the accession number FJ357570.

References

- Attardo GM, Hansen IA, Raikhel AS. Nutritional regulation of vitellogenesis in mosquitoes: implications for anautogeny. *Insect Biochem Mol Biol* 2005;35:661–675. [PubMed: 15894184]
- Attardo GM, Hansen IA, Shiao SH, Raikhel AS. Identification of two cationic amino acid transporters required for nutritional signaling during mosquito reproduction. *J Exp Biol* 2006;209:3071–3078. [PubMed: 16888056]
- Attardo GM, Higgs S, Klingler KA, Vanlandingham DL, Raikhel AS. RNA interference-mediated knockdown of a GATA factor reveals a link to anautogeny in the mosquito *Aedes aegypti*. *Proc Natl Acad Sci U S A* 2003;100:13374–13379. [PubMed: 14595016]
- Avruch J, Long X, Ortiz-Vega S, Rapley J, Papageorgiou A, Dai N. Amino acid regulation of TOR complex 1. *Am J Physiol Endocrinol Metab* 2009;296:E592–E602. [PubMed: 18765678]
- Bai X, Ma D, Liu A, Shen X, Wang QJ, Liu Y, Jiang Y. Rheb activates mTOR by antagonizing its endogenous inhibitor, FKBP38. *Science* 2007;318:977–980. [PubMed: 17991864]
- Castro AF, Rebhun JF, Clark GJ, Quilliam LA. Rheb binds tuberous sclerosis complex 2 (TSC2) and promotes S6 kinase activation in a rapamycin- and farnesylation-dependent manner. *J Biol Chem* 2003;278:32493–32496. [PubMed: 12842888]

- Colombani J, Raisin S, Pantalacci S, Radimerski T, Montagne J, Leopold P. A nutrient sensor mechanism controls *Drosophila* growth. *Cell* 2003;114:739–749. [PubMed: 14505573]
- Fallon AM, Hagedorn HH, Wyatt GR, Laufer H. Activation of vitellogenin synthesis in the mosquito *Aedes aegypti* by ecdysone. *J Insect Physiol* 1974;20:1815–1823. [PubMed: 4411834]
- Gao X, Zhang Y, Arrazola P, Hino O, Kobayashi T, Yeung RS, Ru B, Pan D. Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. *Nat Cell Biol* 2002;4:699–704. [PubMed: 12172555]
- Garami A, Zwartkruis FJ, Nobukuni T, Joaquin M, Roccio M, Stocker H, Kozma SC, Hafen E, Bos JL, Thomas G. Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Mol Cell* 2003;11:1457–1466. [PubMed: 12820960]
- Hannon GJ. RNA interference. *Nature* 2002;418:244–251. [PubMed: 12110901]
- Hansen IA, Attardo GM, Park JH, Peng Q, Raikhel AS. Target of rapamycin-mediated amino acid signaling in mosquito anautogeny. *Proc Natl Acad Sci U S A* 2004;101:10626–10631. [PubMed: 15229322]
- Hansen IA, Attardo GM, Roy SG, Raikhel AS. Target of rapamycin-dependent activation of S6 kinase is a central step in the transduction of nutritional signals during egg development in a mosquito. *J Biol Chem* 2005;280:20565–20572. [PubMed: 15788394]
- Inoki K, Corradetti MN, Guan KL. Dysregulation of the TSC-mTOR pathway in human disease. *Nature Genetics* 2005;37:19–24. [PubMed: 15624019]
- Jacinto E, Hall MN. Tor signalling in bugs, brain and brawn. *Nat Rev Mol Cell Biol* 2003;4:117–126. [PubMed: 12563289]
- Kim E, Goraksha-Hicks P, Li L, Neufeld TP, Guan KL. Regulation of TORC1 by Rag GTPases in nutrient response. *Nature Cell Biology* 2008;10:935–945.
- Li Y, Corradetti MN, Inoki K, Guan KL. TSC2: filling the GAP in the mTOR signaling pathway. *Trends Biochem Sci* 2004;29:32–38. [PubMed: 14729330]
- Long X, Lin Y, Ortiz-Vega S, Yonezawa K, Avruch J. Rheb binds and regulates the mTOR kinase. *Curr Biol* 2005a;15:702–713. [PubMed: 15854902]
- Long X, Ortiz-Vega S, Lin Y, Avruch J. Rheb binding to mammalian target of rapamycin (mTOR) is regulated by amino acid sufficiency. *J Biol Chem* 2005b;280:23433–23436. [PubMed: 15878852]
- Manning BD, Cantley LC. Rheb fills a GAP between TSC and TOR. *Trends Biochem Sci* 2003;28:573–576. [PubMed: 14607085]
- Martin D, Wang SF, Raikhel AS. The vitellogenin gene of the mosquito *Aedes aegypti* is a direct target of ecdysteroid receptor. *Mol Cell Endocrinol* 2001;173:75–86. [PubMed: 11223179]
- Marygold SJ, Leivers SJ. Growth signaling: TSC takes its place. *Curr Biol* 2002;12:R785–R787. [PubMed: 12445406]
- Nave BT, Ouwens M, Withers DJ, Alessi DR, Shepherd PR. Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem J* 1999;344(Pt 2):427–431. [PubMed: 10567225]
- Notredame C, Higgins DG, Heringa J. T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J Mol Biol* 2000;302:205–217. [PubMed: 10964570]
- Panepinto JC, Oliver BG, Amlung TW, Askew DS, Rhodes JC. Expression of the *Aspergillus fumigatus* rheb homologue, rhbA, is induced by nitrogen starvation. *Fungal Genet Biol* 2002;36:207–214. [PubMed: 12135576]
- Park JH, Attardo GM, Hansen IA, Raikhel AS. GATA factor translation is the final downstream step in the amino acid/target-of-rapamycin-mediated vitellogenin gene expression in the anautogenous mosquito *Aedes aegypti*. *J Biol Chem* 2006;281:11167–11176. [PubMed: 16490782]
- Patel PH, Thapar N, Guo L, Martinez M, Maris J, Gau CL, Lengyel JA, Tamanoi F. *Drosophila* Rheb GTPase is required for cell cycle progression and cell growth. *J Cell Sci* 2003;116:3601–3610. [PubMed: 12893813]
- Raikhel AS, Dhadialla TS. Accumulation of yolk proteins in insect oocytes. *Annu Rev Entomol* 1992;37:217–251. [PubMed: 1311540]

- Raikhel, AS.; Brown, M.; Belles, X. Endocrine Control of Reproductive Processes. In: Gilbert, L.; Gill, S.; Iatrou, K., editors. *Comprehensive Molecular Insect Science*, Vol. 3, Endocrinology. Elsevier Press; 2005. p. 433–491.
- Raikhel AS, Kokoza VA, Zhu J, Martin D, Wang SF, Li C, Sun G, Ahmed A, Dittmer N, Attardo G. Molecular biology of mosquito vitellogenesis: from basic studies to genetic engineering of antipathogen immunity. *Insect Biochem Mol Biol* 2002;32:1275–1286. [PubMed: 12225918]
- Raikhel AS, Pratt LH, Lea AO. Monoclonal-Antibodies As Probes for Processing of Yolk Protein in the Mosquito - Production and Characterization. *Journal of Insect Physiology* 1986;32:879–&.
- Raught B, Gingras AC, Sonenberg N. The target of rapamycin (TOR) proteins. *Proc Natl Acad Sci U S A* 2001;98:7037–7044. [PubMed: 11416184]
- Roy SG, Hansen IA, Raikhel AS. Effect of insulin and 20-hydroxyecdysone in the fat body of the yellow fever mosquito, *Aedes aegypti*. *Insect Biochem Mol Biol* 2007;37:1317–1326. [PubMed: 17967350]
- Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, Sabatini DM. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* 2008;320:1496–1501. [PubMed: 18497260]
- Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. *Curr Opin Cell Biol* 2005;17:596–603. [PubMed: 16226444]
- Saucedo LJ, Gao X, Chiarelli DA, Li L, Pan D, Edgar BA. Rheb promotes cell growth as a component of the insulin/TOR signalling network. *Nat Cell Biol* 2003;5:566–571. [PubMed: 12766776]
- Shiao SH, Hansen IA, Zhu J, Sieglaff DH, Raikhel AS. Juvenile hormone connects larval nutrition with target of rapamycin signaling in the mosquito *Aedes aegypti*. *J Insect Physiol* 2008;54:231–239. [PubMed: 17981294]
- Stocker H, Radimerski T, Schindelholtz B, Wittwer F, Belawat P, Daram P, Breuer S, Thomas G, Hafen E. Rheb is an essential regulator of S6K in controlling cell growth in *Drosophila*. *Nat Cell Biol* 2003;5:559–565. [PubMed: 12766775]
- Sun G, Zhu J, Li C, Tu Z, Raikhel AS. Two isoforms of the early E74 gene, an Ets transcription factor homologue, are implicated in the ecdysteroid hierarchy governing vitellogenesis of the mosquito, *Aedes aegypti*. *Mol Cell Endocrinol* 2002;190:147–157. [PubMed: 11997188]
- Uchida K, Moribayashi A, Matsuoka H, Oda T. Effects of mating on oogenesis induced by amino acid infusion, amino acid feeding, or blood feeding in the mosquito *Anopheles stephensi* (Diptera: Culicidae). *J Med Entomol* 2003;40:441–446. [PubMed: 14680108]
- Urano J, Tabancay AP, Yang W, Tamanoi F. The *Saccharomyces cerevisiae* Rheb G-protein is involved in regulating canavanine resistance and arginine uptake. *J Biol Chem* 2000;275:11198–11206. [PubMed: 10753927]
- Wheelock GD, Hagedorn HH. Egg maturation and ecdysiotropic activity in extracts of mosquito (*Aedes aegypti*) heads. *Gen Comp Endocrinol* 1985;60:196–203. [PubMed: 4065529]
- Yamagata K, Sanders LK, Kaufmann WE, Yee W, Barnes CA, Nathans D, Worley PF. Rheb, A Growth Factor-Regulated and Synaptic Activity-Regulated Gene, Encodes A Novel Ras-Related Protein. *Journal of Biological Chemistry* 1994;269:16333–16339. [PubMed: 8206940]
- Zhang H, Stallock JP, Ng JC, Reinhard C, Neufeld TP. Regulation of cellular growth by the *Drosophila* target of rapamycin dTOR. *Genes Dev* 2000;14:2712–2724. [PubMed: 11069888]
- Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, Pan D. Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat Cell Biol* 2003;5:578–581. [PubMed: 12771962]
- Zhu J, Chen L, Raikhel AS. Posttranscriptional control of the competence factor betaFTZ-F1 by juvenile hormone in the mosquito *Aedes aegypti*. *Proc Natl Acad Sci U S A* 2003;100:13338–13343. [PubMed: 14593204]
- Zhu J, Chen L, Sun G, Raikhel AS. The competence factor beta Ftz-F1 potentiates ecdysone receptor activity via recruiting a p160/SRC coactivator. *Mol Cell Biol* 2006;26:9402–9412. [PubMed: 17015464]

Fig. 1. Expression profile of the *Rheb* gene in the fat body of the *Aedes aegypti* female
AaRheb mRNA expression in the fat bodies of pre-vitellogenic and vitellogenic (post blood meal) female mosquitoes at indicated hours. Equal amounts of total RNA from each time point were used to synthesize cDNA. Relative *Rheb* mRNA levels were determined using quantitative PCR. Three groups of three fat bodies were used per each time point. Samples from three biological replicates were analyzed. The term “relative” means that these samples were measured relative to the standard curve of the gene concerned. Means were separated using Tukey-Kramer HSD, with time points sharing the same letter determined not to be significantly different ($P<0.005$).

Fig. 2. RNAi depletion of Rheb inhibits amino-acid-mediated phosphorylation of S6k

Fat bodies were dissected after treatments described below and phosphorylation status of S6k was assessed by means of Western blot analysis using antibodies that detect phosphorylated S6 kinase (*upper panels*). Blots were re-probed with polyclonal antibodies against native S6k as a loading control (*lower panels*). (A) Mosquitoes were injected with dsRheb, ds4EBP or dsMal; fat bodies dissected 5 days after dsRNA injections were incubated in the culture medium containing amino acids (A+) for 3 h and analyzed by means of Western blot. S6k phosphorylation was activated in fat bodies from mosquitoes with 4EBP and Mal depletion backgrounds but not in those after dsRheb injections. *Inset* shows RT-PCR analysis of AaRheb mRNA levels in fat bodies injected with either control *Mal* dsRNA or *AaRheb* dsRNA; the *S7* gene is used as an internal control. (B) RNAi-induced Rheb knockdown significantly decreased S6k phosphorylation *in vivo*. Fat body samples were dissected at indicated time points after a blood meal and subjected to Western blot analyses as described in text.

Fig. 3. *AaRheb* silencing inhibits blood-meal-mediated *Vg* gene expression and *Vg* synthesis *in vivo*

(A) 1- to 2-day-old female mosquitoes were injected with 1 μ g of dsRheb or dsMal. Non-injected wild-type (WT) mosquitoes were also used as a negative control. Five days after dsRNA injections, these mosquitoes were fed with rat blood. Fat bodies were isolated from blood-fed female mosquitoes 24 h post blood meal and transcript levels of *Vg* were analyzed using quantitative PCR. Reactions were performed in triplicate. Values are means $\pm$ SD of triplicate samples from three different mosquito cohorts. Means were separated using the Tukey-Kramer HSD statistical test, with samples sharing the same alphabetical letter determined not to be significantly different ($P < 0.005$). *Inset* shows RT-PCR analysis of *AaRheb* mRNA levels in fat bodies injected with either control *Mal* dsRNA or *AaRheb* dsRNA; the *S7* gene is used as an internal control. (B) Fat bodies were isolated at different time points after a blood meal from mosquitoes treated for RNAi as in (A). Total protein was extracted from groups of three fat bodies (nine for each treatment). Proteins separated by SDS gel electrophoresis were blotted and probed with a mixture of monoclonal antibodies against a 66-kDa *Vg* subunit. An antibody against β -actin was used as a loading control (*lower panel*). The blot shown is representative of three independent experiments.

Fig. 4. RNAi depletion of *AaRheb* interferes with amino acid- and 20-hydroxyecdysone-mediated activation of *Vg* gene expression

(A) Female mosquitoes were injected with 1 μ g of either ds*AaRheb* or a control ds*Mal*; 5 days later, fat bodies were dissected and incubated for 3 h in medium either lacking or containing amino acids. *Vg* mRNA levels were detected using quantitative PCR. Data represent $\pm$ SE of triplicate samples from three separate cohorts of mosquitoes. Means were separated using Tukey-Kramer HSD with samples sharing the same alphabetical letter determined not to be significantly different ($P < 0.005$). (B) Fat bodies from female mosquitoes as in (A) were incubated with various combinations of amino acids and 20E. Expression levels of *Vg* mRNA were analyzed using quantitative PCR. Experiments were

repeated and statistically analyzed as described ($P < 0.005$). (C) Rheb- and Mal-depleted fat bodies were incubated in culture media containing combinations of amino acids and 20E. Protein was isolated and analyzed using monoclonal antibodies against the small Vg subunit (*upper panel*) to assess the level of Vg. An antibody against monoclonal β -actin was used as the control (*lower panel*).

Fig. 5. Rheb is required for mosquito egg development

Female mosquitoes were injected with 1 μ g of dsRNA duplexes of either dsAaRheb or a control dsRNA (dsMal) and were fed blood 72 h later. (A) RNAi-induced depletion of Rheb significantly reduces ovary size in the female *Aedes aegypti*. Comparison of dsMal-injected and dsRheb-injected ovaries isolated 24 h (on the left) and 48 h (on the right) after a blood meal. Ovaries were dissected and photographed under the Nikon SMZ 800. Ovaries from at least ten females for each group were analyzed. Similar observations were obtained from three different batches of mosquitoes. Scale represents 1mm. (B) RNAi-mediated knockdown of *AaRheb* results in a significantly reduced number of eggs laid at the end of a reproductive cycle. Deposition of eggs was induced 3 days after the blood meal by placing a wet filter paper in each tube (one female in one tube). The total number of eggs from each female mosquito was counted after 3–4 days of egg deposition. Ten females were analyzed per group. Three independent cohorts of mosquitoes were evaluated. Because both of our samples (test and control) demonstrated a normal distribution, we used the Student's *t*-test to

determine significant differences in mean egg numbers for knockdown mosquito groups compared with the MAL-injected control group ($P < 0.0001$). *Inset* shows RT-PCR analysis of *AaRheb* expression level in fat bodies injected with control *Mal* dsRNA and *AaRheb* dsRNA as a readout for efficacy of RNAi; the *S7* gene was used as an internal control.