Target of rapamycin-mediated amino acid signaling in mosquito anautogeny

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Mosquitoes generate an enormous burden on human health worldwide. Disease-transmitting species use a reproductive strategy, termed anautogeny, that requires a blood meal to initiate egg maturation. Whereas this strategy is important for driving disease transmission, the molecular mechanisms underlying this phenomenon are still poorly understood. The production of yolk protein precursors (YPPs), a central event in egg maturation, is called vitellogenesis. YPPs are synthesized in the fat body, the insect analogue of the vertebrate liver. Mosquito vitellogenesis is regulated by the steroid hormone 20-hydroxyecdysone (20E). However, 20E alone is not capable of activating vitellogenesis in vivo. Here, we report that amino acid signaling through the nutrient-sensitive target of rapamycin (TOR) pathway is essential for the activation of YPP gene expression. An increase in extracellular amino acid levels, similar to the increase observed after a blood meal, is critical for 20E stimulation of YPP gene expression. Treatment with the TOR kinase inhibitor rapamycin significantly inhibits YPP expression. We used RNA interference to knockdown the expression of two key proteins of the TOR signaling pathway, TOR, and tuberous sclerosis complex 2. Knockdown of TOR inhibited amino acid stimulation while knockdown of tuberous sclerosis complex 2, a negative regulator of TOR signaling, resulted in enhanced YPP expression. Thus, amino acid-based TOR signaling regulates the activation of egg development after a blood meal, an adaptation to the unique life style of mosquitoes.

The mosquito reproductive cycle is a finely tuned system that permits mosquitoes to thrive in a variety of strenuous environmental conditions. In anautogenous mosquitoes, the female reproductive system becomes active only after a blood meal, whereas autogenous mosquitoes are capable of laying a clutch of eggs without a blood meal. Both strategies confer advantages under different environmental conditions. The expression of anautogeny or autogeny can be obligate or facultative, depending on the species (1). Anautogenous mosquitoes are efficient disease vectors because they require frequent host contacts. It is therefore of importance to understand the molecular mechanism underlying anautogeny and integrate this knowledge in the development of future vector- and vector-borne disease control strategies.

The factors controlling the expression of either the autogenous or anautogenous phenotype are due to a mixture of genetic predisposition and environmental cues (2–4). Key differences between anautogenous and autogenous mosquitoes appear to lie in their nutritional status (5–7). Hemolymph amino acid levels play a crucial role in the onset of the reproductive cycle. Within 8 h after a blood meal, the total amino acid concentration in the mosquito hemolymph shows a significant increase that lasts up to 3 days (8). A number of amino acids are essential for oogenesis and a steady infusion of a balanced mixture of amino acids into the hemolymph can stimulate egg development in a variety of mosquito species (9). However, these studies do not address the specific tissues and molecular mechanisms involved in amino acid stimulation of egg development. A central event in insect egg maturation is vitellogenesis, the production of yolk protein precursors (YPPs) in the fat body, the insect analogue of the vertebrate liver (10). After a blood meal, mosquito YPP genes make a transition from a tightly repressed previtellogenic stage to a remarkable level of activation during vitellogenesis (11). The regulatory region of the major YPP gene, Vg, contains binding sites for the ecdysteroid receptor complex (EcR/ultraspiracle), the products of the 20-hydroxyecdysone (20E)-stimulated early genes, E74 and E75, as well as binding sites for GATA-type transcription factors and several factors determining fat body specificity (12). Vg gene expression requires the presence of 20E, which works directly and indirectly through the EcR/ultraspiracle and E74 and E75 (10, 13), respectively. However, in vivo without a blood meal, 20E signaling is not sufficient to activate vitellogenic events in previtellogenic anautogenous mosquitoes (14). In contrast, infusion of a balanced amino acid mixture into previtellogenic anautogenous mosquitoes is sufficient to activate egg development (15).

In a variety of other eukaryotic systems, amino acid signaling is transduced through the target of rapamycin (TOR) signaling pathway (16, 17). The TOR kinase is a central player in an intracellular regulatory network that controls cellular activity according to nutrient availability in eukaryotes. A recent study (18) shows that in larval stages of Drosophila, the fat body operates as a nutrient sensor that restricts growth through a humoral mechanism. This system involves amino acid transporter proteins as well as components of the TOR signaling pathway.

In this report, we demonstrate that amino acid stimulation is crucial for Vg gene expression. Furthermore, we use RNA interference (RNAi) to show the involvement of two key proteins of the TOR signaling pathway in the transduction of amino acid signals. Finally, we show that RNAi knockdown of TOR has a severe impact on mosquito egg development. This work elucidates a major element in the molecular basis of anautogeny.

Materials and Methods

Mosquito Rearing and Fat Body Culture. The Aedes aegypti mosquito strain UGAL/Rockefeller was maintained in laboratory culture as described by Hays and Raikhel (19). The fat body culture system is described in detail elsewhere (19, 20).

Molecular Biology Techniques and Cloning. Standard procedures were used for recombinant DNA manipulations (21). DNA sequences coding for TOR and tuberous sclerosis (TSC)2, a negative regulatory protein of the TOR signaling pathway, were identified in the databases of the Anopheles gambiae genome project and aligned with Drosophila sequences by using the program CLUSTALW (http://clustalw.genome.ad.jp). Highly conserved regions were chosen as template for primers to amplify partial cDNAs of TOR and TSC2 of A. aegypti: TOR Forward:

Abbreviations: YPP, yolk protein precursor; 20E, 20 hydroxyecdysone; EcR, ecdysone receptor; Vg, vitellogenin; RNAi, RNA interference; TOR, target of rapamycin; TSC, tuberous sclerosis complex; ds, double-stranded; AA, Aedes aegypti; Sc, Saccharomyces cerevisiae.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY438003 and AY438004).

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Amino Acids Activate Vg Gene Expression. To ascertain the effect of amino acids as a nutritional signal involved in Vg gene activation, we used an established in vitro tissue culture system for the mosquito fat body (20). The media used in this system contains ~120 mM amino acids per μl, which resembles the natural level of hemolymph amino acids 24 h after a mosquito blood meal (8, 22). In conjunction with the culture system, we used quantitative real-time PCR to measure Vg transcript levels in response to the various treatments as an assessment of the vitellogenic response. Previous work shows that fat bodies in this tissue culture system activate YPP gene expression in response to 20E stimulation (20, 23). To determine whether amino acids have any activating effect, we withdrew them from the media and observed the effect of this withdrawal on Vg expression in fat bodies. To equilibrate the change in osmotic pressure resulting from the withdrawal of amino acids, mannitol was added to balance the osmotic pressure between the two media. In the presence of amino acids, the basal level of Vg mRNA...
increased 23-fold when compared with fat bodies in the absence of amino acids. Withdrawal of amino acids from the media yielded even more dramatic results in the vitellogenic response by the fat body to 20E. Fat bodies exposed to both amino acids and 20E had a 527-fold higher level of Vg mRNA over fat bodies exposed to 20E in absence of amino acids (Fig. 1a).

**Amino Acid Withdrawal Does Not Affect E74 Gene Expression.** To confirm that the withdrawal of amino acids was not compromising transcriptional responsiveness by the fat body in general, we tested the induction of the 20E-inducible early gene E74 (24). We found that E74 is capable of responding to 20E stimulation with and without amino acids, and in fact has a higher basal level and 20E response in the absence of amino acids than in the presence of amino acids (Fig. 1b).

**Rapamycin Inhibits Amino Acid Signaling to the Fat Body.** The immunosuppressive drug rapamycin is a specific inhibitor of TOR and has been used to show TOR’s involvement in nutritional signaling in many other systems. Rapamycin binds to FKBP12, a peptidyl-prolyl isomerase, and this complex binds to TOR and inhibits its kinase activity (25). To determine whether the TOR protein kinase is a part of the nutritional sensor that transduces the amino acid signal to the Vg gene in the fat body, we treated cultured fat bodies with rapamycin (150 nM) in the presence of amino acids and 20E. Rapamycin treatment results in a 75% reduction in the 20E response by Vg (Fig. 1c). Rapamycin treatment of fat bodies in the presence of amino acids without 20E results in total inhibition of the basal response to amino acids (data not shown). To confirm the specificity of the inhibition by rapamycin, we used the drug FK506 as a competitor against rapamycin. FK506 forms complexes with FKBP12 at the same site as rapamycin, but does not have the inhibitory effect on TOR (26). Treatment with 750 nM FK506 alone shows no effect on 20E-mediated induction of Vg. Pre- and continued treatment with 750 nM FK506 in competition with 150 nM rapamycin results in a close to normal response by the Vg gene to 20E signaling (Fig. 1c).

**Cloning of TOR and TSC2 of A. aegypti.** We cloned the cDNAs of two key proteins of the TOR signaling pathway from *A. aegypti*: the TOR kinase (AaTOR) and TSC2 (AaTSC2). The *A. aegypti* AaTOR cDNA (GenBank accession no. AY438003) codes for a protein with 2,444 amino acids and a relative molecular mass of 225 kDa. It is 40% and 28% identical to human TOR and mouse TOR, respectively (Table 1) and the domain sequence is identical in all known TORs. Fig. 2 shows the amino acid identity between the distinct domains of the TOR protein from *A. aegypti* and TOR1 from baker’s yeast, *Saccharomyces cerevisiae*, revealing high conservation in the C-terminal region, which includes the rapamycin/FKBP12-binding domain, the kinase domain, and the C-terminal region. In contrast, the N-terminal region, which contains HEAT repeat elements and the FAT domain, shows a significantly higher diversity. AaTOR mRNA expression was found in all mosquito tissues examined (data not shown).

The TSC2 cDNA (GenBank accession no. AY438004) from *A. aegypti* (AaTSC2) codes for a protein with 2,032 amino acids and a relative molecular mass of 225 kDa. It is 40% and 28% identical to *Drosophila* TSC2 and human TSC2, respectively.

**Amino Acid Signaling Is Controlled by the TOR Pathway.** To investigate the role of AaTOR in Vg gene expression, we took an RNAi approach to knockdown AaTOR mRNA in reproducibly competent but unfed adult female mosquitoes. Isolated fat bodies from RNAi-treated mosquitoes were subjected to our in vitro fat body culture system. AaTOR knockdown resulted in 85% reduction of Vg gene expression after amino acid stimulation compared with mosquitoes treated with control dsRNA (Fig. 3a).

To further confirm the role of TOR signaling in this process, we used the same RNAi approach to knock down TSC2 mRNA. Down-regulation of TSC2 had the opposite effect on Vg expression in mosquitoes than that of TOR. TSC2 depletion causes a 100% increase of the basal Vg expression level of unstimulated fat bodies (Fig. 3b). Furthermore, in TSC2 RNAi-treated fat bodies, Vg gene activation was significantly stronger in response to stimulation by amino acids (3.6-fold).

**TOR Knockdown Inhibits Egg Development in Vivo.** Female mosquitoes received injections of dsRNA 3 and 6 days after emergence. After a 3-day recovery period, a blood meal was given. Ovaries were isolated 24 and 48 h after the blood meal and were examined with a stereo microscope. Deposition of eggs was induced 3 days after the blood meal by placing a wet filter paper in the cage. The eggs were hatched 5 days later, and the percentage of viable progeny was determined.

**Fig. 2.** The C-terminal domains of TOR are highly conserved. Amino acid identity between the TOR kinases of *S. cerevisiae* (ScTOR1) and *A. aegypti* (AaTOR) is shown.

**Table 1.** Amino acid sequence identity between the TOR kinases of different species

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Values shown are the percent amino acid identity. Aa, *Aedes aegypti*; Ag, *Anopheles gambiae*; hypothesis protein XP_3176199; d, *Drosophila melanogaster*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*; At, *Arabidopsis thaliana*.
knockdown, varied. Some of the TOR dsRNA-injected mosquitoes showed no egg development (Fig. 4a). In general, ovaries of mosquitoes injected with TOR dsRNA showed a dramatic reduction in the number and size of ovarioles compared with the dsMAL-injected mosquitoes (Fig. 4b). Ovaries of the latter were similar to those of untreated female mosquitoes (data not shown). TOR dsRNA-injected mosquitoes also showed a significant reduction of the number of eggs deposited, compared with the control (MAL). Furthermore, egg viability dropped from 75% in the dsMAL-injected mosquitoes to 27% in the dsTOR-injected mosquitoes (Fig. 4b).

Discussion

This study identifies the essential elements in the molecular mechanism by which anautogenous mosquitoes activate their reproductive system in response to nutritional stimulation after a blood meal. Previous results (8, 22) have shown the importance of amino acids in the activation of reproduction in anautogenous mosquitoes; however, they did not elucidate the molecular mechanism underlying this process. Our results have clearly demonstrated that amino acids act directly on the fat body tissue to up-regulate gene expression. These experiments confirm that not only are amino acids involved in the regulation of the Vg gene, but amino acid regulation is specifically directed toward the Vg gene. Furthermore, it is apparent from these experiments that amino acids render the Vg gene responsive to 20E (Fig. 1a). Whereas amino acids do not appear to be responsible for the high level of Vg activation, they condition the gene, making it responsive to the 20E-mediated regulatory cascade governing the high level of gene expression.
Amino acid signaling is commonly mediated by the TOR kinase signal transduction pathway (16, 27). TOR is a serine/threonine kinase that is found ubiquitously in eukaryotes. TOR is part of a multiprotein complex of ~2 MDa (28), which is also termed the nutrient-sensitive complex (16). It regulates cell growth and metabolism via transcriptional and translational pathways. Recent findings in Drosophila (18) show that the fat body tissue functions as an amino acid sensor and that nutritional signals are transduced by the TOR signaling pathway. The inhibition of the amino acid-mediated Vg response by rapamycin (Fig. 1c) shows that the TOR kinase is mediating amino acid signaling in the mosquito fat body. In addition, the ability to block this inhibition with a competitor molecule (FK506) shows that this is a specific response and not a result of general toxicity resulting from rapamycin treatment.

To further solidify the evidence that TOR signaling is involved, we cloned the cDNAs of two key enzymes of the TOR signaling pathway from A. aegypti; TOR and TSC2, and performed RNAi knockdown experiments (Fig. 3). Knockdown of AaTOR causes a severe reduction of the amino acid-mediated Vg response (Fig. 3a), resembling the effect caused by rapamycin. TSC2 is a negative regulator of TOR signaling, and like TOR, is highly conserved. The TSC2 tumor suppressor protein forms a complex with TSC1, and this complex inhibits the TOR kinase activity by inactivating the GTPase Rheb (29). Overexpression of the TSC proteins in Drosophila fat body cells has been shown to have a starvation-like effect similar to the suppression of Drosophila TOR activity in this tissue (18). In contrast to the effects of AaTOR knockdown, AaTSC2 knockdown resulted in a significant up-regulation of Vg gene expression after amino acid stimulation. Thus, our data clearly suggest a central role of TOR signaling in the amino acid-stimulated onset of Vg gene expression.

Knockdown of TOR resulted in a significant inhibition of egg development: a reduced number of eggs deposited at the end of the reproductive cycle and their decreased viability (Fig. 4). The in vivo effect of TOR knockdown on egg development should be interpreted with caution because the effect of interrupted TOR signaling may occur not only in the fat body trophocytes but also in other tissues involved in transduction of blood meal-mediated nutritional signals such as the brain and ovaries themselves.

How does TOR regulate Vg gene expression? In yeast, nutritional signals are transferred through TOR to GATA-type transcription factors, which regulate the activity of target genes (30). TOR controls the nuclear translocation of the Gln-3 GATA factor through its phosphorylation and that of an inhibitory cytoplasmic binding partner. Upon TOR signaling, Gln-3 translocates into the nucleus and displaces a GATA repressor protein, resulting in the activation of nitrogen catabolite repression genes (31). We showed previously that the regulatory region of the Vg gene contains multiple GATA-binding sites (12). Furthermore, electrophoretic mobility shift assays have demonstrated a shift in the GATA-binding mobility present in the nucleus within 1 h after blood feeding. A GATA-binding activity with a mobility similar to the vitellogenic nuclear GATA-binding activity is also present in the cytoplasm of previtellogenic fat body cells (D. Martin and A.S.R, unpublished data). This finding suggests a blood meal-generated signal is stimulating the nuclear translocation of this factor. Previously, a GATA factor that acts as a repressor of the Vg gene during the previtellogenic stage was identified in the mosquito (AaGATAr) (32). Although a connection between GATA transcription factors and amino acid-TOR signaling has yet to be demonstrated, TOR’s association with GATA factors in yeast (30) and in mammalian adipocyte cells (33, 34) implies a possible association between TOR and GATA factors in the regulation of mosquito vitellogenesis.
In summary, we have identified that amino acids are a principal signal in mosquito vitellogenic gene expression and that this signal is transduced by the TOR pathway. Evidence suggests that amino acid stimulation of the TOR regulatory cascade causes the fat body of the mosquito female to shift from production and subsequent activation of Vg and other YPF genes (Fig. 5). In turn, this mechanism permits the 20E-mediated gene hierarchy to up-regulate Vg gene expression to the extremely high levels observed during vitellogenesis. Thus, our work has uncovered a mechanism underlying the developmental arrest and blood meal activation of vitellogenesis, a key event in reproduction in anautogenous mosquitoes.

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