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 obligatory 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 autogenic or anautogenic 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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5′GAG CAG TGG CAC GAG GGT TTG GAG GAA GC; *TOR* Reverse: 5′CAA AAC GGA CAC CAG CCG ATA TAG CAC TGG CA; TSC2 Forward: 5′CTG TGG GAG CTG ACG AAG GAT CTG CTC GT; and *TSC2* Reverse: 5′CAG GCT GGC CGC ATT GTA CTT GAT CAG GTT. The 5′ and 3′ ends of the cDNAs were amplified by RACE PCR using the Smart cDNA RACE amplification kit (Becton Dickinson Clontech, Palo Alto, CA). All PCR products were cloned in pCRII-TOPO vector (Invitrogen). Additional experimental procedures and information concerning plasmids and primers are available on request.

RNAi and Real-Time PCR Analysis. For the generation of double-stranded (ds)RNAs, template cDNAs were cloned into the pLitmus 28i vector and dsRNA was produced by *in vitro* transcription with T7-RNA polymerase by using the Hiscribe RNAi transcription kit (New England Biolabs, Beverly, MA). Approximately 1 μg of dsRNA in 0.2 μl of H_2O was injected in the thorax of CO_2-anesthetized female mosquitoes, 2 days after adult emergence. The mosquitoes were cultured for 3 days before further processing.

Total RNA from fat bodies was extracted by using the TRIzol method (GIBCO BRL/Invitrogen). Total RNA was treated with amplification grade Dnase I (GIBCO BRL/Invitrogen) and 1 μg of treated RNA was used in cDNA synthesis reactions by using the Omniscript reverse transcriptase kit (Qiagen, Valencia, CA). cDNA levels in the different samples were quantified by real-time PCR by using Taqman primers/probes for *Vg* and *actin* and SYBR green primers for *E74B*. We used a real-time PCR master mix, iQ Supermix (Bio-Rad) for the Taqman reactions, or the Quantitech SYBR green Mastermix (Qiagen) for the SYBR green reactions. Primers and probes are as follows (all Taqman probes use the Black Hole Quencher and were synthesized by Qiagen): Actin Forward: 5′-ATC ATT GCT CCA CCA GAA CG; Actin Reverse: 5′-AAG GTA GAT AGA GAA GCC AAG; Hex-Labeled actin probe: 5′-ACT CCG TCT GGA TCG GTG GCT CC; *Vg* Forward: 5′-ATG CAC CGT CTG CCA TC; *Vg* Reverse: 5′-GTT CGT AGT TGG AAA GCT CG; Texas red-labeled *Vg* probe: 5′-AAG CCC CGC AAC CGT CCG TAC T; E74B Forward: 5′-GAC CTC GTT CGC AAA CAC CTC; and E74B Reverse: 5′-AAG CCA CCT GTT GAT CGT CTT C. Final reaction concentration for all primers and probes was 250 nM. Total reaction volumes were 20 μl and were run in 96-well PCR plates (Bio-Rad). All reactions were run in duplicate by using 2 μl of cDNA per reaction. Reactions were run on an iCycler real-time PCR machine (Bio-Rad). Standard curves used to quantify relative gene concentrations were made from tenfold serial dilutions of cDNA pools containing high concentrations of the gene of interest or from a dilution of a plasmid standard. The program used for amplifying the reactions was as follows: (*i*) melting: 95°C for 3 min; (*ii*) melting: 95°C for 10 sec; (*iii*) annealing 59°C for 45 sec (florescence recorded); and (*iv*) repeat to step *ii* for 50 cycles. Real-time data were collected by iCycler iQ real-time detection system software, V.3.0 for Windows. Data were analyzed in Microsoft Excel.

Results

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 nM 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

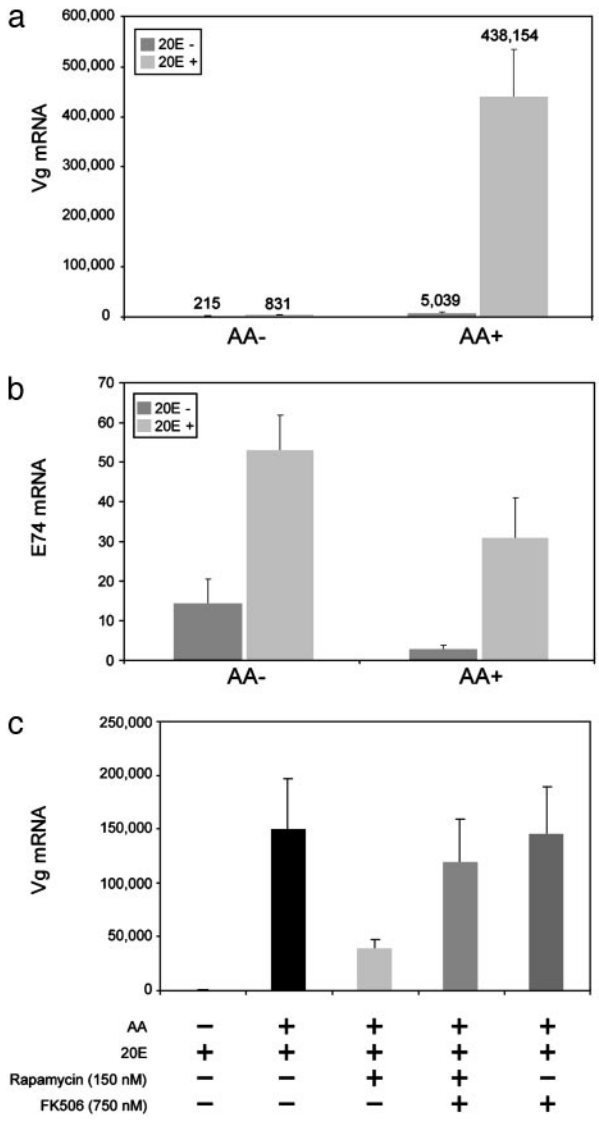

Fig. 1. *Vg* gene responsiveness to 20E depends on the presence of amino acids and can be inhibited by the TOR kinase antagonist rapamycin. (*a*) *Vg* requires amino acids to respond to 20E. Real-time PCR analysis of *Vg* mRNA from *in vitro*-cultured mosquito fat bodies with and without amino acids in the presence and absence of 20E. Fat bodies from 3- to 5-day-old mosquitoes were incubated with the appropriate treatment in culture for 6 h and were then collected. Total RNA was collected from groups of six fat bodies. cDNA was synthesized from equal amounts of DNase I-treated total RNA. Real-time PCRs were performed in duplicate. Data were normalized by real-time PCR analysis of *actin* levels in the cDNA samples. Data represent means ± SE of triplicate samples. (*b*) Early gene *E74* does not require amino acids to respond to 20E. RNA samples were from the same experiment as in *a* and real-time PCR was performed as above with *E74*-specific primers. (*c*) Rapamycin antagonizes amino acid-based responsiveness to 20E. The rapamycin competitor FK506 can reverse antagonism of this response. Fat bodies were cultured as above with the exception of a 1-h pretreatment in either untreated physiological saline or saline containing the appropriate concentration of rapamycin or FK506. RNA isolation and real-time PCR analysis were as in *a*.

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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. 1*a*).

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. 1*b*).

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. 1*c*). 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. 1*c*).

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 279 kDa. The TOR sequence is highly conserved with 62% and 53% overall amino acid identity to *Drosophila* TOR and human 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 bakers' yeast, *Saccharomyces cerevisiae*, revealing high conservation in the C-terminal region, which includes

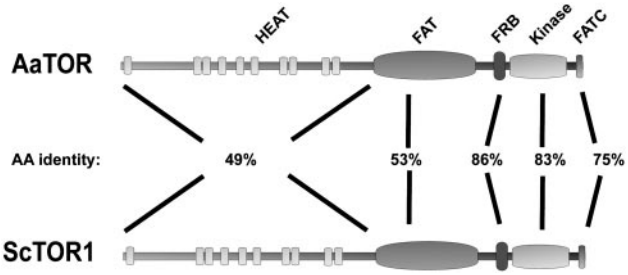

Fig. 2. The C-terminal domains of TOR are highly conserved. Amino acid identity between the TOR kinases of *S. cerevisiae* (ScTOR1) and *A. aegypti* (AaTOR) is shown.

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 reproductively 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. 3*a*).

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. 3*b*). 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.

The ovarian phenotype, observed after RNAi-mediated *TOR*

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

	AaTOR	AgTOR	dTOR	HsTOR	MmTOR	SpTOR1	ScTOR1	AtTOR
AaTOR	100	74	62	53	54	45	41	37
AgTOR		100	60	53	53	44	40	37
dTOR			100	52	53	44	41	38
HsTOR				100	92	46	43	38
MmTOR					100	46	43	38
SpTOR1						100	50	45
ScTOR1							100	41
AtTOR								100

Values shown are the percent amino acid identity. Aa, *Aedes aegypti*; Ag, *Anopheles gambiae* hypothetical protein XP_317619; d, *Drosophila melanogaster*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*; At, *Arabidopsis thaliana*.

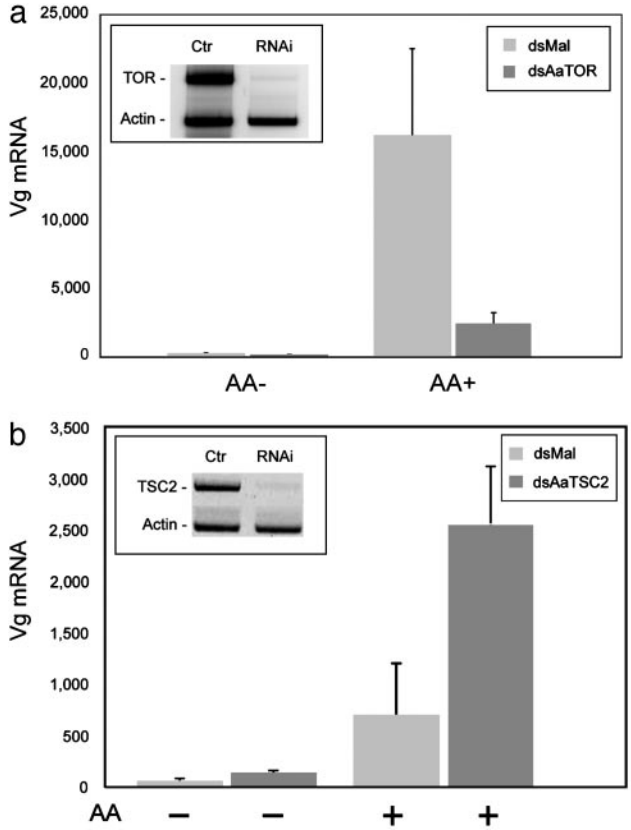

Fig. 3. The amino acid signal is transduced to the *Vg* gene via the TOR signaling pathway. (*a*) RNAi-mediated knockdown of *AaTOR* inhibits *Vg* mRNA expression. Female mosquitoes were injected with 1 μg of dsRNA duplexes of either ds*AaTOR* or a control dsRNA derived from the noncoding region of a bacterial gene (*dsMAL*) and incubated for 72 h. Fat bodies were dissected and subjected to *in vitro* fat body culture with medium lacking or containing amino acids for 6 h. *Vg* mRNA levels were determined by real-time PCR as described above. RT-PCR analysis shows *AaTOR* expression in fat bodies injected with control *MAL* dsRNA and *AaTOR* dsRNA; the actin gene is used as an internal control. (*b*) RNAi-mediated knockdown of *AaTSC2* increases *Vg* gene expression. As above, fat bodies of RNAi-treated mosquitoes were stimulated with amino acids in *in vitro* fat body culture for 6 h. Fat body culture and gene expression analysis was performed as in *a*.

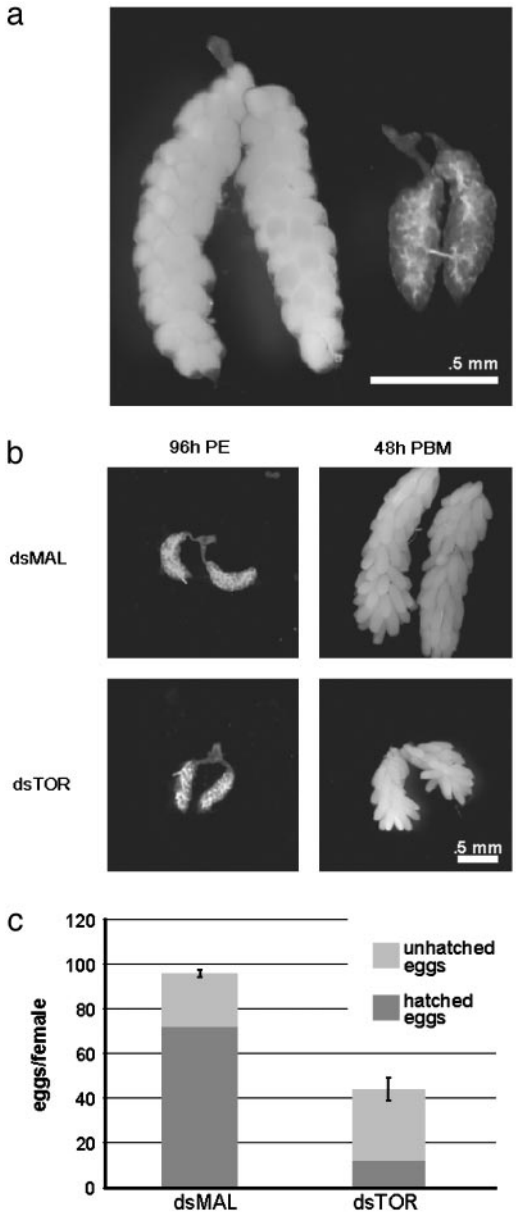

Fig. 4. TOR knockdown inhibits egg development *in vivo*. Female mosquitoes were injected with 1 μg of dsRNA duplexes of either ds*AaTOR* or a control dsRNA (dsMAL) and were fed on blood 72 h later. (*a*) RNAi-mediated knockdown of AaTOR results in inhibition of egg development. Comparison of ovaries, isolated 24 h after a blood meal. The left ovary was isolated from a control (dsMAL)-injected mosquito, and the right ovary was isolated from a mosquito that received a ds*AaTOR* injection. (*b*) RNAi-mediated knockdown of *AaTOR* results in a significantly reduced number and size of follicles. Ovaries were dissected and photographed 48 h PBM (post-blood meal). (*c*) RNAi-mediated knockdown of *AaTOR* results in a significantly reduced number of eggs laid at the end of a reproductive cycle and lower egg viability. Egg hatching was induced 5 days PBM for 3 h, and the percentage of hatched eggs was determined with a stereo microscope. A total of 75% of the dsMAL-injected eggs and 27% of the ds*TOR* injected eggs hatched.

knockdown, varied. Some of the *TOR* dsRNA-injected mosquitoes showed no egg development (Fig. 4*a*). In general, ovaries of mosquitoes injected with *TOR* dsRNA showed a dramatic reduction in the number and size of ovarioles compared with the ds*MAL*-injected mosquitoes (Fig. 4*b*). 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 ds*MAL*-injected mosquitoes to 27% in the ds*TOR*-injected mosquitoes (Fig. 4*b*).

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. 1*a*). 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.

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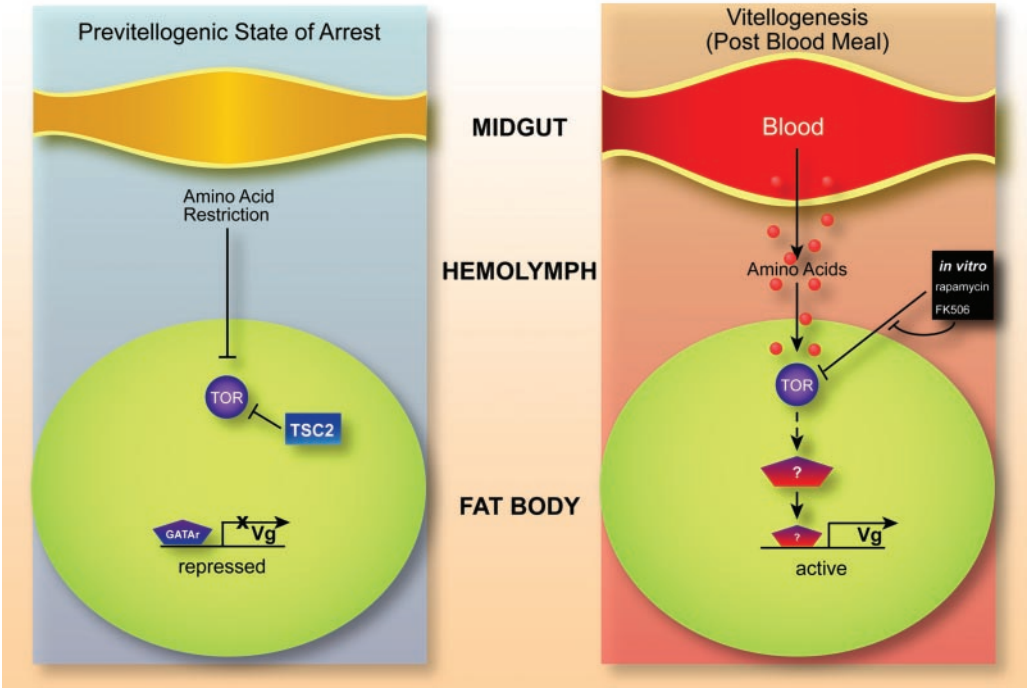

Fig. 5. A model for *Vg* gene activation in the mosquito fat body after blood feeding. During the previtellogenic state of arrest (*Left*) low amino acid levels in the hemolymph keep *Vg* gene expression in the fat body repressed. The TOR kinase is inactive and TSC2 blocks TOR signaling. The GATAr transcription factor binds and inactivates the *Vg* promoter (35). After blood feeding, the amino acid concentration in the hemolymph rises and activates TOR signaling (*Right*). GATAr is presumably replaced by an activating GATA factor at the *Vg* promoter, which makes it accessible to hormonal activation by 20E. *Vg* activation can be blocked *in vitro* by inhibiting TOR with rapamycin (black box).

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. 1*c*) 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. 3*a*), 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 the previtellogenic state of arrest to post-blood feeding derepression and subsequent activation of *Vg* and other *YPP* 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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