Target of Rapamycin-dependent Activation of S6 Kinase Is a Central Step in the Transduction of Nutritional Signals during Egg Development in a Mosquito*

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Female mosquitoes are effective disease vectors, because they take blood from vertebrate hosts to obtain nutrients for egg development. Amino acid signaling via the target of rapamycin (TOR) pathway has been identified as a key requirement for the activation of egg development after a blood meal. We report the characterization of the TOR kinase and one of its major downstream targets, S6 kinase, of the yellow fever mosquito *Aedes aegypti* during egg development in adult females. Both TOR and S6K mRNA are expressed at high levels in the ovaries and in lower levels in fat body and other tissues. After a blood meal, the subcellular localization of TOR shifts from the cytoplasm to the plasma membrane of fat body cells. By detecting phosphothreonine 388 of mosquito S6 kinase, we show that TOR activity strongly increases in fat body and ovaries after a blood meal in vivo. Furthermore, phosphorylation of S6 kinase increases in *in vitro* cultured fat bodies after stimulation with amino acids. This increase is sensitive to the TOR inhibitor rapamycin in a concentration-dependent manner but not to the phosphatidylinositol 3-kinase/ phosphatidylinositol 3-kinase-related kinase inhibitor LY294002, the MAPK inhibitor PD98059, or the translational inhibitor cycloheximide. RNA interference-mediated reduction of S6 kinase strongly inhibits the amino acid-induced up-regulation of the major yolk protein vitellogenin in *in vitro* and effectively disrupts egg development after a blood meal in *in vivo*. Our data show that TOR-dependent activation of S6 kinase is a central step in the transduction of nutritional information during egg development in mosquitoes.

The role of mosquitoes as vectors of devastating diseases like malaria, yellow fever, and dengue fever is linked to their reproductive strategy, which includes the uptake of vertebrate blood to obtain nutrients for egg development. Amino acids derived from the blood meal are used by the mosquito fat body tissue, the insect analogue of the vertebrate liver, to synthesize yolk proteins, which are secreted into the hemolymph and subsequently taken up by the ovaries and deposited in the eggs; this process is termed vitellogenesis (1). As a consequence of the unique mosquito lifestyle, expression of the genes encoding yolk protein precursors (YPP(s)) is tightly connected with the uptake of blood.

In contrast to *Drosophila*, in which YPP genes are expressed in both the fat body and the ovarian follicle cells, mosquitoes express YPP genes exclusively in the fat body. An analysis of the promoter region of the major YPP gene, vitellogenin (*Vg*), revealed binding sites for the ecdysone receptor complex (ecdysone receptor-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 other factors determining fat body specificity (2). Before the mosquito takes a blood meal, YPP genes are kept in a state of arrest, presumably by a GATA-type transcription factor, AaGATAr, which recruits the universal co-repressor protein CtBP to the promoter regions of these genes (3).

After a blood meal, the YPP genes shift from a tightly repressed previtellogenic stage to a remarkable level of activation during active vitellogenesis. The ecdysteroid hormone 20E is a major activator of YPP gene expression (4). It acts directly on *Vg* gene expression through the ecdysone receptor complex (20E-ecdysone receptor-ultraspiracle) and indirectly through *E74* (5), and *E75* (6) respectively.

We recently showed that a preceding stimulation of the fat body is necessary to render the Vg gene responsive to the 20E signal (7). We discovered that amino acids serve as a critical signal enabling effective 20E stimulation of Vg expression. Female mosquitoes utilize the target of rapamycin (TOR)-signaling pathway to transduce amino acid nutritional information to YPP genes in the fat body. Both treatment of fat bodies with the TOR inhibitor rapamycin in *in vitro* and RNA interference (RNAi)-mediated reduction of the TOR protein result in a severe down-regulation of Vg gene expression after amino acid stimulation compared with control mosquitoes. In contrast, RNAi-mediated knockdown of the tuberous sclerosis complex-2 protein, a negative regulator of TOR, enhances the level of Vg gene expression. Importantly, RNAi-mediated reduction of TOR results in severely reduced numbers of deposited eggs and viable progeny after a blood meal is given. This data suggests that the change of amino acid levels after a blood meal triggers the TOR signaling cascade and subsequently activates YPP gene expression.

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† The abbreviations used are: YPP, yolk protein precursor; RNAi, RNA interference; S6K, S6 kinase; TOR, target of rapamycin; Vg, vitellogenin; RACE, rapid amplification of cDNA ends; PBS, phosphate-buffered saline; PI 3K, phosphatidylinositol 3-kinase; 20E, 20-hydroxyecdysone; FAT domain, a protein domain found in ERAP, ATM, and TRRAP proteins; APS, *Aedes* physiological saline; MAPK, mitogen-activated protein kinase; PBM, post-blood meal.

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The key enzyme of the highly conserved TOR signaling cascade is the TOR kinase. This protein emerged as a central controller of cell growth in eukaryotes (8–10). TOR is a serine/threonine kinase, which apparently integrates signals of the insulin receptor or insulin-like growth factor receptor pathways with nutritional signals (11). TOR signaling affects all aspects of gene expression, including transcription, translation, and protein stability. Major downstream targets of TOR are components of the translation machinery, particularly proteins responsible for ribosome recruitment to 5'-capped mRNA: 4-EBP, S6-kinase (S6K), and eIF4G (8).

Mosquitoes adapted the highly conserved TOR signaling cascade to their unique lifestyle and use it to process nutritional information in the fat body of adult females. An outstanding question that remains is which players act downstream of TOR in the mosquito fat body.

In this paper, we characterize TOR and its downstream target S6K of Aedes aegypti in adult females and demonstrate TOR-dependent activation of S6K via phosphorylation at threonine 389 after a blood meal in the fat body and ovaries. Furthermore, we show that RNAi-mediated reduction of S6K effectively blocks mosquito egg development after a blood meal. The results of our studies suggest a central role for TOR/S6K-mediated nutritional signaling during mosquito egg development in ovaries and fat body tissue.

MATERIALS AND METHODS

A. aegypti Rearing and Fat Body Culture—The A. aegypti mosquito strain UGAL/Rockefeller was maintained in laboratory culture as described by Hays and Raikhel (12). The fat body culture system is described in detail elsewhere (12, 13).

RNA Isolation—For expression profiling, tissue-specific RNAs were isolated by dissection of female mosquitoes at various points in time after a blood meal. Tissues of 50 individual mosquitoes were pooled. For quantitative PCR, three groups of three fat bodies were used for each treatment. Total RNA was isolated by means of a commercially available modification (TRIzol, Invitrogen) of the one-step phenol/guanidine thiocyanate method (14).

Cloning and Sequencing of the TOR and S6 Kinase cDNA of A. aegypti—Standard procedures were used for recombinant DNA manipulations (15). Cloning and sequencing of TOR from A. aegypti is described elsewhere (7). Expressed sequence tag cDNA sequences coding for parts of A. aegypti S6 kinase were identified in the databases of the Institute for Genomic Research. The 5'- and 3'-ends of the cDNAs were amplified by rapid amplification of cDNA ends (RACE) PCR using the Smart cDNA RACE amplification kit (Clontech). All PCR products were cloned in pCRII-TOPO vector (Invitrogen).

Sequence Analysis—The full-length cDNA and deduced amino acid sequences of AaTOR and AaS6K were compared using the BLAST tool (16) at the National Center for Biotechnology Information (NCBI). Sequence alignments were performed using the CLUSTALW software (17).

Quantitative PCR—Relative cDNA levels were quantified by real time PCR using TaqMan primers/probes for TOR, S6K, and actin. We used iQ Supermix (Bio-Rad) for the TaqMan reactions. Primers and probes were as follows (all TaqMan probes used the Black Hole Quencher and were synthesized by Qiagen (Qiagen, Valencia, CA.): actin forward, 5'-ATCATGGCTCCACCAGCAAGC; actin reverse, 5'-AA- GGTGAATGAAAGCCAGAAG; hex-labeled actin probe, 5'-ACTCCGTC- CTGGATCTTCT; TOR forward, 5'-CGTGTCAGTTGTTGCCTACACGA; TOR reverse, 5'-CGATCGATGCAGGCTC; TOR forward, 5'-ACGATGGAAGACGGT- GTGATCTC; TOR reverse, 5'-CGATCGTGTCCTAGGCTACACGA; Texas Red-labeled TOR probe, 5'-ACGCCAGAAGCTGCGATGACGAA; S6K forward, 5'-TGGTAGCAGAAGAGGG; S6K reverse, 5'-GGCGGCCCAGTGGGTCATG; and Texas Red-labeled S6K probe, 5'-TCGCCACATACCCGCGCCAGA. Reactions were carried out as described previously (7).

Immunolocalization of TOR Proteins—Organs selected for immunostaining were fixed overnight at 4 °C with 4% paraformaldehyde in PBS. After extensive washing with PBS, the samples were submitted to a graded alcohol series (20, 40, 60, 80, 100, 80, 60, 40, and 20%), PBS for not shorter than 15 min each). After multiple washings with 0.5× PAT (PBS, 1% albumin, 0.5% Triton X-100), the samples were treated with 3% goat serum in 0.5× PAT for 2 h at room temperature and then washed three times with 0.5× PAT. Subsequent incubation with anti-serum (number 2972, Cell Signaling Technology, Beverly, MA, 1:100 in PAT) lasted 2 days at 4 °C. After extensive washing with 0.5× PAT, the tissues were incubated overnight at 4 °C with Alexa 488-conjugated affinity purified goat IgG (number A11034, Molecular Probes, Eugene, OR, 1:100). After thoroughly washing, the samples were stained with 4',6-diamidino-2-phenylindole for 1 h at room temperature. After extensive washing with PAT, the samples were ready for observations. The samples were mounted with Gel/Mount (Biomega, Foster City, CA).

RESULTS

Sequence Features of AaTOR—All TOR proteins have a highly conserved domain structure containing N-terminal HEAT repeats followed by a FAT domain named after the three protein groups sharing this domain, FRAF, ATM, TRRAP (20), the FK506-binding protein 12-binding domain, the kinase domain, and the FATC domain at the C terminus (Fig. 1A). The FK506-binding protein 12-domain, the kinase domain, and the FATC domain are highly conserved (Fig. 1B).

AaTOR Immunolocalization—We investigated the localization of TOR protein in fat bodies and ovaries using a polyclonal antiserum against nTOR (Fig. 2). In fat body tissue of 3-day-old unfed mosquitoes, a general diffuse pattern of immunoreactivity was found in all cells (Fig. 2B). In contrast, 4 h after a blood meal this pattern of immunoreactivity had shifted to the plasma membrane region (Fig. 2E). Strong immunoreactivity was also found in the cytoplasm of the ovarian follicle cells of mosquitoes (Fig. 2H).

AaTOR mRNA Is Expressed in Multiple Tissues—The expression pattern of AaTOR in adult female mosquitoes (72 h after the adult emergence) was investigated by quantitative
PCR in various organs before and then 4, 24, and 48 h after a blood meal (Fig. 3). In unfed mosquitoes, AaTOR mRNA is abundant at relatively low concentrations in the carcass, fat body, midgut, and malpighian tubules, and little change was noted after a blood meal. Higher concentrations of TOR mRNA were found in the ovaries, and relative levels were elevated at 4 h and 24 h (2-fold) post-blood meal (PBM).

Sequence Features of AaS6 Kinase—The cDNA sequence of A. aegypti S6 kinase was identified by a combination of EST-mining and RACE-PCR. The full-length open reading frame predicts a protein of 550 amino acids and 62 kDa (GenBank™ accession number AY700377). We named the protein A. aegypti S6 kinase (AaS6K). The S6 kinase sequence is highly conserved, with 68 and 64% overall amino acid identity to Drosophila S6 kinase and human S6 kinase 1, respectively. The protein contains two conserved domains, a catalytic domain typical for serine/threonine protein kinases and an extension domain, typical for this type of kinase (Fig. 4A). The N and C terminus of AaS6K show less conservation in their amino acid sequence (Fig. 4B). The threonine residue at position 388 is
analogous to threonine 389 of the human S6K1 (threonine 412 of human S6K2).

**AaS6K mRNA Expression Profile**—The expression pattern of AaS6K in adult female mosquitoes (72 h after the adult emergence) was investigated by quantitative PCR in various organs before and then 4, 24, and 48 h after a blood meal (Fig. 5). The highest mRNA levels were found in the ovaries, followed by the midgut, fat body, malpighian tubules, and carcass. No dramatic changes in the expression levels were observed after a blood meal.

**Enhanced Phosphorylation of S6K in Fat Body and Ovaries after a Blood Meal**—We examined the activity of the TOR kinase in the fat body of female mosquitoes using an antibody against the phosphorylated form of S6K. Fat bodies and ovaries were dissected from female mosquitoes before and at different points in time after a blood meal, immediately homogenized in ice-cold cracking buffer, and subjected to Western blot analysis. We found that phosphorylation of S6K rises dramatically after a blood meal in the fat body and even more strongly in the ovaries (Fig. 6).

**Amino Acid Stimulation Induces TOR-mediated S6K Phosphorylation in Cultured Fat Bodies**—We examined the activity of the TOR kinase in the fat body of female mosquitoes using an in vitro fat body culture assay. Fat bodies of female mosquitoes, 72 h after eclosion, were isolated, and the samples were kept in Aedes physiological saline (APS) for 1 h at room temperature. After this, the APS was replaced by a medium either with or without amino acids. After 3 h of incubation time, the fat bodies were homogenized in cracking buffer and subjected to Western blot analysis. We found a strong increase of threonine 388-phosphorylated S6K after stimulation with amino acids (Fig. 7A).

**Rapamycin, but Not PD98059, LY294002, or Cycloheximide Suppresses Phosphorylation of S6K**—The signaling events involved in the up-regulation of S6K phosphorylation in the mosquito fat body after stimulation with amino acids were dissected using pharmacological inhibitors. The involvement of the TOR pathway was first investigated using the TOR-specific inhibitor rapamycin. Cultured fat bodies were pretreated in APS containing rapamycin (0, 10, 50, 150 nM) for 1 h and then stimulated with medium containing amino acids and different concentrations of rapamycin for 2 h. The addition of 150 nM rapamycin resulted in the suppression of amino acid-induced increases of phospho-S6K. Rapamycin completely inhibited AaS6K threonine 388 phosphorylation at a concentration of 150 nM (Fig. 7B).

To investigate the possible involvement of PI 3K signaling (typically associated with insulin signaling) in the phosphorylation of S6K after amino acid stimulation, we used LY294002 a broad spectrum inhibitor of a number of PI 3K-related kinase and all PI 3K isoforms. Cultured fat bodies were pretreated in APS containing LY294002 (0, 1, 10, 100 nM) for 1 h and then stimulated with medium containing amino acids and varying concentrations of LY294002 for 2 h. LY294002 has no influence on S6K activation, as indicated by the absence of detectable changes in phospho-S6K contents (Fig. 7C).

Next, we investigated the possible involvement of MAPK pathway in the amino acid-induced phosphorylation of S6 kinase using the MAPK-specific inhibitor PD98059. Cultured fat bodies were pretreated in APS containing PD98059 (0, 10, 50, 150 nM) for 1 h and then stimulated with medium containing amino acids and different concentrations of PD98059 for 2 h. PD98059 had no effect on amino acid-stimulated phosphorylation of S6 kinase threonine 388 (Fig. 7D).

**FIG. 2.** Subcellular localization of TOR in fat body and ovaries. Blood meal activation of the fat body triggers localization of TOR immunoreactivity at the plasma membrane of fat body trophocytes. A–C, fat body tissue of unfed mosquito females. D–F, fat body tissue of blood-fed mosquitoes 3 h PBM. G–I, oocytes of unfed mosquitoes. A, D, and G, DAPI (4',6-diamidino-2-phenylindole) staining. B, E, and H, immunofluorescence with α-mTOR antibody. C, F, and I, merge.

**FIG. 3.** AaTOR mRNA expression in different tissues of female mosquitoes without and 4, 24, and 48 h PBM. Relative TOR mRNA levels were determined by real time PCR.
To determine whether phosphorylation of S6 kinase after amino acid stimulation involves newly translated factors, we used the translational inhibitor cycloheximide to block translation in the cultured fat bodies. The cultured fat bodies were pretreated in AMP containing cycloheximide (0, 10, 50, 150 μM) for 1 h and then stimulated with medium containing amino acids and different concentrations of cycloheximide for 2 h. No differences in the phosphorylation of S6 kinase were observed after treatment with increasing concentrations of cycloheximide (Fig. 7E).

A Functional PI 3K Is Present in the Mosquito Fat Body—Immunoprecipitation of PI 3-kinase from mosquito fat body lysates using an antibody against the PI 3-kinase p110 subunit resulted in the concentration of a 110-kDa protein (Fig. 8A). The precipitated enzyme showed PI 3-kinase activity (production of the second messenger PI(3,4,5)P3 from PI(4,5)P2), and this activity was blocked strongly by the addition of LY294002 to the reaction buffer (Fig. 8B).

RNAi-mediated Reduction of AaS6K Inhibits Vitellogenin Gene Activation after Amino Acid Stimulation—Female mosquitoes, 3 days after eclosion, were injected with dsRNA against S6K and a control dsRNA (MAL). A second round of injections was performed 3 days later. After a 72-h recovery period, fat bodies were isolated and kept in APS for 1 h at room temperature.

FIG. 4. The AaS6K protein. A, domain structure of AaS6K. B, comparison of the amino acid sequences of S6K of A. aegypti (Aa), Drosophila (Dm), and human (hs), performed with ClustalW. The protein domains are highlighted with different colors. The GenBank accession numbers for the S6K sequences are: A. aegypti, AY700377; D. melanogaster, AAC47312; H. sapiens, P23443.
levels were determined by real time PCR. A small number of eggs. RNAi-treated mosquitoes started vitellogenesis and developed any signs of yolk deposition. Approximately 10% of the S6K revealed that they were not enlarged, and they did not show later, and the mosquitoes were dissected to inspect the ovaries.\n
The majority of mosquitoes injected with S6K dsRNA did not induced 5 days after the blood meal by placing a wet filter paper in the cage. The number of eggs was determined 24 h recovery period, a blood meal was given. Deposition of eggs was incubation with medium containing amino acids resulted in a strong up-regulation of Vg mRNA in \textit{AaS6K} Thr(P)-388 content was assessed using immunoblot analysis. The analysis was performed using antibodies against the Thr(P)-388 of S6K (upper panels). A second blot was probed with an antibody against S6K as loading control (lower panels).

RNAi-mediated Reduction of \textit{AaS6K} Inhibits Mosquito Egg Development after a Blood Meal—Female mosquitoes received injections of dsRNA 3 and 6 days after emergence. After a recovery period, a blood meal was given. Deposition of eggs was induced 5 days after the blood meal by placing a wet filter paper in the cage. The number of eggs was determined 24 h later, and the mosquitoes were dissected to inspect the ovaries. The majority of mosquitoes injected with S6K dsRNA did not develop or deposit any eggs (Fig. 9B). Inspection of the oocytes revealed that they were not enlarged, and they did not show any signs of yolk deposition. Approximately 10% of the S6K RNAi-treated mosquitoes started vitellogenesis and developed a small number of eggs.

**FIG. 5.** \textit{AaS6K} mRNA expression in different tissues of female mosquitoes without and 4, 24, and 48 h PBM. Relative S6K mRNA levels were determined by real time PCR.\n
**FIG. 6.** Blood meal-induced activation of TOR signaling in the fat body. Fat bodies were isolated at different points in time after a blood meal, and \textit{AaS6K} Thr(P)-388 content was assessed using immunoblot analysis. The analysis was performed using antibodies against the Thr(P)-388 of S6K (upper panels). A second blot was probed with an antibody against S6K as loading control (lower panels).

Mosquitoes are undoubtedly the most dangerous animals to humans because of the fact that they spread diseases like malaria, yellow fever, and dengue fever, which affecting huge numbers of people worldwide. The reproductive biology of mosquitoes is tightly linked to their ability to transmit disease pathogens. A detailed understanding of how the reproductive processes are regulated on a molecular level may reveal new ways to disrupt the transmission of these pathogens.

In the first 24 h after a blood meal, the mosquito fat body increases its number of ribosomes 4-fold and starts converting the amino acids from the blood meal into huge amounts of YPPs (21, 22). In the ovaries, the oocytes take up the YPPs by receptor-mediated endocytosis (23). This process is called vitellogenesis, and the aim of our project is to unravel the molecular mechanisms and signaling events underlying this process.

The steroid hormone 20E is the main regulator of YPP gene expression in mosquitoes (24). A second type of regulator are the free amino acids in the hemolymph, which increase dramatically after a blood meal (25). In a recent study, we showed that amino acids activate the TOR signaling cascade, which transduces the amino acid signal to the YPP genes (7). These findings have added another so far unknown function to the list of processes in which TOR signaling is involved.

In \textit{Drosophila}, TOR has been shown to be required for normal growth and proliferation during the larval development of the fly (26, 27). In the larval stages of \textit{Drosophila}, the fat body operates as a nutrient sensor that restricts growth through a humoral mechanism (28). \textit{Drosophila} TOR has a central role in the regulation of starvation-induced autophagy in the \textit{Drosophila} fat body (29, 30). Furthermore, inhibition of TOR signaling in the \textit{Drosophila} fat body causes life span extension in the flies (31).

Many components of the TOR signaling pathway (TOR, raptor, tuberous sclerosis complexes 1 and 2, and RHEB) are highly conserved and occur in organisms from primitive eukaryotes like yeast up to vertebrates. Other components occur only in higher organisms. For example, S6K has no homologue in plants, yeast, or \textit{Caenorhabditis elegans}, but it is found in arthropods and vertebrates. The amino acid sequence of TOR kinase proteins is highly conserved, especially in the kinase and the FK506-binding protein binding region (Fig. 1). Even the domain structure shows no changes between primitive unicellular eukaryotes and vertebrates.

TOR is thought to be a cytoplasmic protein localized to intracellular membranes (32, 33). mTOR has been reported to shuttle between the cytoplasm and nucleus of mammalian cells (34). The results of our immunohistochemical analysis have shown a diffuse pattern of \textit{AaTOR} in the cytoplasm of fat body cells of unfed mosquitoes (Fig. 2b). A dramatic change in \textit{Aa}-TOR localization occurs after a blood meal. Four hours after the mosquito takes blood, the diffuse expression pattern of \textit{AaTOR} disappears and TOR immunoreactivity is located near the plasma membrane of the fat body cells (Fig. 2e). At
FIG. 7. Rapamycin but not LY294002, PD98059, or cycloheximide inhibits amino acid stimulation of AaS6K phosphorylation in in vitro fat body culture. Fat bodies were isolated and incubated in fat body culture for 1 h at room temperature. Western blot analysis was performed using antibodies against the Thr(P)-388 of S6K (upper panels). A second blot was probed with an antibody against S6K as loading control (lower panels). A, fat bodies were incubated with medium without amino acids (left lane) and with amino acids (right lane). B, rapamycin was added to the culture medium in increasing concentrations. C, LY294002 was added in increasing concentrations. D, PD98059 was added in increasing concentrations. E, cycloheximide was added in increasing concentrations.

FIG. 8. PI 3-kinase activity in mosquito fat bodies. Groups of 30 fat bodies were isolated and stimulated with 17 μM bovine insulin for 10 min at 37 °C. Immunoprecipitation of PI 3-kinase and an enzyme-linked immunosorbent assay of PI 3-kinase activity was performed following the manufacturers protocol. A, a PI 3-kinase antibody (AB) with broad reactivity against the p110 subunit of different species was used to isolate PI 3-kinase from mosquito fat bodies. The immunoprecipitate was boiled in NuPAGE LDS Sample Buffer (Invitrogen) and analyzed by Western blotting with the same antibody used for precipitation. B, the production of PI(3,4,5)P3 from PI(4,5)P2, was assayed via competitive enzyme-linked immunosorbent assay. 100 μM LY294002 was added to the reaction buffer where indicated. Data represent means ± S.E. of triplicate samples.

FIG. 9. RNAi-mediated knockdown of AaS6K inhibits Vg mRNA expression in vitro and egg development in vivo. Female mosquitoes were injected with 1 μg of dsRNA duplexes of either dsAaS6K or a control dsRNA derived from the noncoding region of a bacterial gene (dsMAL) and incubated for 72 h. A, fat bodies were dissected and subjected to in vitro fat body culture with medium either lacking or containing amino acids for 6 h. Vg mRNA levels were determined by real time PCR. Data represent means ± S.E. of triplicate samples. B, after a 3-day recovery period, a blood meal was given. Deposition of eggs was induced 5 days PBM.
this stage of development, the fat body plasma membrane is a dynamic surface mediating the secretion of massive amounts of yolk protein as well as amino acid uptake. Our data suggest that TOR might be involved in the regulation of these processes.

Little is known about the expression patterns of TOR proteins in different tissues of multicellular organisms. The general opinion is that TOR is a housekeeping gene expressed in all tissues of multicellular animals. In contrast, Arabidopsis TOR is expressed only in growing tissues and not in differentiated organs (35). We found relatively high levels of TOR mRNA in the ovaries and lower levels in the other tested tissues of adult female mosquitoes (Fig. 3). mRNA levels did not change dramatically after a blood meal. We assume that the rise of TOR mRNA in the developing ovaries may be because of the deposition of maternal TOR mRNA in the oocytes.

Major downstream targets of mTOR have been identified as components of the translational machinery: the eukaryotic initiation factor 4E-binding protein, S6K, and eIF4G (8). In the current model of mTOR function, a change in nutrient conditions causes a conformational change in a complex containing mTOR, mLST8, and Raptor, which changes the ability of mTOR to interact with its substrate, e.g. S6K or 4E-binding protein (8).

To monitor TOR activity in mosquitoes, we cloned the homologue of S6K from A. aegypti (AaS6K). Like TOR, this protein is highly conserved in its kinase domain (Fig. 4). The region surrounding threonine 388, located in the kinase extension domain, is also highly conserved. Threonine 388 of AaS6K is the analogous site to Thr-389 of the human p70 S6K1, which is a major phosphorylation site. Like AaTOR, AaS6K was expressed in all tissues examined. The highest concentration was found in the ovaries (Fig. 5). No dramatic changes of S6K mRNA levels were observed after a blood meal.

Motivated by the high degree of amino acid identity between mosquito and human S6K, we used a phosphorylation-specific antibody that recognizes human S6K only when phosphorylated at threonine 389, to detect Thr(P)-388 AaS6K in protein extracts gathered from mosquito fat bodies. The antibody recognizes a phosphoprotein with a molecular mass of ~62 kDa, which appears in the fat bodies and ovaries of mosquitoes after a blood meal (Fig. 6). This band was also found in cultured fat bodies that were stimulated by amino acids but not in the unstimulated control (Fig. 7A). Importantly, the band is abolished after treatment of the fat bodies with rapamycin, leaving little doubt that the detected protein is indeed Thr(P)-388 AaS6K (Fig. 7B). The finding that cycloheximide does not inhibit S6K phosphorylation shows that this process is not dependent upon protein translation. In contrast, the activation of the Vg gene after amino acid stimulation, a process we assume to be downstream of S6K activation, is strictly translation dependent.2

We did not find any impact on phosphorylation of AaS6K by inhibitors of the insulin signaling pathway, the MAP kinase pathway, or an inhibitor of the translational machinery. Treatment of fat bodies with LY294002 before and during amino acid stimulation did not inhibit S6K phosphorylation, an indication that the insulin signaling pathway is not involved in the activation of AaS6K by amino acids. Therefore an auto- or paracrine mechanism using insulin signaling appears to be not involved in the activation of AaS6K by amino acids. In vivo, the situation is likely to be more complex. Preliminary results show that Vg gene expression can be enhanced by bovine insulin,3 which is an indication that some sort of a growth factor and the insulin signaling pathway may be involved in this process. Furthermore, we have found that the mosquito fat body possesses functional PI 3-kinase (Fig. 8) an additional hint that insulin signaling might play a role in the regulation of vitellogenic gene expression. This aspect of regulation warrants further investigation.

In conclusion, the results of our present work indicate that TOR-dependent phosphorylation of S6K is a key factor for the onset of reproductive events taking place in female mosquitoes. S6K becomes activated by TOR in the developing ovaries as well as in the fat body, which subsequently starts to produce yolk proteins. It is more than likely that nutrient-dependent TOR and S6K signaling control egg development not only in mosquitoes but also in Drosophila and other insects.

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