



## TOR signaling is required for amino acid stimulation of early trypsin protein synthesis in the midgut of *Aedes aegypti* mosquitoes

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### ABSTRACT

Blood meal digestion in mosquitoes occurs in two phases, an early phase that is translationally regulated, and a late phase that is transcriptionally regulated. Early trypsin is a well-characterized serine endoprotease that is representative of other early phase proteases in the midgut that are only synthesized after feeding. Since the kinase Target of Rapamycin (TOR) has been implicated as a nutrient sensor in other systems, including the mosquito fat body, we tested if TOR signaling is involved in early trypsin protein synthesis in the mosquito midgut in response to feeding. We found that ingestion of an amino acid meal by female mosquitoes induces early trypsin protein synthesis, coincident with phosphorylation of two known TOR target proteins, p70S6 kinase (S6K) and the translational repressor 4E-Binding Protein (4E-BP). Moreover, *in vitro* culturing of midguts from unfed mosquitoes led to amino acid-dependent phosphorylation of S6K and 4E-BP which could be blocked by treatment with rapamycin, a TOR-specific inhibitor. Lastly, by injecting mosquitoes with TOR double stranded RNA (dsRNA) or rapamycin, we demonstrated that TOR signaling was required *in vivo* for both phosphorylation of S6K and 4E-BP in the midgut, and for translation of early trypsin mRNA in response to amino acid feeding. It may be possible to target the TOR signaling pathway in the midgut to inhibit blood meal digestion, and thereby, decrease fecundity and the spread of mosquito borne diseases.

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### 1. Introduction

The adult *Aedes aegypti* female mosquito requires a blood meal in order to complete the reproductive cycle. Blood is a very unique meal, consisting primarily of protein with only small amounts of lipid and carbohydrate. Efficient degradation of proteins into amino acids is important in blood meal digestion because amino acids are required for yolk protein synthesis and they provide metabolic energy for completion of the gonotrophic cycle (Briegleb, 2003). Moreover, much of the reduced carbon available from blood meal amino acids is converted to lipid and carbohydrate as an energy reserve for the next gonotrophic cycle (Zhou et al., 2004).

The primary endoprotease responsible for blood digestion in the mosquito midgut is trypsin (Noriega et al., 1999a). Other proteases such as chymotrypsin (Jiang et al., 1997), and the exopeptidases amino-peptidase and carboxypeptidase (Noriega et al., 2002), are also involved in the digestion of the blood meal. Trypsin is expressed in two phases, an early phase and a late phase. The early phase is characterized by the expression of early trypsin which is

transcribed in the midgut before feeding, but only expressed as protein after the ingestion of a blood meal (Noriega et al., 1996). The late phase proteases are transcribed and translated approximately 8 h after feeding and are responsible for the bulk of digestion. One of the chymotrypsins is transcribed before feeding, but as with early trypsin, is only translated at detectable levels after feeding (Jiang et al., 1997). Studies have shown that a blood meal is not required for early trypsin translation since ingestion of a homogeneous protein meal, or simply free amino acids, is sufficient to induce early trypsin synthesis (Noriega et al., 1999a). Importantly, ingestion of a meal containing only sugar or a saline solution is not capable of stimulating early trypsin translation (Noriega et al., 1999a). Taken together, these results indicate that amino acids are necessary and sufficient to activate early trypsin protein synthesis in the mosquito midgut.

Regulation of protein synthesis by amino acids often occurs at the level of translational initiation (Proud, 2002; Sonenberg and Dever, 2003). Two proteins known to control rates of protein synthesis are the translational repressor 4E-BP and the kinase S6K, each of which is regulated by phosphorylation. The 4E-BP protein functions by inhibiting the activity of the translation initiation factor eIF4E which is required for recruitment of the cap binding complex to the 5' terminus of mRNA transcripts. Phosphorylation of

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4E-BP by TOR protein kinase inhibits its association with eIF4E, thereby, leading to increased rates of cap-dependent translational initiation (Proud, 2002). In contrast, S6K phosphorylates the ribosomal protein S6 which is involved in recruiting ribosomes to mRNA transcripts containing a 5' tract of pyrimidine residues (5'TOP) in the upstream untranslated region (Kimball and Jefferson, 2004; Jefferies et al., 1997). Studies have shown that the activity of S6K is stimulated by TOR-mediated phosphorylation, which in turn, enhances translation of mRNA transcripts with 5'TOP sequences (Kimball and Jefferson, 2004).

TOR kinase is a component of the TORC1 protein complex which contains a scaffold protein called raptor that is responsible for the rapamycin sensitivity of TORC1 (Oshiro et al., 2004). TOR has been shown to be activated hormonally in vertebrates by insulin pathway signaling and by AMP Kinase (Arsham and Neufeld, 2006). In addition, TOR is activated by elevated amino acid concentrations, possibly through the class III PI3 kinase Vps34 (Nobukuni et al., 2005; Backer, 2008). The TOR signaling pathway in *Drosophila melanogaster* has been shown to be controlled by the insulin signaling pathway (Lizcano et al., 2003; Miron et al., 2003), as well as, by the flux of amino acid concentrations (Zhang et al., 2000). Studies in mosquitoes have recently shown that TOR regulates yolk protein synthesis in the fat body and oocyte maturation in the ovaries (Hansen et al., 2004, 2005). Regulation of yolk protein transcription in the mosquito fat body is controlled by TOR-mediated phosphorylation of S6K which leads to translation of the GATA transcription factor (Park et al., 2006). Nothing is known about TOR signaling in the mosquito midgut in response to feeding, nor if the activities of 4E-BP and S6K are controlled by TOR-mediated phosphorylation in this tissue.

Anautogenous mosquitoes, such as *Ae. aegypti*, must be able to fully digest the blood meal in order to obtain the necessary nutrients to complete the gonotrophic cycle (Attardo et al., 2006; Hansen et al., 2004). Moreover, high levels of ammonia that are produced by protein deamination need to be rapidly and efficiently reduced to prevent toxicity (Scaraffia et al., 2008). Translational control of early trypsin synthesis is a key step in the first phase of digestion that occurs within the first 3 h of feeding (Noriega and Wells, 1999b). Therefore, disruption of early trypsin translation in blood fed mosquitoes could be a strategy for vector control. To better understand how early trypsin translation is controlled by amino acid signaling, we investigated the role of TOR in the mosquito midgut with regard to regulation of 4E-BP and S6K phosphorylation and early trypsin protein synthesis.

## 2. Materials and methods

### 2.1. Mosquito rearing

*Ae. aegypti* (Rockefeller strain) were raised in a 16 h light/8 h dark photoperiod at 28 °C and 80% humidity. Female adults were maintained with 3% or 10% sucrose solution in a soaked cotton pad placed on the mosquito cage. Experimental mosquitoes were artificially fed a porcine blood meal containing 5 mM ATP, or an amino acid meal as noted. Amino acid meals consisted of M199 media (Invitrogen, Carlsbad, CA) containing 5 mM ATP, 10 mM HEPES, 40 µl per ml 100× MEM non-essential amino acids and 80 µl per ml MEM essential amino acids (MP Biomedicals, Solon, OH), adjusted to pH 7.2. The addition of amino acids approximates the amino acid levels used in Noriega et al. (1999a).

### 2.2. Generation of an early trypsin antibody

An early trypsin rabbit polyclonal antibody was generated by Sigma-Genosys (St. Louis, MO) using a synthetic peptide antigen corresponding to the region from Arg127 to Thr140 of the

*Ae. aegypti* early trypsin protein. Serum was obtained and the antibody was purified by affinity chromatography using the peptide antigen coupled to a Sulfo-link column (Pierce, Rockford, IL). The antibody eluate was dialyzed against CNBr-Sepharose binding buffer (GE Healthcare, Piscataway, NJ) and purified a second time using a CNBr-Sepharose affinity column containing full-length early trypsin protein produced in *Escherichia coli* using the pET-32a system (Novagen, Madison, WI) as described previously (Lu et al., 2006).

### 2.3. Western blotting

Midgut extracts were obtained by gently mincing five midguts with a small Kontes pestle in 100 µl phosphate buffered saline (PBS) containing 1 mM AEBBSF (4-[2-Aminoethyl] benzenesulfonyl fluoride hydrochloride) and 5 mM DTT. Samples to be analyzed for phosphorylated 4E-BP or S6K also contained a phosphatase inhibitor cocktail (Calbiochem, La Jolla, CA). The samples were then centrifuged at 16,000 × g for 5 min and an equivalent volume of 2× SDS-PAGE sample buffer was added to the supernatant. After boiling for 5 min, 1–5 midgut equivalents were loaded per lane on a 12% or 15% SDS-PAGE gel and the gel was processed for Western blotting using standard procedures. A 1:1000 dilution of purified anti-early trypsin antibody was used as primary antibody, and a 1:3000 dilution of goat-anti-rabbit IgG conjugated to horse radish peroxidase (Bio-Rad, Hercules, CA) was used as the secondary antibody. For Western blot analysis of phosphorylated 4E-BP and S6K proteins, we used 1:1000 dilutions of anti-phospho-4E-BP Thr37/46 antibody (Cell Signal Technology #9659S, Danvers, MA), anti-*Drosophila* 4E-BP antibody (d4E-BP) kindly provided by Nahum Sonenberg (McGill University), anti-phospho S6K Thr412 antibody (Upstate #07-018, Lake Placid, NY), or S6K antibody (Santa Cruz Biotechnology, #sc-230, Santa Cruz, CA), and appropriate secondary antibodies. A replica gel was run as a loading control and Western blotted with a mouse monoclonal antibody against β-tubulin (Sigma, St. Louis, MO, #T5168) at a concentration of 1:5000. Blots were developed with Lumi-Light (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Representative Western blots from multiple experiments are presented.

### 2.4. In vitro midgut cultures

Dissected midguts were cultured in individual wells of a 96-well plate containing 40 µl amino acid free M199 media that was formulated in the lab using all of the M199 constituents contained in commercial preparations (Invitrogen, Carlsbad, CA), but lacking amino acids. For the rapamycin experiments, midguts were pre-treated at 30 °C in a humid chamber for 1 h with or without the TOR inhibitor rapamycin (EMD Biosciences, La Jolla, CA) at various concentrations as described in section 3. Pre-treated midguts were then transferred to a new 96-well plate containing M199 media with amino acids, and the same differing concentrations of rapamycin for 90 min at 30 °C. Midguts for each rapamycin culture point were processed in groups of 10 and the phosphorylation state was determined using phospho-specific antibodies and Western blotting as described above.

To measure general translation, midguts were cultured in three groups of 10 as described above for 1 h in either amino acid free M199 media alone, or the same media containing 20 µM rapamycin or 10 µg/ml puromycin. The midguts were then transferred to fresh 96-well plates containing M199 media with amino acids and the same inhibitors, as well as, 0.5 µl per well [<sup>35</sup>S] methionine/cysteine (GE Healthcare, Piscataway, NJ; specific activity 1000 Ci/millimole at 10 milliCuries/ml). After a 90 min incubation, each group of 10 midguts was homogenized in 0.5 ml water and 0.5 ml of 20% ice cold TCA was added and the tubes and vortexed for 30 s. The

mixture was filtered through a glass fiber filter (millipore) and washed with 5 ml 10% TCA, followed by a wash with 5 ml anhydrous ethanol. The filters were dried overnight and subjected to liquid scintillation counting.

### 2.5. Inhibition of TOR signaling in mosquitoes by dsRNA and rapamycin injections

Double stranded TOR RNA (dsRNA) was constructed using the same procedure described in Lu et al. (2006). A portion of the 3' end of the TOR transcript was PCR amplified using midgut RNA isolated from unfed mosquitoes with the primers TOR-F1 5'-GCCGTGCTGGAAGCTTTC-3' and TOR-R1 5'-GATTTCCGGTGTC-TACCAGAAAGG-3'. This PCR product was then used as a template to add T7 promoters to each strand using the primers TOR-FT7 5'-TAATACGACTCACTATAGGGGCCGTGCTGGAAGCTTTC-3' and TOR-RT7 5'-TAATACGACTCACTATAGGGGATTTCCGGTGCTACCAGAAAGG-3'. After gel purification of the second PCR product, TOR dsRNA was synthesized using the Maxi-Script RNAi kit (Ambion, Austin, TX). Control luciferase dsRNA was constructed as described previously (Lu et al., 2006). Newly emerged mosquitoes were injected six times with 5 mg/ml TOR or Luciferase (LUC) dsRNA using a Drummond Nanoject injector (Drummond Scientific Company, Broomall, PA) set at 69 nl/injection. Alternatively, mosquitoes were injected with 69 nl of 20  $\mu$ M rapamycin dissolved in PBS 1 h before feeding. All mosquitoes were maintained on glucose for 3 days and then half were fed an amino acid meal. Midguts were isolated after 24 h and RNA transcripts and protein were analyzed by quantitative RT-PCR and Western blotting, respectively. The size of developing oocytes was determined in unfed and blood fed mosquitoes 24 h after feeding (4 days total after injection). Note that amino acid fed mosquitoes do not complete the gonotrophic cycle (JEP, unpublished data).

### 2.6. Quantitative real-time reverse transcriptase polymerase chain reaction (QRT-PCR)

Messenger RNA levels were determined after cDNA synthesis by quantitative real-time PCR analysis as previously described (Scaraffia et al., 2005). Early trypsin RNA levels were determined using primers ET-F1 5'-GGTGGCATCATAGTGAAAGTCA-3' and ET-R1 5'-GAAACGGTGCACATAGCTCCAT-3'. TOR RNA levels were determined using the primers TOR-F1 5'-CGTGAAGAGCCCTCGTC-3' and TOR-R1 5'-CACGTGCATGACGCTTTC-3'. Levels of early trypsin and TOR transcripts were normalized to ribosomal S7 protein gene transcripts using the primers AaS7RP F5'-ACCGCCGTCTACGATGCA-3' and AaS7RP R5'-ATGGTGGTCTGCTGGTCTT-3'. QRT-PCR was carried in the 96-well 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) in conjunction with the Start Universal SYBR Green Master Mix (Roche Applied Science). Data analysis was performed using the ABI Prism 7300 SDS Software (v.1.2.2; Applied Biosystems). The statistical analysis was completed using GraphPad Prism (version 4.02; GraphPad Software, Inc., San Diego, CA).

### 2.7. Egg size determination

Twenty-four hours after feeding on a porcine blood meal, the size of developing oocytes was determined as described previously (Lu et al., 2006).

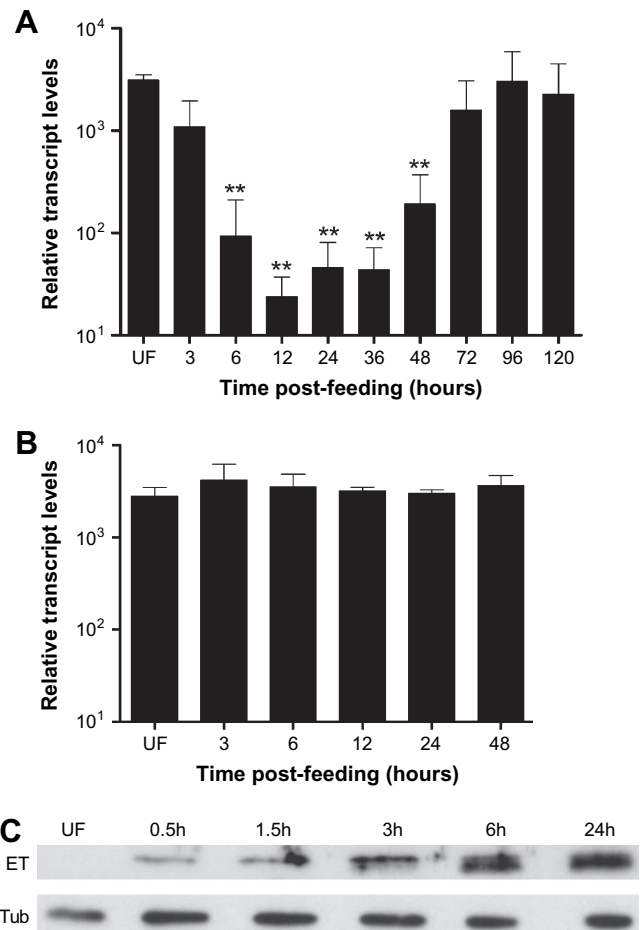
### 2.8. Statistics

Statistical analysis was performed using GraphPad InStat (San Diego, CA). ANOVA or Student's *t* test was used with significance determined as  $p < 0.05$ .

## 3. Results

### 3.1. Expression pattern of early trypsin in the midgut

The translation of early trypsin mRNA in the midgut of blood fed *Ae. aegypti* mosquitoes is dependent on the presence of pre-existing transcripts. Previous studies using semi-quantitative Northern blotting from protein and blood fed *Ae. aegypti* mosquitoes indicated that early trypsin transcript levels are initially high in the midgut, but rapidly fall following a meal (Kalhok et al., 1993; Noriega et al., 1996). To better characterize early trypsin transcript levels in the midguts of unfed and blood fed mosquitoes, we used quantitative real-time PCR to measure early trypsin mRNA levels in three different cohorts of mosquitoes over a period of 3–120 h post-feeding. As shown in Fig. 1A, early trypsin transcript levels were



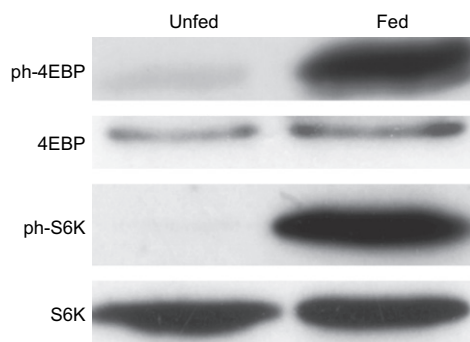
**Fig. 1.** Differential expression of early trypsin RNA and protein levels in *Ae. aegypti* mosquitoes in response to feeding. (A) Time course of early trypsin RNA levels in the midgut of unfed (UF) and blood fed mosquitoes as determined by quantitative real-time RT-PCR. Each bar represents the mean and standard deviation of replica PCR results taken from three independent cohorts of mosquitoes. Early trypsin RNA levels are expressed as relative values compared to endogenous transcript levels of the ribosomal S7 protein gene using 120 h as the maximum amount. Data was analyzed by ANOVA with  $**p < 0.01$  comparing early trypsin transcript levels in fed versus unfed mosquitoes. (B) Early trypsin transcript levels in unfed and amino acid fed mosquitoes measured over a 48 h period using QRT-PCR and the same relative transcript scale based on S7 transcripts as in (A). Data is presented as mean and standard deviation of three mosquito cohorts fed and dissected in different experiments. (C) Midgut early trypsin protein levels in amino acid fed mosquitoes based on Western blotting using an anti-early trypsin rabbit polyclonal antibody. The loading control for the Western blot was an anti-tubulin antibody. This is a representative Western blot from multiple independent experiments. Note that this particular early trypsin antibody recognized numerous non-specific ~25–35 kDa proteins in the midguts of blood fed mosquitoes that precluded its use in Western blotting using protein extracts from these mosquitoes (data not shown).

high prior to feeding, but by 6 h post-feeding, transcript levels were reduced ~15-fold and continued to decline until 12 h when they were ~50-fold lower in the midguts of fed mosquitoes compared to unfed mosquitoes. By 72 h post-feeding, early trypsin transcript levels were equivalent to that of unfed mosquitoes. These results are similar to those reported by Noriega et al. (1996) in which *Ae. aegypti* mosquitoes were fed a meal consisting only of gamma globulin protein.

Although it was known that early trypsin protein expression is activated by 3 h post-feeding an amino acid meal (Noriega et al., 1996), it was not known if amino acid feeding altered early trypsin mRNA levels, or if early trypsin protein synthesis occurred prior to 3 h. To examine these parameters, we generated a polyclonal antibody directed against a 14 amino acid early trypsin peptide (Arg127–Thr140) and used the affinity-purified antibody in Western blotting experiments. As shown in Fig. 1, amino acid feeding had no effect on early trypsin transcript levels (Fig. 1B), however, early trypsin protein levels were increased within 30 min of feeding and remained high for 24 h (Fig. 1C). These results indicate that ingestion of amino acids rapidly induces a regulatory signal in the mosquito midgut that stimulates the translation of early trypsin mRNA, and moreover, that the reduction of early trypsin transcript levels post blood meal feeding is not recapitulated by an amino acid meal.

### 3.2. Phosphorylation of 4E-BP and S6K in response to amino acid feeding

Based on TOR-mediated translational control studies in other systems (Gingras et al., 2001), including the mosquito fat body (Hansen et al., 2004, 2005), we hypothesized that early trypsin protein synthesis is controlled by TOR signaling. To test this idea, we examined the phosphorylation status of two known TOR target proteins, 4E-BP and S6K, in mosquito midguts using phospho-specific antibodies and Western blot analysis. The phospho-specific 4E-BP antibody detects phosphorylation at Thr37 and Thr 46 in human TOR at positions that are conserved in the mosquito protein. Similarly, the phospho-specific S6K antibody detects phosphorylation at the conserved Thr412 residue in both the human and mosquito protein. As seen in Fig. 2, both 4E-BP and S6K are only minimally phosphorylated in unfed mosquito midguts, but by 90 min post-feeding an amino acid meal, both 4E-BP and S6K are highly phosphorylated. Western blotting with control antibodies that detect both phosphorylated and unphosphorylated forms of the 4E-BP and S6K proteins, revealed that increased levels of



**Fig. 2.** Amino acid feeding induces phosphorylation of 4E-BP and S6K in the mosquito midgut. The antibodies used in this Western blot were ph-4E-BP, a phospho-specific 4E-BP antibody that detects phosphorylation at Thr37 and Thr46; d4E-BP, a *Drosophila melanogaster* anti-4E-BP antibody that detects both phosphorylated and unphosphorylated 4E-BP; ph-S6K, a phospho-specific antibody that detects phosphorylated S6K at Thr412; and S6K which detects both phosphorylated and unphosphorylated S6K. This is a representative Western blot from multiple independent experiments.

phosphorylated 4E-BP and S6K proteins are due to phosphorylation rather than altered protein expression.

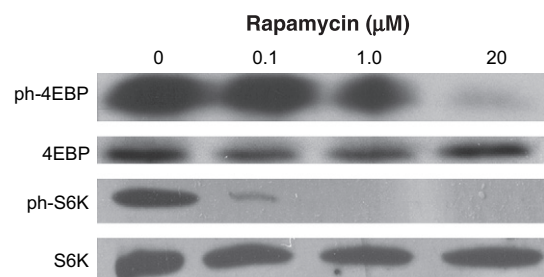
### 3.3. TOR signaling in an *in vitro* midgut culture system

To better control for TOR signaling within the midgut, we used an *in vitro* organ culture system containing dissected midguts from unfed female mosquitoes cultured in M199 media with or without amino acids (Zieler and Dvorak, 2000). After pre-culturing midguts in amino acid deficient media, the midguts were placed in amino acid rich media to induce phosphorylation of 4E-BP and S6K. As shown in Fig. 3, the amino acid induction of 4E-BP and S6K phosphorylation could be inhibited by rapamycin, a specific inhibitor of TOR activity (Gingras et al., 2001). This result confirms that TOR plays a role in regulating protein synthesis in the mosquito midgut through phosphorylation of the 4E-BP and S6K proteins. Interestingly, significantly higher doses of rapamycin were required to inhibit 4E-BP phosphorylation at the amino terminal residues Thr37/46 in response to amino acids than were needed to inhibit S6K phosphorylation at Thr412 (20  $\mu$ M compared to 0.1  $\mu$ M). This is similar to the reduced effects of rapamycin on the human 4E-BP protein as compared to S6K (Averous and Proud, 2006).

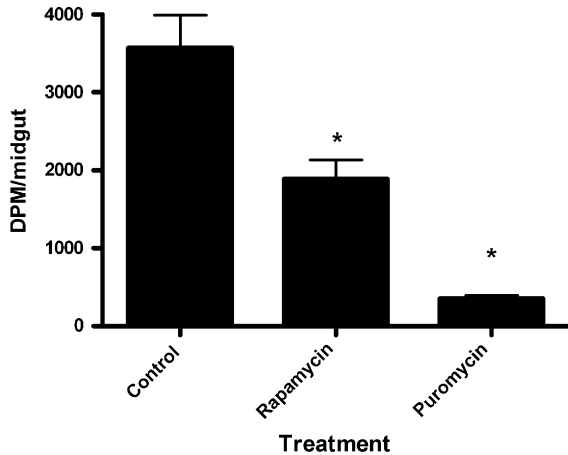
To determine the relative contribution of TOR signaling to global protein synthesis in isolated mosquito midguts, we measured total protein synthesis in amino acid rich media containing [<sup>35</sup>S]-labeled methionine and cysteine with or without inhibitors. As shown in Fig. 4, incubation of isolated midguts in either 20  $\mu$ M rapamycin, or 10  $\mu$ g/ml of puromycin, a polypeptide chain terminator, resulted in a 53% and 90% inhibition, respectively, of radioactive incorporation in TCA precipitable counts. These data suggests that TOR signaling is responsible for approximately half of all protein synthesis in the mosquito midgut in response to amino acid stimulation.

### 3.4. Inhibition of TOR activity *in vivo* blocks early trypsin synthesis

In order to test if TOR signaling is required for early trypsin synthesis in intact mosquitoes, we injected mosquitoes with dsRNA directed against TOR, or as a control, dsRNA directed against firefly luciferase (LUC). As shown in Fig. 5A, 4 days after injection, the level of TOR transcript in the midguts of unfed mosquitoes was reduced by over 90% as compared to uninjected mosquitoes, or mosquitoes injected with LUC dsRNA. Importantly, mosquitoes injected with TOR dsRNA had significantly reduced egg size after blood meal feeding (Fig. 5B), confirming that the TOR RNAi knock-down was functionally relevant (Hansen et al., 2004). The penetrance of the TOR RNAi knock-down in the midgut was investigated by monitoring the level of 4E-BP and S6K phosphorylation 3 h after an



**Fig. 3.** Rapamycin inhibits 4E-BP and S6K phosphorylation in isolated midguts cultured in amino acid rich media. Isolated midguts from unfed mosquitoes were cultured in amino acid free M199 media in the presence or the absence of various concentrations of rapamycin for 60 min and then transferred to fresh M199 media containing amino acids and the same concentrations of rapamycin for an additional 90 min. Protein extracts were prepared and analyzed by Western blotting as described in Fig. 2. This is a representative Western blot from multiple independent experiments.



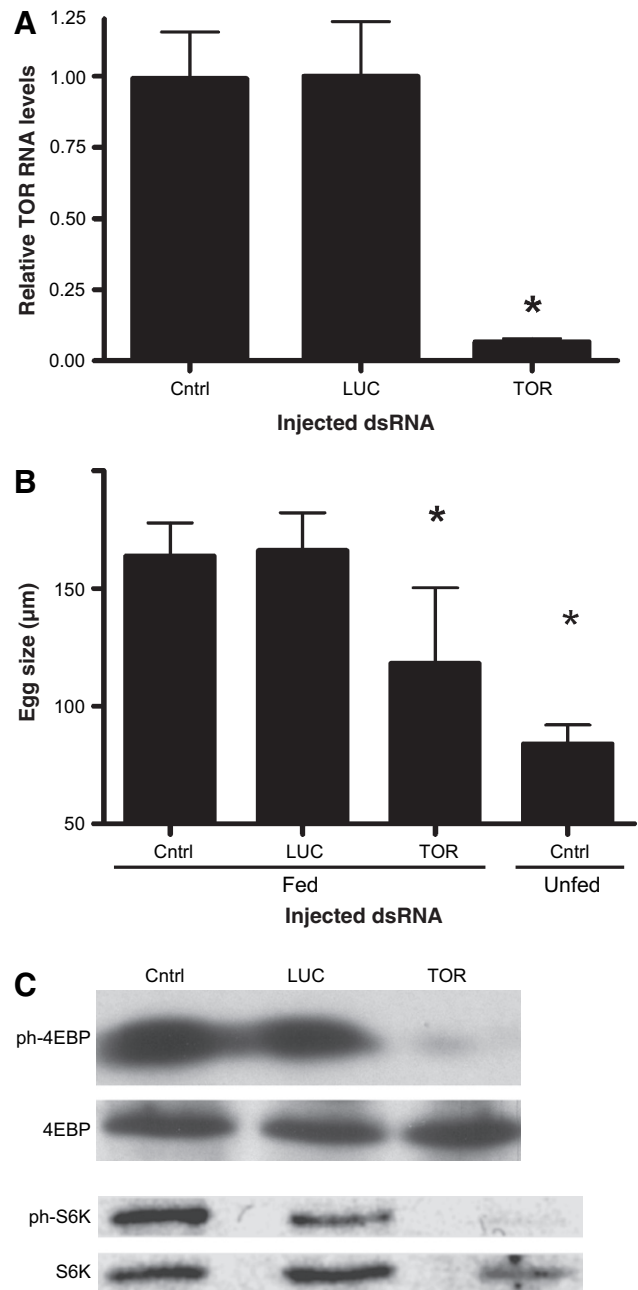
**Fig. 4.** The TOR signaling pathway accounts for half of the amino acid induced translation in isolated midguts from unfed mosquitoes. Midguts were cultured for 60 min in amino acid free M199 media in the absence of any inhibitors, or in the presence of 20  $\mu$ M rapamycin or 10  $\mu$ g/ml puromycin, a general translational inhibitor. The midguts were then transferred to fresh M199 media containing amino acids and the same concentrations of inhibitors. This media also contained [ $^{35}$ S] methionine/cysteine as a trace label to follow protein synthesis. Incubation continued for 90 min after which the pooled midguts were homogenized and TCA precipitable protein was analyzed by scintillation counting. Data is expressed as the mean and standard deviation of disintegrations per minute per midgut from three independent experiments. Asterisks (\*) indicate that the data point is significantly different ( $p < 0.05$ ) from the control sample.

amino acid meal. As shown in Fig. 5C, 4E-BP phosphorylation in the midguts of injected mosquitoes was dramatically reduced by TOR dsRNA injections, but not LUC dsRNA, thereby demonstrating that TOR signaling in the mosquito midgut is required for amino acid induced 4E-BP phosphorylation. A similar trend was found for S6K in that TOR dsRNA injected mosquitoes had lower levels of phosphorylated S6K than did amino acid fed mosquitoes injected with LUC dsRNA.

Based on the data in Fig. 5 showing that injection of dsRNA TOR led to inhibition of TOR signaling in the midgut, we analyzed the effect of TOR RNAi on early trypsin transcription and translation. These studies revealed that TOR dsRNA had no effect on the level of early trypsin transcripts in the midgut of unfed mosquitoes (Fig. 6A). However, injection of TOR dsRNA did significantly reduce early trypsin protein levels 3 h after an amino acid meal as compared to dsRNA LUC injected mosquitoes (Fig. 6B). Indeed, the low level of early trypsin protein in dsRNA TOR injected mosquitoes was comparable to mosquitoes that had been injected with 69 nM of 20  $\mu$ M of the TOR inhibitor rapamycin in PBS 1 h before feeding. Taken together, the *in vitro* and *in vivo* results show for the first time that amino acid induction of early trypsin synthesis in the midgut of *Ae. aegypti* mosquitoes requires signaling through the TOR pathway.

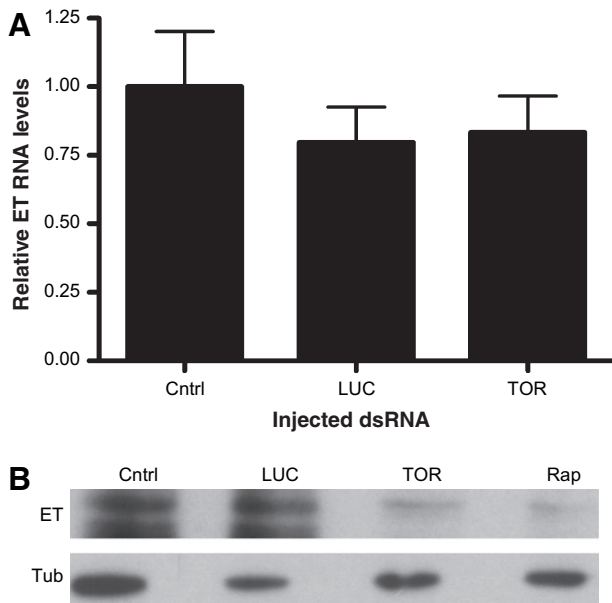
#### 4. Discussion

Management of mosquito borne disease at the vector level is currently being approached in two ways. First, insecticides are used to reduce mosquito populations in infected areas and thereby reduce pathogen transmission. Second, plans are underway to replace wild mosquito populations with transgenic mosquitoes that are genetically resistant to pathogen infection (James, 2003). Although transgenesis is an elegant approach that could revolutionize how mosquito borne diseases are controlled, insecticidal agents that reduce mosquito numbers may be more cost efficient and logistically feasible. Use of broad spectrum insecticides such as DDT (dichloro-diphenyl-trichloroethane), are not without risks because they can lead to insect resistance and harmful side effects



**Fig. 5.** TOR signaling is required for 4E-BP phosphorylation in the midguts of amino acid fed mosquitoes. (A) TOR RNA levels were determined by quantitative RT-PCR in the midguts of different cohorts of unfed mosquitoes 6 days post-injection. Mean early trypsin RNA levels relative to S7 ribosomal protein gene transcripts were determined using midgut RNA from control (uninjected), and TOR or LUC injected mosquitoes. Asterisk (\*) indicates that TOR RNA levels were significantly different ( $p < 0.05$ ) from the non-injected or LUC injected control mosquitoes. (B) Effect of TOR dsRNA injection on egg development as determined by egg size. Egg size was determined using a microscopic micrometer. Data is expressed as the average size of eggs from 10 different mosquitoes per sample point using more than one cohort of mosquitoes. The mean egg sizes were significantly different (\*) between the LUC and TOR injected mosquitoes, as well as, between the TOR injected and unfed controls ( $p < 0.05$ ). (C) Effect of TOR dsRNA injection on the phosphorylation of 4E-BP and S6K as determined by Western blotting. Protein extracts were prepared 90 min after amino acid feeding and analyzed with the same antibodies described in Fig. 2. Representative Western blots from multiple experiments are shown. The S6K antibody was used on the same filter as the ph-S6K antibody following an antibody-stripping step.

to the environment. As an alternative, we are attempting to identify mosquito-selective metabolic targets that could be exploited to reduce mosquito fecundity with minimal risk to other organisms. An understanding of blood meal metabolism in female mosquitoes



**Fig. 6.** TOR signaling is required for early trypsin protein synthesis in amino acid fed mosquitoes. (A) Injection of TOR dsRNA had no effect on early trypsin transcript levels in unfed mosquitoes based on quantitative RT-PCR. (B) Injection of TOR dsRNA or rapamycin decreased early trypsin protein levels in amino acid fed mosquitoes. Western blotting was performed the same as in Fig. 1. The doublet band seen here likely reflects the presence of unprocessed zymogen. This is a representative Western blot from multiple independent experiments.

may provide these targets as female mosquitoes must maximize energy recovery from the blood meal without succumbing to nitrogen toxicity. Our previous work in this area has identified some of the key parameters in blood meal metabolism in *Ae. aegypti* (Scaraffia et al., 2005, 2008; Zhou et al., 2004). We have now extended these studies to include an analysis of regulatory pathways that control the first step in blood meal metabolism, namely, activation of midgut proteases by translational control.

We used quantitative methods to measure early trypsin transcript levels before and after blood meal feeding and found that RNA levels decrease as much as 50-fold by 12 h (Fig. 1A). This pattern of early trypsin gene expression in unfed and fed *Ae. aegypti* mosquitoes is qualitatively similar to earlier reports using Northern blotting (Kalhok et al., 1993; Noriega et al., 1996). We also analyzed early trypsin protein levels by Western blotting to determine the effect of amino acid feeding over a 24 h period. Consistent with a rapid signaling response in the mosquito midgut, we observed early trypsin protein expression within 30 min of feeding that reached a peak by 6 h post-feeding (Fig. 1B). Interestingly, trypsin protein levels remained high up to 24 h post-feeding which likely reflects the elevated levels of early trypsin transcripts in these amino acid fed mosquitoes (Fig. 1B).

In order to investigate the mechanism underlying translational induction of early trypsin protein synthesis, we focused on TOR activation which has been shown to be important in vitellogenesis in the mosquito fat body following a blood meal (Hansen et al., 2004, 2005; Park et al., 2006). We reasoned that TOR signaling might be the upstream activator of early trypsin synthesis in the midgut because amino acids are a known nutrient stimulus for TOR signaling (Hansen et al., 2004). Moreover, the 5' untranslated region of the early trypsin mRNA transcript contains a pyrimidine-rich region that is similar to other pyrimidine-rich regions that have been shown to be responsive to TOR signaling through S6K (Gingras et al., 2001). Indeed, we found that amino acid feeding induced S6K phosphorylation *in vitro* and *in vivo*, and that S6K phosphorylation

was mediated by TOR based on its inhibition by both rapamycin and TOR RNAi.

TOR is also known to activate translation through phosphorylation of the translational repressor protein 4E-BP (Gingras et al., 2001; Miron et al., 2003). Our bioinformatic analysis of the *Ae. aegypti* genome identified a single 4E-BP gene, and expression analyses revealed that 4E-BP is differentially expressed in response to blood meal feeding in the midgut, fat body, and ovary (data not shown). We examined 4E-BP phosphorylation in the midguts of unfed and amino acid fed mosquitoes, and consistent with the downstream effects of TOR signaling on S6K and 4E-BP, we found that 4E-BP phosphorylation was also TOR-mediated *in vitro* and *in vivo*. Taken together, these results confirm that amino acids function as nutrient signals that stimulate TOR activity in the mosquito midgut.

Lastly, we investigated more directly whether TOR signaling is required for early trypsin protein synthesis in amino acid fed mosquitoes. As shown in Fig. 6, injection of TOR dsRNA had no effect on early trypsin transcript levels, but it greatly reduced early trypsin protein levels, and importantly, also significantly reduced egg size in blood fed mosquitoes (Fig. 5). This finding reveals the existence of a signaling link between amino acids, the TOR/4E-BP/S6K pathway, and early trypsin synthesis in the midgut of *Ae. aegypti* mosquitoes. Note, however, that early trypsin translation does not occur when isolated midguts from unfed mosquitoes are cultured with amino acids *in vitro* (data not shown), despite the fact that the TOR pathway is activated in this same culture system (Figs. 2 and 3). Therefore, we can only conclude that TOR activity is necessary, but not sufficient, to induce translation of early trypsin mRNA.

How might this information be used to develop new targets for vector control? Since TOR activity is an important factor in the initiation of digestive processes in the mosquito midgut, disruption of this pathway may be an important target in vector control. The fact that TOR is also required for the activation of vitellogenesis and egg maturation in *Ae. aegypti* (Hansen et al., 2004), makes it even more attractive as it is involved in several key processes that are dependent on blood meal feeding. If agents can be developed that specifically target mosquito TOR, or other TOR pathway components that are mosquito-selective, then it may be possible to control mosquito populations by disrupting blood meal digestion and reproductive processes.

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## References

- Arsham, A.M., Neufeld, T.P., 2006. Thinking globally and acting locally with TOR. *Curr. Opin. Cell Biol.* 18, 589–597.
- Attardo, G.M., Hansen, I.A., Shiao, S.H., Raikhel, A.S., 2006. Identification of two cationic amino acid transporters required for nutritional signaling during mosquito reproduction. *J. Exp. Biol.* 209, 3071–3078.
- Averous, J., Proud, C.G., 2006. When translation meets transformation: the mTOR story. *Oncogene* 25, 6423–6435.
- Backer, J.M., 2008. The regulation and function of Class III PI3Ks: novel roles for Vps34. *Biochem. J.* 410, 1–17.
- Briegleb, H., 2003. Physiological bases of mosquito ecology. *J. Vector Ecol.* 28, 1–11.
- Gingras, A.C., Raught, B., Sonenberg, N., 2001. Regulation of translation initiation by FRAP/mTOR. *Genes Dev.* 15, 807–826.
- Hansen, I.A., Attardo, G.M., Park, J.H., Peng, Q., Raikhel, A.S., 2004. Target of rapamycin-mediated amino acid signaling in mosquito anaotogeny. *Proc. Natl. Acad. Sci. U.S.A.* 101, 10626–10631.

- Hansen, I.A., Attardo, G.M., Roy, S.G., Raikhel, A.S., 2005. 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.* 280, 20565–20572.
- James, A.A., 2003. Blocking malaria parasite invasion of mosquito salivary glands. *J. Exp. Biol.* 206, 3817–3821.
- Jefferies, H.B., Fumagalli, S., Dennis, P.B., Reinhard, C., Pearson, R.B., Thomas, G., 1997. Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k. *EMBO J.* 16, 3693–3704.
- Jiang, Q., Hall, M., Noriega, F.G., Wells, M., 1997. cDNA cloning and pattern of expression of an adult, female-specific chymotrypsin from *Aedes aegypti* midgut. *Insect Biochem. Mol. Biol.* 27, 283–289.
- Kalhok, S.E., Tabak, L.M., Prosser, D.E., Brook, W., Downe, A.E., White, B.N., 1993. Isolation, sequencing and characterization of two cDNA clones coding for trypsin-like enzymes from the midgut of *Aedes aegypti*. *Insect Mol. Biol.* 2, 71–79.
- Kimball, S.R., Jefferson, L.S., 2004. Molecular mechanisms through which amino acids mediate signaling through the mammalian target of rapamycin. *Curr. Opin. Clin. Nutr. Metab. Care* 7, 39–44.
- Lizcano, J.M., Alrubaie, S., Kieloch, A., Deak, M., Leever, S.J., Alessi, D.R., 2003. Insulin-induced *Drosophila* S6 kinase activation requires phosphoinositide 3-kinase and protein kinase B. *Biochem. J.* 374, 297–306.
- Lu, S.J., Pennington, J.E., Stonehouse, A.R., Mobula, M.M., Wells, M.A., 2006. Reevaluation of the role of early trypsin activity in the transcriptional activation of the late trypsin gene in the mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol.* 36, 336–343.
- Miron, M., Lasko, P., Sonenberg, N., 2003. Signaling from Akt to FRAP/TOR targets both 4E-BP and S6K in *Drosophila melanogaster*. *Mol. Cell. Biol.* 23, 9117–9126.
- Nobukuni, T., Joaquin, M., Roccio, M., Dann, S.G., Kim, S.Y., Gulati, P., Byfield, M.P., Backer, J.M., Natt, F., Bos, J.L., Zwartkruis, F.J., Thomas, G., 2005. Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc. Natl. Acad. Sci. U.S.A.* 102, 14238–14243.
- Noriega, F.G., Pennington, J.E., Barillas-Mury, C., Wang, X.Y., Wells, M.A., 1996. *Aedes aegypti* midgut early trypsin is post-transcriptionally regulated by blood feeding. *Insect Mol. Biol.* 5, 25–29.
- Noriega, F.G., Colonna, A.E., Wells, M.A., 1999a. Increase in the size of the amino acid pool is sufficient to activate translation of early trypsin mRNA in *Aedes aegypti* midgut. *Insect Biochem. Mol. Biol.* 29, 243–247.
- Noriega, F.G., Wells, M.A., 1999b. A molecular view of trypsin synthesis in the midgut of *Aedes aegypti*. *J. Insect Physiol.* 45, 613–620.
- Noriega, F.G., Edgar, K.A., Bechet, R., Wells, M.A., 2002. Midgut exopeptidase activities in *Aedes aegypti* are induced by blood feeding. *J. Insect Physiol.* 48, 205–212.
- Oshiro, N., Yoshino, K., Hidayat, S., Tokunaga, C., Hara, K., Eguchi, S., Avruch, J., Yonezawa, K., 2004. Dissociation of raptor from mTOR is a mechanism of rapamycin-induced inhibition of mTOR function. *Genes Cells* 9, 359–366.
- Park, J.H., Attardo, G.M., Hansen, I.A., Raikhel, A.S., 2006. 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.* 281, 11167–11176.
- Proud, C.G., 2002. Regulation of mammalian translation factors by nutrients. *Eur. J. Biochem.* 269, 5338–5349.
- Scaraffia, P.Y., Isoe, J., Murillo, A., Wells, M.A., 2005. Ammonia metabolism in *Aedes aegypti*. *Insect Biochem. Mol. Biol.* 35, 491–503.
- Scaraffia, P.Y., Tan, G., Isoe, J., Wysocki, V.H., Wells, M.A., Miesfeld, R.L., 2008. Discovery of an alternate metabolic pathway for urea synthesis in adult *Aedes aegypti* mosquitoes. *Proc. Natl. Acad. Sci. U.S.A.* 105, 518–523.
- Sonenberg, N., Dever, T.E., 2003. Eukaryotic translation initiation factors and regulators. *Curr. Opin. Struct. Biol.* 13, 56–63.
- Zhang, H., Stallock, J.P., Ng, J.C., Reinhard, C., Neufeld, T.P., 2000. Regulation of cellular growth by the *Drosophila* target of rapamycin dTOR. *Genes Dev.* 14, 2712–2724.
- Zhou, G., Flowers, M., Friedrich, K., Horton, J., Pennington, J., Wells, M.A., 2004. Metabolic fate of [<sup>14</sup>C]-labeled meal protein amino acids in *Aedes aegypti* mosquitoes. *J. Insect Physiol.* 50, 337–349.
- Zieler, H., Dvorak, J.A., 2000. Invasion in vitro of mosquito midgut cells by the malaria parasite proceeds by a conserved mechanism and results in death of the invaded midgut cells. *Proc. Natl. Acad. Sci. U.S.A.* 97, 11516–11521.