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Identification of two cationic amino acid transporters required for nutritional signaling during mosquito reproduction

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Summary

The defining characteristic of anautogenous mosquitoes is their requirement for a blood meal to initiate reproduction. The need for blood drives the association of vector and host, and is the primary reason why anautogenous mosquitoes are effective disease vectors. During mosquito vitellogenesis, a key process in reproduction, yolk protein precursor (YPP) gene expression is activated specifically in the fat body, the insect analogue of the vertebrate liver. We have demonstrated that blood meal derived amino acids (AAs) activate YPP genes *via* the target of rapamycin (TOR)-signal transduction pathway. Here we show, by stimulating fat bodies with balanced AA solutions lacking individual AAs, that specific cationic and branched AAs are essential for activation of the *vitellogenin* (*vg*) gene, the major YPP gene. Treatment of fat bodies with AA uptake inhibitors results in a strong inhibition of AA-induced *vg* gene expression proving that an active transport mechanism is necessary to transduce the AA signal. We identified two cationic AA transporters (CATs) in the fat body of *Aedes aegypti* females – *Aa slimfast* and *iCAT2*. RNAi knockdown of *slimfast* and *iCAT2* results in a strong decrease in the response to AAs by the *vg* gene similar to that seen due to TOR inhibition. These data demonstrate that active uptake of specific AAs plays a key role in nutritional signaling during the onset of vitellogenic gene expression in mosquitoes and it is mediated by two cationic AA transporters.

Key words: TOR, vitellogenesis, slif, iCAT2, *Aedes aegypti*, fat body.

Introduction

Anautogenous mosquito species require vertebrate blood to begin their first gonotrophic cycle whereas autogenous mosquitoes undergo theirs without a blood meal. This requirement places strong evolutionary pressure upon anautogenous mosquitoes to make frequent host contacts and as a consequence makes them ideal disease vectors. The understanding of mosquito reproductive biology, the process responsible for driving disease transmission, is an important component in the development of novel strategies for use in mosquito-borne disease control (Attardo et al., 2005). Significant physiological differences are documented between autogenous and anautogenous mosquitoes. Typically, autogenous mosquitoes emerge from the pupal stage with larger stores of nutrients and higher titers of hemolymph amino acids (AAs) (Su and Mulla, 1997a; Su and Mulla, 1997b).

Egg development in female mosquitoes begins with vitellogenesis, the tissue specific expression, synthesis and secretion of yolk protein precursors (YPPs) by the fat body. The secreted proteins are then transported to the ovaries where they are incorporated into the developing oocytes (Raikhel et al., 2002; Raikhel and Dhadialla, 1992).

In *Aedes aegypti* (the anautogenous species used in this work), after pupation and a 3-day preparation period, the mosquito enters a previtellogenic state of arrest during which yolk protein precursor (YPP) gene transcription is repressed until stimulation by a blood meal (Attardo et al., 2003; Martin et al., 2001a). After blood feeding, transcription of YPP genes is upregulated in the fat body. Expression of the major YPP gene, *vitellogenin* (*vg*) peaks at around 24 h and subsides between 36 and 48 h after a blood meal (PBM). *vg* is both directly and indirectly regulated by the steroid hormone 20-hydroxyecdysone (20E), the titers of which positively correlate with *vg* expression during vitellogenesis (Deitsch et al., 1995; Kokoza et al., 2001; Martin et al., 2001b). 20E acts as the primary signal regulating *vg* expression. However, 20E activation of *vg* appears to be conditional. Exogenous treatment of competent mosquitoes with physiological levels of 20E does not result in activation of vitellogenesis (Lea, 1982).

Studies concerning the effects of AA levels upon egg development in mosquitoes have shown that a number of AAs are essential for oogenesis (Lea et al., 1956; Uchida, 1998; Uchida et al., 2001). Analysis of the dynamics of hemolymph AA concentrations shows significant increases in total AA concentration within 8 h post blood meal. The increased AA concentrations last until 3 days post blood meal (Uchida et al., 1990). Our recent work has demonstrated that AAs act directly upon the fat body to activate basal expression of the *vg* gene and that without them 20E is incapable of activating *vg* (Hansen et al., 2004). Furthermore, we discovered that the nutritionally regulated TOR (target of rapamycin) kinase signal transduction pathway mediates the AA signal. The TOR kinase is a serine/threonine kinase, which is ubiquitously expressed in eukaryotes (Raught et al., 2001). It has been well characterized in its role as a nutrient sensor in multiple systems including *Saccharomyces cerevisiae*, *Drosophila melanogaster* and vertebrate cells (Colombani et al., 2003; Cooper, 2002; Lynch et al., 2000).

In the *D. melanogaster* fat body, TOR-mediated AA signaling regulates the growth rate of the whole organism. This system was identified during a search for growth phenotypes by a transposon-mediated mutagenesis study. The disrupted gene was found to be a cationic AA transporter (CAT) called *slimfast* (*slif*). Disruption of *slif* in the fat body resulted in global inhibition of growth as well as a sensitivity to arginine starvation. The phenotypic effects of *slif* knockdown resemble those of rapamycin treatment, an inhibitor of the TOR pathway (Colombani et al., 2003). Two proton-assisted AA transporters (PAT) that genetically interact with TOR have been identified in *Drosophila* (Goberdhan et al., 2005). Among them, the gene *pathetic* (*path*) encodes a low capacity/high affinity transporter, which has been suggested to play a role as an AA sensor in the TOR pathway.

In this work we continue our characterization of AA signaling in mosquito vitellogenesis by analysis of the effect that specific AAs have upon vitellogenesis, and determine which are essential and non-essential for this process. We also expand our analysis to include the involvement of amino acid transport in this system through the cloning and characterization of the *A. aegypti* homologue of *slif* and a novel cationic AA transporter *iCAT2*.

Materials and methods

Animals

Aedes aegypti L. mosquitoes were reared, fed and dissected as described (Deitsch et al., 1995).

Fat body culture

The fat body tissue culture system was described previously (Deitsch et al., 1995; Raikhel et al., 1997). Media in which AAs were removed was supplemented with an equal molar amount of mannitol (Sigma-Aldrich Chemicals, St Louis, MO, USA), to compensate for changes in osmotic pressure. Fat bodies were incubated for 3 h at 27°C before collection and processing.

Molecular biology techniques and cloning

Standard procedures were used for recombinant DNA manipulations (Ausubel et al., 1991). DNA sequences homologous to *D. melanogaster slif* and *iCAT2* were identified in the *An. gambiae* genome project database and aligned using ClustalW (http://clustalw.genome.ad.jp/). Highly conserved regions were chosen as a template for primers to amplify partial cDNAs of the *A. aegypti* homologues from fat body cDNA. 5′- and 3′-ends of the cDNAs were amplified by rapid amplification of cDNA ends (RACE) PCR using the Smart cDNA RACE Amplification Kit (BD Clontech, Palo Alto, CA, USA). All PCR products were cloned in pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA). Analysis of primary predicted protein structure was performed at EXPASY (http://us.expasy.org/). Transmembrane domain prediction was created using the TMHMM software at (http://bioweb.uwlax.edu/Default.htm).

Sequence alignment and phylogenetic analysis

Sequence alignments were performed with ClustalW (default parameters) using the following amino acid sequences: *Aedes aegypti* Slimfast (AaSlif) AAT73699; *Anopheles gambiae* Slif (AgSlif) EAA09921; *Drosophila pseudoobscura* Slif (DpSlif) EAL30882; *Drosophila melanogaster* slif (DmSlif) NP_730765; *Apis melifera* Slif (AmSlif) XP_393144; *Drosophila melanogaster* iCAT2 (DmCAT2) AAF49292; *Apis melifera* iCAT2 (AmCAT2) XP_393753; *Drosophila pseudoobscura* iCAT2 (DpCAT2) EAL29695; *Anopheles gambiae* iCAT2 (AgCAT2) EAA09874; *Gallus gallus* CAT1 (GgCAT1) XP_417116; *Rattus norvegicus* CAT1 (RnCAT1) P30823; *Homo sapiens* CAT1 (HsCAT1) AAH69358; *Mus musculus* CAT1(MmCAT1) NP_031539; *Rattus norvegicus* CAT2 (RnCAT2) NP_072141; *Mus musculus* CAT2 (MmCAT2) NP_031540; *Homo sapiens* CAT2 (HsCAT2) NP_001008539; *Gallus gallus* CAT2 (GgCAT2) XP_420685; *Xenopus laevis* CAT2 (XlCAT2) AAH78099; *Danio rerio* CAT2 (DrCAT2) AAH86843; *Mus musculus* CAT3 (MmCAT3) AAH50195; *Rattus norvegicus* CAT3 (RnCAT3) NP_058913; *Homo sapiens* CAT3 (HsCAT3) AAL37184; *Tetraodon nigroviridis* CAT3 (TnCAT3) CAG11735; *Danio rerio* CAT3 (DrCAT3) AAH85672.

DAMBE was used to perform phylogenetic analysis of the data set (Xia and Xie, 2001). A putative cationic amino acid transporter of *Arabidopsis thaliana* (AAN18189) was used as outgroup. Bootstrap values (1000 replicates are indicated on the nodes of the Bootstrap N-J tree).

Real-time PCR analysis

cDNA synthesis and quantification of specific mRNAs was performed as previously described (Hansen et al., 2004). Primers and probes were synthesized by Operon (Operon, Huntsville, AL, USA): *slif* sense, CTG GTT GGC TTC GTG AT; *slif* antisense, CTC TAG TTG ACT TTC CGA C; *slif* probe, (6-FAM) CAT TCG ACA TTC GGT TCT TGG CTC CG (BHQ1-Q); *iCAT* sense, GGT ACG CTG ATG GCG TAC ACT G; *iCAT* antisense, TCC TGA CGC AGG ATA CGT

TGA A; *iCAT* probe, (AminoC6+TxRed) CCA CAA ACT GCC TCC ATC CC (BHQ2a-TxRed).

RNA interference

Generation of double-stranded RNAs (dsRNA) was accomplished by cloning *slif* and *iCAT2* template cDNAs into the pLitmus 28i vector. dsRNA for the nonfunctional portion of the bacterial gene MAL was utilized as a negative control. dsRNA was produced by *in vitro* transcription with T7-RNA polymerase using the Hiscribe RNAi Transcription Kit (New England Biolabs, Beverly, MA, USA). Approximately 0.5–1 μg of dsRNA in 0.3–0.5 μl of H_2O was injected into the thorax of CO_2-anesthetized 1 day-old female mosquitoes. The mosquitoes were allowed to recover for 5 days before further processing.

dsRNA was produced to a 774 nucleotide (nt) region of *slif*, spanning from nt 1192 to nt 1965, a 1137 bp fragment of *iCAT2* spaning from nt 1046 to nt 2182, and to a 912 bp fragment of a neutral AA transporter (NAT) spanning from nt 324 to 1235 using the MEGAscript® T7 Kit (Ambion, Austin, TX, USA). The primers used to generate the template cDNAs for dsRNA synthesis were: *slif* sense, gga gtt tgc cgc ttt cac gat cgg gtg gaa tct; *slif* antisense, agc gcg ttc gga gat ttg gca atg ttc agg ttg aac; *iCAT2* sense, aga tag ctc cca tgg agt ggg act tca tgt cca gc; *iCAT2* antisense, gca gca ggc cga gaa ggg tac cgg cca ggg tca ag; *NAT* sense, gtg cta tgc aga act ggg cac gg; *NAT* antisense, aac gac tcg acg ata ctg ctg tag gtg a. The effectiveness of the knockdown was tested using RT-PCR: *slif* sense: atg gac aaa ttc ttc aag gcc ctc tgc cgc aaa aaa cca; *slif* antisense: cta cgc ctt ttc gag tcc tac cat gca gaa cgg att ctc tag t; *iCAT2* sense: atg tcc acc ccc tca tgc tgg aag att ctg acg cga aag aaa att; *iCAT2* antisense: cag atg acg tca gtg ccc ata ctt gag ttt ggt cca tcc ggg gcg; *NAT* sense: ggg aat ttt cat ctc gcc gaa gg; *NAT* antisense: cac gat cag gaa cgc aca gat gat g.

Results

Specific amino acids are essential for vg activation

To determine the role of individual AAs in the regulation of *vg* gene transcription, we performed experiments using 22 different culture media formulations. In 20 of these media individual AAs were removed to determine which are essential for hormonal activation of *vg* (Fig. 1). We identified 10 AAs which, when withdrawn resulted in a dramatic reduction (more than 90% decrease) in the response by *vg* to 10^{-6} mol l^{-1} 20E induction relative to complete medium. Based upon the severity of the effect caused by their withdrawal, these AAs have been labeled essential to vitellogenesis. The essential AAs are listed here in order from most to least essential: leucine>tryptophan>methionine>valine>histidine>lysine>phenylalanine>arginine>asparagine>threonine. In addition, three AAs, cysteine, glycine and isoleucine resulted in a significant decrease in the response by *vg* to 20E (50–80% decrease). Withdrawal of tyrosine, aspartic acid, serine, proline, glutamine, alanine and glutamic acid resulted in a low to non-statistically significant reduction in response by *vg* (30–0% decrease).

Based upon the properties of the AAs identified as essential, we recognized some patterns occurring in this group. All the cationic AAs and two of the three branched chain AAs were found to be essential. The last branched chain amino acid isoleucine, also caused significant decrease in *vg* expression, but the result was not as dramatic as that seen with leucine and valine.

Vg gene activation by amino acid depends upon electrochemical gradients required for amino acid transport

Many amino acid transporters require electrochemical gradients across membranes to function. Therefore, we used the Na^+/K^+-ATPase inhibitor ouabain (10^{-5} mol l^{-1}) and the V-ATPase inhibitor bafilomycin A1 (10^{-6} mol l^{-1}) to shut down the Na^+ and the proton gradient, respectively, at the plasma membrane of fat body cells. Treatment of the fat bodies with both drugs resulted in a strong inhibition of AA-induced *vg* gene expression (Fig. 2A). By contrast, ouabain and bafilomycin A1 did not inhibit the upregulation of the early gene E74 after stimulation with 20E (Fig. 2B).

Cloning and characterization of two cationic amino acid transporter cDNAs from Aedes aegypti fat body

Next, we isolated the cDNAs of cationic AA transporters

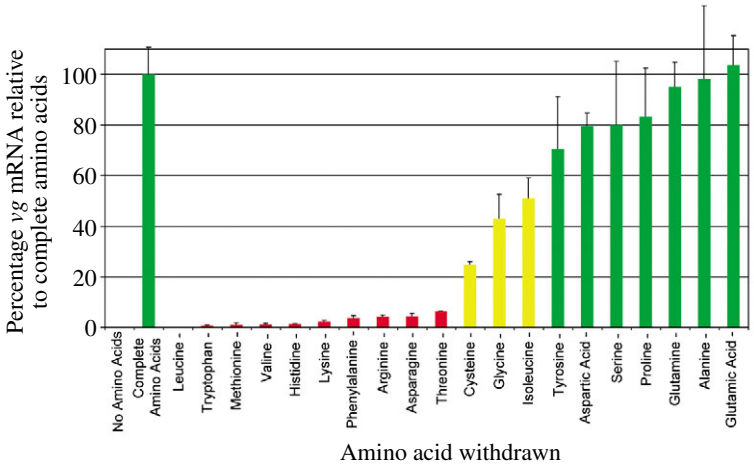


Fig. 1. Specific amino acids (AAs) are essential for 20-hydroxyecdysone (20E)-activation of the *vitellogenin* (*vg*) gene. Fat bodies from 3- to 5-day-old mosquitoes were cultured for 3 h at 27°C in media lacking individual AAs in the presence of 20E (10^{-6} mol l^{-1}). Total RNA was isolated from three groups of six fat bodies per treatment. cDNA was synthesized from equal amounts of DNase I-treated total RNA. Real-time PCR was used to quantify levels of *vg* mRNA. Data was normalized by real-time PCR analysis of *actin* levels in the cDNA samples. *vg* production in responses to 20E stimulation in the 'withdrawal' medias are presented as the mean percentage (± s.e.m. of triplicate samples) relative to the response observed in the control medium containing complete AAs.

expressed in the *A. aegypti* fat body. EST mining and BLAST analysis of the *Drosophila* and *Anopheles* genome databases revealed two potential proteins with homology to vertebrate cationic AA acid transporters.

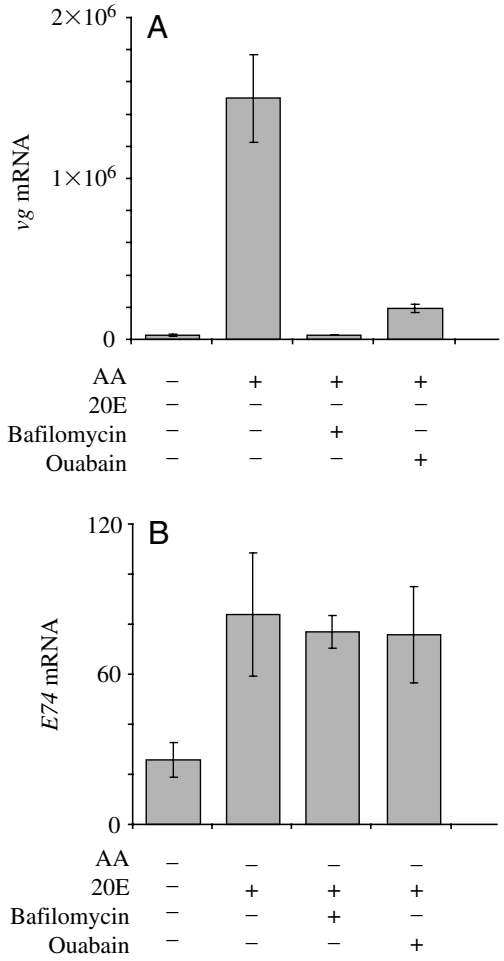


Fig. 2. Amino acid stimulation of the *vg* gene depends on Na^+/K^+-ATPase and V-ATPase activity. Treatment with 10^{-6} mol l^{-1} bafilomycin A1, a specific inhibitor of vacuolar type H^+-ATPase, and 10^{-5} mol l^{-1} ouabain, an inhibitor of Na^+/K^+-ATPase, results in a significant reduction of the *vg* gene expression. (A) Fat bodies of 3- to 5-day-old mosquitoes were dissected and incubated in *Aedes* physiological saline (APS) with different treatments as indicated. After 1 h the APS was replaced with media containing different combinations of amino acids (AAs), bafilomycin A1 and ouabain, and the fat bodies were incubated at room temperature for 6 h. Total RNA was isolated from three groups of three fat bodies per treatment. cDNA was synthesized from equal amounts of DNase I-treated total RNA. Gene expression was analyzed using *vg*-specific real-time PCR primers. Values are means ± s.e.m. of triplicate samples. (B) Treatment with bafilomycin A1 or ouabain does not inhibit 20-hydroxyecdysone (20E) activation of the *E74* early gene (E74B isoform). As a control, fat bodies were dissected and pre-treated in APS as described above. After 1 h the APS was replaced with media containing different combinations of 10^{-6} mol l^{-1} 20E, 10^{-6} mol l^{-1} bafilomycin A1 and 10^{-5} mol l^{-1} ouabain. The fat bodies were incubated at room temperature for 6 h and processed as described above. Gene expression was analyzed using *E74*-specific real-time PCR primers. Values are means ± s.e.m. of triplicate samples.

One of these, *slimfast* (*slif*), has been described in *Drosophila* as a cationic AA transporter (Colombani et al., 2003). The *A. aegypti slif* (*Aaslif*) cDNA (AY654299) consists of 2256 nucleotides and codes for a protein consisting of 428 AAs with a predicted molecular mass of 64 kDa. *Aaslif* is 68% identical to a predicted *An. gambiae* homologue (XM_314535) and 52% identical to *D. melanogaster slif*. Protein folding prediction algorithms predicted that *Aaslif* contains a total of 14 trans-membrane helices.

We cloned a second cationic AA transporter and named it *Aedes aegypti* insect cationic amino acid transporter 2 (AaiCAT2; DQ099901). We have chosen the name iCAT to clearly distinguish between the vertebrate CAT2 and the insect transporter. The iCAT2 cDNA consists of 2993 bp and encodes a protein of 605 AAs. The predicted molecular mass of iCAT2 is 66 kDa and it contains 14 trans-membrane helices. AaiCAT2 is 75% identical to its predicted *An. gambiae* homologue (EAA09874) and 46% identical to a predicted *D. melanogaster* homologue (AAF49292).

Phylogenetic analysis placed the insect CAT homologues in two groups that lie outside the well-characterized mammalian CAT1, 2 and 3 proteins (Fig. 3), indicating that variants of the mammalian CAT proteins probably arose from a common precursor after divergence from insects. In accordance with this, *slif* and *iCAT2* are more closely related to each other than to any of the vertebrate CAT proteins.

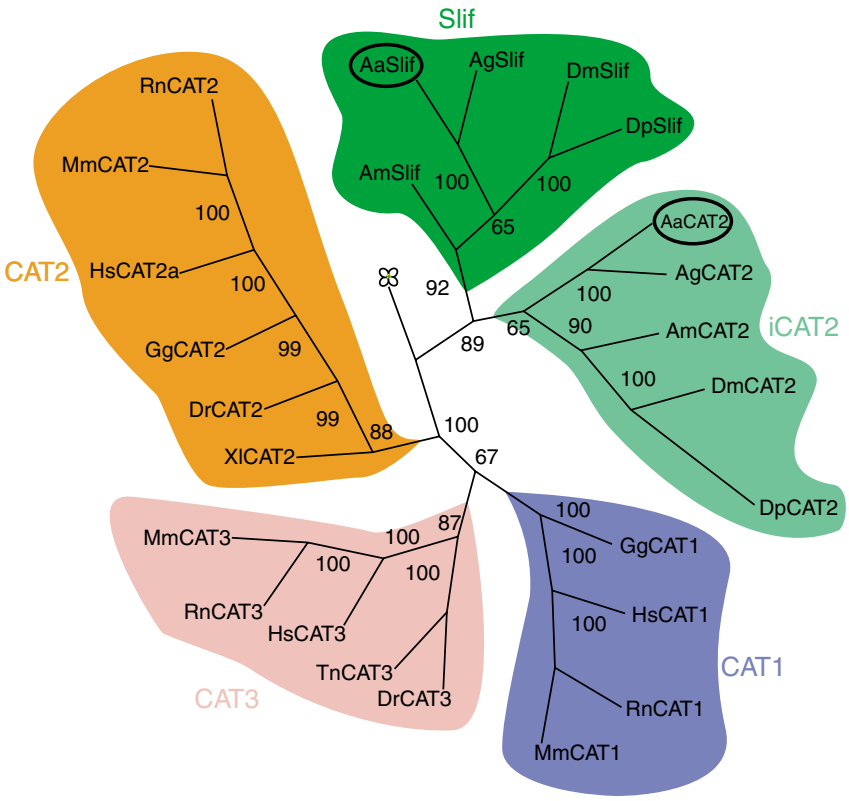
Aaslif and AaiCAT2 mRNA expression profiles

We investigated the expression patterns of the two CAT mRNAs in fat bodies of female mosquitoes at different stages of the first vitellogenic cycle by quantitative PCR (Fig. 4). The level of Aaslif mRNA was high in fat bodies of newly emerged females but dropped during the first 12 h after emergence to a basal expression level that changed little during the first 3 days of the adult life. No changes in *Aaslif* expression occurred immediately after a blood meal, however, we found a fourfold increase in *Aaslif* mRNA from 36 to 72 h post blood meal (PBM), at a time when vitellogenesis was terminated.

By contrast, the levels of *AaiCAT2* mRNA was not elevated directly after emergence and stayed at a basal level during the previtellogenic period. However, there was a dramatic increase in the *AaiCAT2* mRNA in female fat bodies following blood feeding. The peak of the *AaiCAT2* mRNA declined by 24 h PBM, to its basal level.

RNAi-mediated knockdown of cationic amino acid transporters results in strong inhibition of AA-induced vg gene expression

Various dsRNAs were injected into 1 day-old non-blood fed mosquitoes. After 5 days of recovery the mosquitoes were dissected and fat bodies were cultured in the presence and absence of AAs. Relative levels of *vg* mRNA in the fat bodies were determined using real-time PCR. Gene transcript knockdown was confirmed by RT-PCR analysis (Fig. 5A,B)



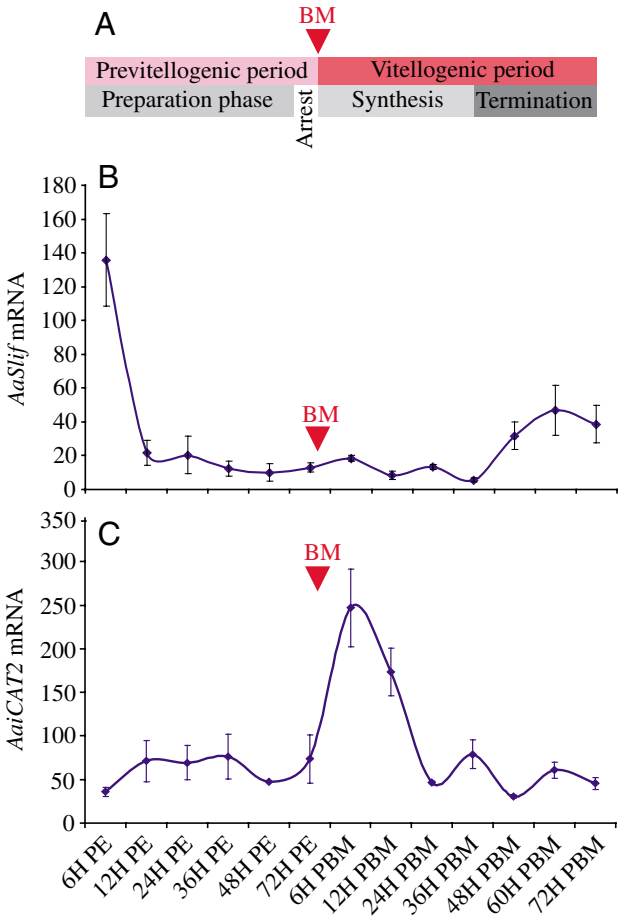


Fig. 3. Dendrogram/bootstrap analysis of insect and vertebrate cationic amino acid transporters. Sequence similarity was assessed using amino acid alignments in ClustalW and a rooted tree was calculated by the neighbor-joining method. Confidence values were derived by bootstrapping the dataset, using 1000 replicates. The alignment was visualized using Treeview 1.6.6 (Page, 1996).

In MAL- and NAT-injected control mosquitoes, there was no effect on AA-dependent activation of the *vg* gene and the *vg* mRNA level increased in fat bodies incubated in the presence of AAs (Fig. 5A). However, knockdown of either *slif* or *iCAT2* resulted in a significant downregulation of the *vg* gene expression. When we injected dRNAs for both *slif* or *iCAT2* simultaneously, knockdown of both CAT mRNAs did not lead to a stronger inhibition than the knockdown of a single transporter protein (Fig. 5A).

Discussion

Amino acids derived from digested vertebrate blood have several functions in female mosquitoes. They are the building blocks for yolk protein precursors that are synthesized by the fat body after blood feeding, secreted into the hemolymph and subsequently taken up into and stored by the vitellogenic oocytes as protein reserves for the developing embryo (Raikhel et al., 2002). Furthermore, a high percentage of blood meal derived AAs are used for energy production *via* oxidation or for energy storage *via* gluconeogenesis and lipogenesis (Zhou et al., 2004). In addition, AAs function as blood meal-derived signaling molecules that affect the fat body-specific expression of yolk protein genes *via* the nutrient sensitive TOR/S6K signaling pathway (Hansen et al., 2004; Hansen et al., 2005).

Here, we demonstrate that in *A. aegypti*, vitellogenic gene expression is dependent upon a number of specific AAs. We

Fig. 4. Expression of *Aaslif* and *AaiCAT2* mRNA. Relative mRNA levels in fat body tissue of female mosquitoes at different points of the first reproductive cycle were determined by real-time PCR. (A) Schematic diagram of the different phases of female mosquito fat bodies during the first reproductive cycle. The previtellogenic period starts with a 3 day preparation period during which the mosquito fat body gains competence to respond to 20-hydroxyecdysone (20E) and produce yolk protein precursor (YPPs). This is followed by a state-of-arrest that lasts until a blood meal (BM) is taken. The vitellogenic period is divided into a synthesis phase during which the YPPs are produced, and a termination phase, during which the fat body undergoes remodeling and returns to its previtellogenic state of being a store for lipid, protein and glycogen reserves (Raikhel and Dhadialla, 1992). (B) *Aaslif* mRNA expression profile. (C) *AaiCAT2* mRNA expression profile. PBM, post blood meal.

tested the effect of media lacking specific AAs *versus* a complete balanced AA medium on *vg* gene expression in mosquito fat bodies (Fig. 1). Interestingly, the AAs found to be essential for vitellogenesis include the eight AAs shown to be essential for growth in mammals (Rose, 1976) with the exception of isoleucine, which was on the borderline for being essential for vitellogenesis. One AA found to be essential for vitellogenesis, but not for mammalian growth was asparagine. Comparison of AAs essential for *A. aegypti vg* gene expression and those essential for complete egg development in *A. aegypti* and *Culex pipiens* has revealed a high degree of conservation of the AAs essential for these processes. The only exception again was asparagine, which was found to be essential for vitellogenesis in this assay but not for egg development when omitted from an artificial blood meal in *Aedes* (Lea et al., 1956; Uchida, 1992). Studies examining the requirements of AAs for larval growth show that the same AAs essential for vitellogenesis are also essential for larval growth (Sing and Brown, 1957) (Fig. 1).

Both cationic and branched chain AAs have been implicated in nutritional signaling *via* the TOR kinase pathway, and leucine in particular has been shown in a number of studies to be especially important (Colombani et al., 2003; Jacinto and Hall, 2003; Kimball and Jefferson, 2004; Lynch et al., 2000). The fact that the lack of leucine has the greatest effect upon *vg* gene expression provides further confirmation that the AA/TOR/S6K signaling pathway is regulating this system. The results from the AA withdrawal experiments show that exclusion of either arginine or lysine from the fat body severely inhibits vitellogenic activation even when the other 19 AAs are present. Most likely, these two AAs, as well as the other AAs found to be essential, are working through a specific detection system linked to the TOR pathway. The requirement of these specific AAs for nutritional signaling is likely conserved in evolution.

A number of AAs were identified as non-essential for TOR-mediated activation of *vg* gene expression in the mosquito fat body. Hemolymph concentrations of some of these AAs may be high before a blood meal making them poor signaling molecules. Indeed, proline, alanine and glutamic acid are at a high concentration relative to the other free AAs in the hemolymph of competent pre-blood fed mosquitoes (Goldstrohm et al., 2003). *A. aegypti* may use proline for nitrogen storage before and after blood meal. This finding is in agreement with the fact that these AAs also appear to be non-essential for vitellogenic activation.

The mechanism of how AAs activate the TOR pathway remains unknown. Amino acids are taken up by cells *via* a specific system of plasma membrane transporter proteins. The various transporters possess substrate selectivity. Every transporter transfers a specific subset of AAs through the membrane. AA transporters, if not a direct sensor for AAs are thought to be at least indirectly involved in sensing AAs. There are four proposed general mechanisms by which AA transport might regulate nutritional signaling. One possibility is that the transporter acts as a receptor at the top of a signal transduction pathway. In yeast, an AA-sensing protein, Ssy1, has been identified. While it is not definite that this protein actually transports AAs, it is necessary for detection and is structurally related to other AA transporters (Poulsen et al., 2005). The second possibility is that AAs flowing into and other solutes flowing out of the cell results in physiological changes in membrane polarity, cellular volume, pH and salt concentrations, which are then detected by the cell. Changes in cell volume have been attributed to potentiate S6 kinase phosphorylation in hepatocytes (Van Sluijters et al., 2000). A third possibility is that an intracellular receptor is detecting increased concentration of AAs, charged tRNAs, or AA metabolites. In yeast the protein GCN2 (a regulator of the translation factor eIF2B) is activated by uncharged tRNA (Zhang et al., 2002). Fourth and finally, if AAs are sensed by an extra cellular receptor, nearby transporters affecting local extra cellular AA concentrations could regulate these receptors by their level of transport activity. This type of

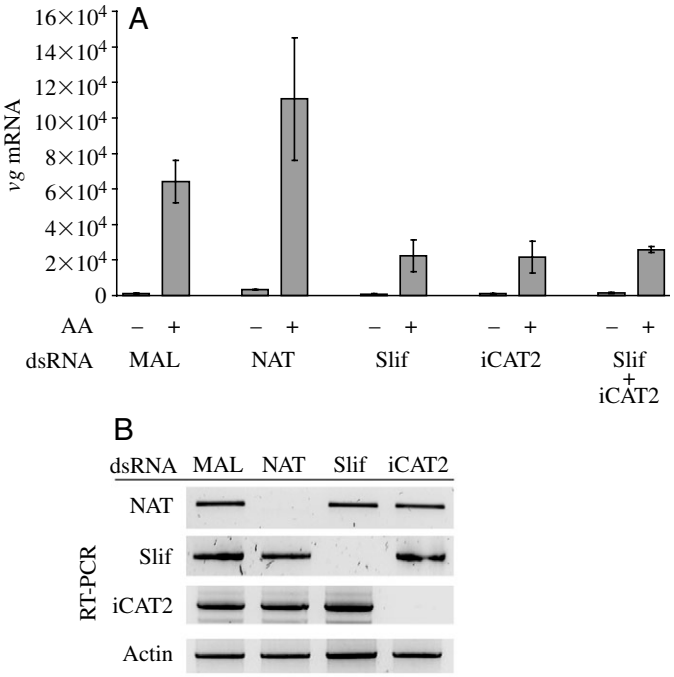


Fig. 5. RNAi-mediated knockdown of *Aaslif* and *AaiCAT2* inhibits amino acid stimulation of the *vg* gene. 1 day-old mosquitoes were injected with 0.6–1.0 μg of the following dsRNAs: the non-coding region of a control bacterial gene (MAL), the coding region of neutral amino acid transporter (NAT), the coding region of the *Aaslif* gene (Slif), the coding region of the *AaiCAT2* gene (iCAT2), or a 1:1 mixture of both *Aaslif* and *AaiCAT2* dsRNAs. Mosquitoes were allowed to recover for 5 days. Fat bodies from these mosquitoes were then dissected and cultured in either the presence or absence of amino acids (AAs) for 6 h. Total RNA was isolated from three groups of six fat bodies per treatment. cDNA was synthesized from equal amounts of DNase I-treated total RNA. (A) Gene expression was analyzed using *vg*-specific real-time PCR primers. Data were normalized by real-time PCR analysis of *actin* levels in the cDNA samples. Values are means ± s.e.m. of triplicate samples. (B) Knockdown of transporter genes was confirmed by RT-PCR analysis of the same cDNA used for the analysis in A.

mechanism occurs in the central nervous system as seen when the neurotransmitter glutamate is removed from the synaptic cleft due to rapid uptake by transporters (Hand and Rouleau, 2002; Hyde et al., 2003).

Many transporters are energized by electrochemical gradients along the plasma membrane. These gradients are maintained by primary ATP-driven pumps, such as V-ATPase, and secondary mineral ion transporters, such as Na^+/K^+-ATPase (Christensen, 1990). To test our hypothesis that active AA transport into the fat body cells is necessary to induce *vg* gene expression we disrupted the Na^+ and proton gradients at the fat body plasma membrane with specific inhibitors (Fig. 2A). Bafilomycin and ouabain inhibit membrane V-ATPase and Na^+/K^+-ATPase, respectively. Thereby these drugs de-energize the plasma membrane and reduce AA transporter activity in a manner similar to that of depletion of ATP (Boudko et al., 2005). De-energizing the fat body plasma membrane by these inhibitors resulted in inhibition of the AA-induced *vg* gene expression, suggesting that active transport of AAs through the fat body plasma membrane is necessary for the induction of *vg* gene expression. By contrast, neither inhibitor inhibits the upregulation of the early gene E74 after the stimulation with 20E (Fig. 2B). This indicated that the active transport at the plasma membrane of fat body cells was selectively required for the AA-induced *vg* gene expression.

Our previous work has shown, that in the fat body of *Aedes aegypti* the AA signal is transduced to the *vg* gene *via* the TOR/S6K signaling pathway (Hansen et al., 2004; Hansen et al., 2005). In *Drosophila* an amino acid transporter has been identified as part of a nutrient sensor mechanism in the fat body that controls global growth in the fly (Colombani et al., 2003). This transporter, *slimfast* (*slif*), is closely related to the cationic AA transporter family of proteins (CATs) in mammals. CAT proteins are defined as transporters exhibiting affinities and translocation rates for cationic AAs, which are higher than or equivalent to those for other types of AAs. There are different cationic AA transport systems and the CAT proteins specifically are members of the y+ system of cationic transporters. The y+ system is specific for the basic AAs arginine and lysine. y+ transporters are typically pH independent and transport activity is stimulated by membrane hyperpolarization. These transporters are stimulated by the presence of amino acids on the *trans* side of the membrane (Closs, 2002; Deves and Boyd, 1998).

Based on sequence homology we identified two CATs in the *An. gambiae* and *Drosophila* databases and subsequently cloned the *A. aegypti* homologues of these proteins. The first one is the mosquito homologue to *Drosophila slif* (Colombani et al., 2003) and was therefore termed *Aaslif*. The second, so far undescribed CAT was called *AaiCAT2* for *A. aegypti* insect cationic amino acid transporter 2. Phylogenetic analysis revealed that the two insect CATs (iCATs) are more similar to each other than to the vertebrate CATs (Fig. 3). The two iCAT and the three vertebrate CAT families form separate phylogenetic clusters.

We determined the mRNA expression profiles of *Aaslif* and *AaiCAT2* in fat bodies during the previtellogenic period and during the vitellogenic period (see Fig. 4A). The mRNA expression profiles are surprisingly different (Fig. 4B,C). The *AaSlif* mRNA level is highly elevated directly after emergence and drops to a basal level between 6 and 12 h post eclosion; it rises again at 48 h PBM and are still elevated at 72 h PBM, the last time point we measured. By contrast, *AaiCAT2* mRNA levels stay at a basal level during the entire previtellogenic period but are elevated after activation of vitellogenesis by blood feeding. These differences suggest that these two transporters have different functions during metamorphosis and vitellogenesis. The presence of high levels of transcript for *AaSlif* in newly eclosed mosquitoes and low levels of expression during previtellogenic and postvitellogenic periods suggests that this protein plays a role in amino acid transport during development and metamorphosis rather then during mosquito reproduction. However, *slif* may play a role as amino acid sensor in adult female mosquitoes. By contrast, the expression profile of *AaiCAT2* suggests that it has more of a functional role in the fat body in response to increasing levels of AAs in the hemolymph after a blood meal. Its expression increases significantly after blood feeding, indicating that the mosquito is responding to higher levels of hemolymph AAs by producing this transporter. Based on these results, we can hypothesize that the *AaiCAT2* protein is produced after a blood meal to allow the fat body to absorb the AAs needed for yolk protein production during vitellogenesis.

To test if *slif* and *iCAT2* are obligatory for AA signaling to the mosquito fat body we performed RNAi knockdown experiments (Fig. 5). We used dsRNA against *slif* and *iCAT2* alone and then in combination. We utilized dsRNA against a neutral AA transporter (NAT) (Jin et al., 2003) as a control. Knockdown of either *slif* or *iCAT2* causes a severe reduction of the AA-mediated *vg* response. The knockdown effect is similar to that of TOR inactivation by either rapamycin or RNAi and the effect of S6 kinase knockdown (Hansen et al., 2004; Hansen et al., 2005). Downregulation of the cationic AA transporter *slif* has been reported to affect TOR signaling in the *Drosophila* fat body of (Colombani et al., 2003). By contrast, knockdown of *NAT* did not cause any effect, indicating the required specificity of both iCATs. Knockdown of both *slif* and *iCAT2* together had no stronger effect than knockdown of the single transporter, indicating the lack of a synergistic action of these CATs. Thus, both transporters probably have different specificity and may be responsible for the uptake of different essential AAs that participate in signaling to the fat body. A detailed analysis of protein expression levels and the specificity of these two molecular carriers will help to assess this hypothesis.

In summary, the results presented in this paper stress the importance of AAs as signaling molecules for the onset of vitellogenesis in the mosquito fat body after a blood meal. Specific AAs are essential for successful activation of vitellogenic gene expression whereas others are not. Electrochemical gradients needed for active AA transport across the fat body plasma membrane are also required. Furthermore, we identified two CATs that are obligatory for this process. Further analysis of this system will provide insight

into the molecular mechanics of mosquito reproduction and the reproduction of other blood feeding insects.

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Database Deposition: The sequences reported in this paper have been deposited in the GenBank™ database with the accession numbers AY654299 (*AaSlif*) and DQ099901 (*AaiCAT2*).

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