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Characterization of a blood-meal-responsive proton-dependent amino acid transporter in the disease vector, *Aedes aegypti*

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SUMMARY

After anautogenous mosquitoes ingest the required blood meal, proteins in it are rapidly cleaved, yielding a large pool of amino acids. Transport of these amino acids into gut epithelial cells and their subsequent translocation into other tissues is critical for oogenesis and other physiological processes. We have identified a proton amino acid transporter (PAT) in *Aedes aegypti* (AaePAT1, AAEL007191) which facilitates this transport and is expressed in epithelial cell membranes of larval caecae and the adult midgut. *AaePAT1* encodes a 475 amino acid protein showing high similarity to *Anopheles gambiae* AGAP009896, *Culex pipiens* CPIJ011438 and *Drosophila melanogaster* CG7888. When expressed in *Xenopus* oocytes the transport kinetics showed AaePAT1 is a low affinity transporter with low substrate specificity, having K_m and V_{max} values of about 7.2 mmol l^{-1} and 69 pmol oocyte^{-1} min^{-1}, respectively, for glutamine. A number of other amino acids are also transported by this PAT. In female adult midgut, AaePAT1 transcript levels were induced after ingestion of a blood meal.

Key words: proton-dependent amino acid transporter, midgut, insect epithelia, blood-meal induced protein.

INTRODUCTION

Blood feeding by female mosquitoes plays a crucial role in the biology of many mosquito species. In these anautogenous mosquitoes a blood meal is required to initiate oogenesis (Clements, 1992). Among other signaling events, once the female mosquito ingests blood, the ovaries produce ecdysone, signaling initiation of vitellogenesis (Fallon et al., 1974; Swevers et al., 2005), resulting in subsequent egg production. Besides this essential requirement for reproduction, in infected mosquitoes the blood meal also can transmit both parasitic and viral diseases (Beaty and Marquardt, 1996; Clements, 1992). For example, many viral diseases, including dengue, yellow fever and chikungunya, are spread by the mosquito, *Aedes aegypti* (Black et al., 2002; Chhabra et al., 2008).

Proteins in the blood meal are processed by midgut proteases that are upregulated following ingestion (Barillas-Mury et al., 1995; Noriega et al., 2002; Noriega et al., 1996), resulting in a pool of amino acids available for transport into midgut cells. Following transport into these cells and their subsequent translocation into the hemolymph, amino acids are then used for protein biosynthesis in many tissues, including the fat body. For example, it is estimated that approximately 12% of the amino acids from the blood meal are used for vitellogenin synthesis (Zhou et al., 2004a; Zhou et al., 2004b). Consequently, efficient amino acid transport into midgut cells is essential for nutrition, metabolism and development of the mosquito (Clements, 1992). Additionally in virally infected mosquitoes, these amino acids are also utilized for synthesis of viral proteins (Molina-Cruz et al., 2005).

Although it is clear that the mosquito midgut is involved in amino acid uptake, to date only a limited number of amino acid transporters have been functionally characterized in mosquitoes (Attardo et al., 2006; Boudko et al., 2005b; Jin et al., 2003; Meleshkevitch et al., 2006; Okech et al., 2008). However, sequencing of the *Drosophila melanogaster*, *Anopheles gambiae*, *Ae. aegypti*, *Tribolium castaneum*, *Apis mellifera* and *Bombyx mori* genomes (Adams et al., 2000; Consortium, 2008; Holt et al., 2002; Honeybee Genome Sequencing Consortium, 2006; Nene et al., 2007; Richards et al., 2008) and the recently completed *Culex pipiens* genome (Broad Institute and The Institute for Genomic Research), show that amino acid transporters in these insect species are relatively conserved. Phylogenetic analyses of these transporters by Boudko et al. (Boudko et al., 2005a) show that orthologues of insect amino acid transporters group together. Although there is sequence evidence for the presence of a repertoire of amino acid transporters in mosquitoes, there is much less evidence on the functional role of these transporters in mosquito physiology. Here we analyzed a proton-dependent amino acid transporter that belongs to a transporter class which has not been characterized in mosquitoes.

The SLC36 family of transporters consists of proton-dependent amino acid transporters (PATs) facilitating the symport of protons and amino acids usually in a 1:1 stoichiometry (Boll et al., 2002; Sagne et al., 2001). The activity of this family of transporters is independent of Na^+, K^+ and Cl^-, but demonstrates dependence on pH, with amino acid uptake resulting in acidification of the cell (Abbot et al., 2006; Boll et al., 2002). Several PATs have been identified in vertebrates, and they fall into four PAT subclasses, but only PAT1 and PAT2 subclasses from human, mouse and rat have been characterized (Boll et al., 2003a; Boll et al., 2002; Chen et al., 2003a; Chen et al., 2003b; Kennedy et al., 2005; Sagne et al., 2001). PAT1 is a low affinity transporter with the ability to transport many small chain amino acids as well as γ-aminobutyric acid (Boll et al., 2003a; Boll et al., 2002). By contrast, PAT2 is a high affinity transporter with higher substrate selectivity and less sensitivity to pH changes. PAT1 has been identified in lysosomes and on the apical membrane of intestinal epithelial cells, where it is involved in amino acid absorption across the membrane (Sagne et al., 2001). PAT1 mRNA is found in all tissues, but PAT2 mRNA seems to be expressed in the lung, heart, kidney and muscle (Boll et al., 2003a; Boll et al., 2002; Broer, 2008; Kennedy et al., 2005).

In this report we present the cloning, functional characterization and tissue distribution of a proton amino acid transporter from the disease vector *Aedes aegypti*. This transporter, designated AaePAT1, is a low affinity transporter with low substrate specificity. AaePAT1 transports many amino acids at lower pH and it is localized to the apical membrane of the midgut and caecae but not in other tissues examined. Furthermore, AaePAT1 expression is induced by a blood meal and thus it likely to play a role in the transport of amino acids from the pool of amino acids into midgut epithelial cells.

MATERIALS AND METHODS

Cloning of the AaePAT1

In previous studies we demonstrated significantly increased transcript levels of an amino acid transporter following a blood meal (Sanders et al., 2003). These studies used microarrays made from partially sequenced cDNAs isolated from the midgut and Malpighian tubules of adult female *Ae. aegypti* (Linnaeus). To obtain the full-length cDNA clone we screened a cDNA library using methods previously published (Jin et al., 2003; Ross and Gill, 1996). The library consisted of size-selected (>2 kb) cDNAs that were cloned into the pSPORT1 vector and electroporated into DH10B cells (Invitrogen, Carlsbad, CA, USA). The clone obtained was fully sequenced using both vector- and sequence-specific primers to obtain the full-length cDNA sequence and to deduce the amino acid sequence of the open reading frame (ORF).

Preparation of cRNA

To analyze *AaePAT1* transport properties the predicted ORF from the cDNA clone was amplified by polymerase chain reaction (PCR) using sequence-specific primers adding *Bam*HI and *Xba*I restriction sites at the 5′and 3′ end, respectively. The PCR product was first cloned into PCR2.1 vector using a TA-TOPO Kit (Invitrogen). The selected clone was sequenced to confirm the absence of any PCR-introduced mutations, following which the *Bam*HI–*Xba*I fragment was subcloned into the expression vector pGH19. The resulting plasmid was linearized using the *Not*I restriction site downstream of the ORF, and *in vitro* transcribed by T7 RNA polymerase using mMESSAGE mMACHINE T7 Kit (Ambion, Austin, TX, USA). cRNA was extracted once with an equal volume of phenol:chloroform (1:1), precipitated with isopropanol, resuspended in nuclease-free water at a final concentration of 1–1.5 $\mu g\,\mu l^{-1}$, and stored at –80°C.

Transport assays in *Xenopus laevis* oocytes

Stage V and VI oocytes from *Xenopus laevis* were dissected and treated with collagenase Type 1A for 75 min in Ca^{2+}-free Barth's Solution (82.5 $mmol\,l^{-1}$ NaCl, 2 $mmol\,l^{-1}$ KCl, 1 $mmol\,l^{-1}$ $MgCl_2$, and 10 $mmol\,l^{-1}$ Hepes, pH 7.4). The oocytes were kept in ND96 (96 $mmol\,l^{-1}$ NaCl, 2 $mmol\,l^{-1}$ KCl, 1 $mmol\,l^{-1}$ $MgCl_2$, 1 $mmol\,l^{-1}$ $CaCl_2$, 10 $mmol\,l^{-1}$ Hepes and 50 $mmol\,l^{-1}$ Tris, pH 7.4) supplemented with gentamycin at 18°C overnight. The oocytes were then injected with ~30 ng cRNA, following which the oocytes were incubated in ND96 with gentamycin at 18°C for 4–5 days for AaePAT1 expression. The oocytes were incubated for 5 min at room temperature in uptake solution (100 $mmol\,l^{-1}$ NaCl, 2 $mmol\,l^{-1}$ KCl, 1 $mmol\,l^{-1}$ $MgCl_2$, 1 $mmol\,l^{-1}$ $CaCl_2$, 10 $mmol\,l^{-1}$ Hepes and 50 $mmol\,l^{-1}$ Tris, pH 7.4) prior to recording or uptake assays.

Amino acid transport was measured by incubating each oocyte (5 oocytes/test) in 300 µl uptake solution with 1 $mmol\,l^{-1}$ L-amino acid and 50 $nmol\,l^{-1}$ of the corresponding ^{3}H-labeled L-amino acid as a tracer for 7 min. Oocytes were washed three times with non-radioactive uptake solution and lysed with 200 µl 2% SDS. The specific activity of glutamine used was 54 $Ci\,mmol\,l^{-1}$, with 0.54 µCi used for each oocyte. Glutamine uptake in each oocyte was in the range of 800–1000 d.p.m. For the other amino acids used, specific activities ranged from a low of 26 $Ci\,mmol\,l^{-1}$ (serine) to a high of 197 $Ci\,mmol\,l^{-1}$ (leucine). Data collection and analysis was performed as previously described (Jin et al., 2003; Umesh et al., 2003).

Electrophysiological properties of AaePAT1 were determined as previously described (Umesh et al., 2003) using a two electrode voltage clamp (TEV-200, Dagan Instruments, Minneapolis, MN, USA) interfaced to a computer with a Digidata 1200 A/D controller using the pCLAMP 6.0 program suite (Axon Instruments, Union City, CA, USA). Amino acid substrates were applied over a 9 s exposure in 150 µl volumes using a Rheodyne injection valve. Current responses were recorded at room temperature and data acquired by Clampfit 8.0 (Axon Instruments) and analyzed by ORIGIN 6.0 (MicroCal Software, Northampton, MA, USA).

RNA isolations

Total RNA was isolated from blood-fed and non-blood-fed adult female midguts using the TRIzol reagent (Invitrogen). Approximately 25 midguts were homogenized with 1 ml TRIzol reagent. RNA was cleaned using a RNAeasy Kit (Qiagen, Valencia, CA, USA) and quantified using a DNA Quant spectrophotometer.

Quantitative real time polymerase chain reaction (qPCR)

Total RNA (500 ng) from midguts of both blood-fed and non-blood-fed adult female mosquitoes was reverse transcribed using oligo(dT) (500 ng) and SuperScript II (200 i.u.) followed by an incubation with *E. coli* RNase H (2 i.u.) following the manufacturer's protocol (Invitrogen). cDNA obtained (25 ng) was then used for qPCR amplification. Forward and reverse primers (5 $pmol\,\mu l^{-1}$ in a 25 µl total reaction volume) used for qPCR were: for AaePAT1 – 5′-CCAACAACTATGTGCTGG-3′ (T_m, melting temperature 58°C) and 5′-GTAGGTGCACAGAATGCC-3′ (T_m 59°C); V-ATPase B subunit – 5′-CCGTCAATCGTACCATTTCGG-3′ (T_m 62°C) and 5′-GGTCTTGTAGGTCAGTCGTGG-3′ (Tm 64°C); 18S – 5′-CCTTCAACAAGGATCAAGTGG-3′ (T_m 60°C) and 5′-GGA-GTAGCACCCGTGTTGG-3′ (T_m 64°C). 18S RNA was used as a reference gene to normalize control and experimental samples. Amplified product sizes for these three transcripts were 230, 98 and 133 bp, respectively. The product sizes were confirmed by running a 1% agarose gel, and no products were obtained without reverse transcriptase as a control, confirming no genomic DNA amplification. PCR amplification efficiency was calculated to be 0.93, 0.99 and 0.90 for AaePAT1, V-ATPase B subunit and 18S qPCR primers, respectively. QPCR was performed in an ABI 7700 (Applied Biosystems, Foster city, CA, USA) thermocycler using Brilliant SYBR Green QPCR Master Mix (Qiagen). All samples were placed in a GeneMate semi-skirt 96-well PCR plate (ISC BioExpress, Kaysville, UT, USA) and capped with 8 caps/strip of optical caps (Applied Biosystems). Fold changes between transcript levels in blood-fed samples compared with that of non-blood-fed samples were calculated by the comparative quantification cycle (C_q) method (Heid et al., 1996). The controls, without reverse transcriptase, showed C_q values of 40, indicating no amplification of product.

Preparation of antibody

A synthetic peptide of 15 sequence-specific amino acids plus an amino-terminal cysteine (CETDYNPYEHRHVEHP, amino acids 46–60) was synthesized by Synthetic Biomolecules (San Diego, CA,

USA). The peptide was conjugated to a maleimide-activated keyhole limpet hemocyanin (KLH) (Pierce, Rockford, Il, USA). The KLH-coupled peptide, following removal of free uncoupled peptide, was injected into rabbits for antibody production. Purified serum was used for western blot and immunohistochemistry.

Western blot

Fourth instar larval midguts and gastric caecae were isolated and homogenized in buffer [50 mmol l^{-1} Tris-HCl pH 7.4, 300 mmol l^{-1} NaCl, 5 mmol l^{-1} EDTA and 1% (w/v) Triton X-100] containing complete protease inhibitor cocktail (Roche), 0.2 mmol l^{-1} PMSF (Sigma, St Louis, MO, USA), 100 mmol l^{-1} NEM (N-ethylmaleimide) (Sigma), and 50 µmol l^{-1} MG-132 (EMD Biosciences, Gibbstown, NJ, USA). The homogenate was then centrifuged at 2000 ***g*** for 5 min at 4°C. The supernatant was transferred and centrifuged again at 16000 ***g*** for 20 min at 4°C. The supernatant (20 µg) was then mixed with 6× sample loading buffer, boiled and then loaded in to SDS-polyacrylamide gel (10%) and electrotransferred to PVDF membranes. After blocking, the membranes were incubated for 2.5 h with anti-AaePAT1 antibody (1:500 dilution) followed by incubation with anti-rabbit horseradish peroxidase (HRP, 1:5000) secondary antibody (goat anti-rabbit, Sigma). Blots were developed by enhanced chemiluminescence (ECL™, GE Healthcare, Piscataway, NJ, USA).

Immunolocalization

Since, in our hands, immunolocalization of proteins in blood-fed mosquitoes gives less defined cellular structures we opted to perform immunohistochemistry using tissues from larvae and non-blood-fed adults. Isolated midguts and Malpighian tubules were fixed in 4% paraformaldehyde in PBS pH 7.4 overnight and then dehydrated in an ethanol series as previously described (Patrick et al., 2006; Pullikuth et al., 2006). The samples were then cleared with xylene and embedded in Paraplast. Several 8 µm transverse sections were obtained and incubated either with whole serum from rabbits immunized with AaePAT1 peptide or this serum preincubated with excess AaePAT1 peptide or with pre-immune serum (1:100 dilution). After washing, the sections were incubated with Cy3-conjugated goat anti-rabbit IgG (Jackson Immuno Research, West Grove, PA, USA). For detection of actin, sections were also incubated with Alexa-Fluor-488–phalloidin. The sections were examined by fluorescence microscopy using a Zeiss Axiphot or a confocal microscope with a helium–neon laser (Zeiss LSM510 Axioplan 2, Institute for Integrative genome Biology, University of California, Riverside, CA, USA). The images were analyzed using LSM510 software (Zeiss) and imported into Adobe Photoshop 6.0 for assembly and labeling.

RESULTS

Blood feeding by female anautogenous mosquitoes is required for oogenesis (Clements, 1992). To analyze the mosquito's response to a blood meal, we previously demonstrated that the transcript levels of many genes are significantly altered following a blood meal. One of the genes that showed significant changes in DNA microarray experiments codes for a putative amino acid transporter (Sanders et al., 2003).

Structural features of AaePAT1

To functionally characterize this transporter the full-length clone was isolated from a midgut–Malpighian tubule cDNA library utilizing an approach described previously (Jin et al., 2003; Ross and Gill, 1996). The 2205 bp cDNA isolated has an open reading frame of 1428 bp encodes a 475 amino acid protein (GenBank Accession number GQ231545). The cloned transporter is homologous to proton-dependent amino acid transporters (PAT), previously identified in vertebrates (Boll et al., 2003b; Boll et al., 2002; Chen et al., 2003a) and thus named AaePAT1. The sequence is identical to a predicted ORF, AAEL007191, obtained from genome sequencing (Nene et al., 2007), whereas another ORF, AAEL008913, has a single amino acid change, E246 to K.

AaePAT1 is predicted to have 10 transmembrane domains (THMHH 2.0 server) with both the carboxy- and amino-termini being extracellular. AaePAT1 is also predicted to have one *N*-glycosylation site at amino acid 64 (NetNGlyc 1.0 server).

The *AaePAT1* (AAEL007191) gene is 8.5 kb in length having six exons that encode the ORF. In *Drosophila*, the orthologous gene, CG7888, is predicted to have three alternative spliced products (FB2008_10, released November 19, 2008) giving rise to two different proteins with an alternative N-terminus, suggesting alternative splicing may be a possibility in mosquitoes.

A CLUSTALW phylogenetic analysis of dipteran PATs was conducted using the *Drosophila*, *Anopheles*, *Aedes* annotations (Adams et al., 2000; Holt et al., 2002; Nene et al., 2007) and the *Culex* genome available on line (Vectorbase). Of these, AaePAT1 is most similar to uncharacterized transporters from *Culex pipiens* CPIJ011438 (87% identity), *Anopheles gambiae*, AGAP009896 (84% identical) and *Drosophila melanogaster*, CG7888 (69% identity). Although not proved, these transporters are probably orthologues of AaePAT1. The phylogenetic analysis also suggests all insects for which sequences are available have one copy of the gene, thus it is probable that the two genes identified in *Aedes aegypti* (AAEL007191 and AAEL008913) are polymorphs.

In the *Ae. aegypti* genome it is predicted that there could be 14 additional PATs, whereas in the *C. pipiens* and *An. gambiae* genomes and the *D. melanogaster* genome 12 and eight PATs are predicted, respectively; all of these fall into six or seven clusters, with none apparently being mosquito specific.

Transport properties of AaePAT1

Uptake of glutamine into *Xenopus laevis* oocytes was used as a measure of AaePAT1 transport capacity. Transport by AaePAT1 in injected oocytes were compared to that of the uninjected negative control. Water-injected oocytes showed no difference from uninjected oocytes (data not shown), therefore uninjected oocytes were used as the negative control in subsequent experiments.

Uptake by AaePAT1was found to be time-dependent, with linear uptake when assays were conducted for 6–9 min (Fig. 1A). Hence, subsequently, uptake was measured using a 7 min assay. Transport assays showed AaePAT1 is a low affinity transporter of glutamine. Transport was saturable and the dose–response relationship followed Michaelis–Menten kinetics. K_m and V_{max} values of about 7.2 mmol l^{-1} and 69 pmol oocyte^{-1} min^{-1} were obtained, respectively (Fig. 1B).

AaePAT1 was determined to be electrogenic using two-electrode voltage clamp electrophysiology experiments. *Xenopus laevis* oocytes expressing AaePAT1 displayed a low inward current when 10 mmol l^{-1} glutamine and 500 µmol l^{-1} cysteine, serine or proline were added to the uptake buffer (Fig. 2).

Transport of glutamine by AaePAT1 was also analyzed in media with or without Na^+, K^+ or Cl^- (Fig. 3A). Removal of these ions did not affect uptake levels of glutamine. However, decreasing the pH of the media caused an increase in glutamine uptake (Fig. 3B). Since AaePAT1 is predicted to be a proton amino acid transporter, the

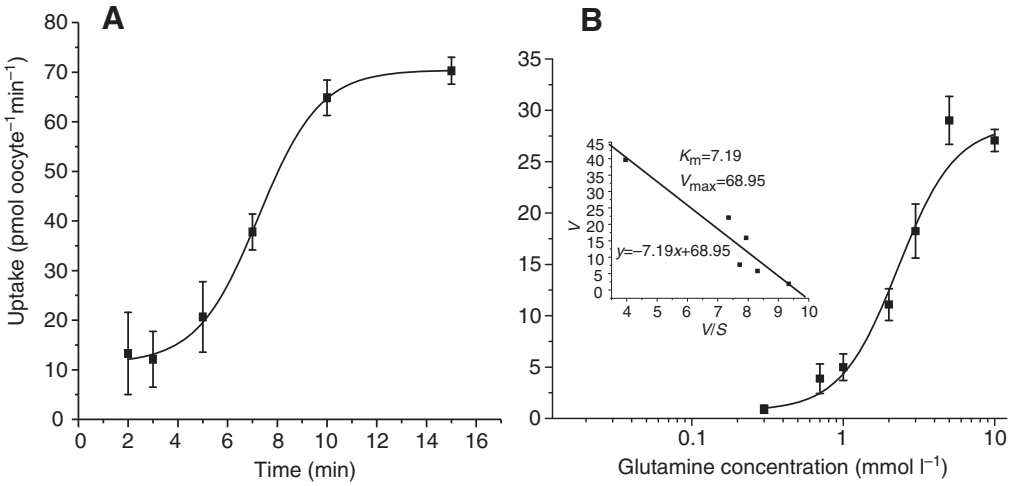

Fig. 1. Kinetics of glutamine uptake by AaePAT1. (A) The rate of uptake over time was examined using 5 mmol l^{-1} L-glutamine with 50 nmol l^{-1} [^{3}H]L-glutamine as a tracer in an assay for 2, 3, 5, 7, 10, and 15 min. Each experiment was done using five oocytes. Values are means ± standard errors. (B) The effect of concentration on the rate of uptake was examined using L-glutamine at concentrations of 0.3, 0.7, 1, 2, 3, 5 and 10 mmol l^{-1} with 50 nmol l^{-1} [^{3}H]L-glutamine as a tracer. (Inset) Michaelis–Menten kinetics was fitted to experimental data using Origin 6.0. K_m and V_{max} values are from the inset. S is substrate concentration.

observed electrogenic properties are probably the result of excess protons entering the cell during the transport cycle.

Functional analysis was continued with inhibition (or competition) assays to analyze the substrate selectivity of the transporter. AaePAT1 appears to have wide substrate selectivity because a variety of amino acids, including serine, aspartic acid, alanine, tryptophan, glycine, cysteine, proline, lysine and leucine can inhibit glutamine transport (Fig. 4A). To confirm whether each of the amino acids that inhibited glutamine uptake is transported, a number of these amino acids were individually tested using radiolabeled substrates (Fig. 4B). Glutamine, tryptophan, serine and alanine were rapidly taken up, while glutamic acid and proline were transported at lower rates. Leucine and lysine were not transported in these assays.

Quantitative PCR

Microarray experiments showed the AaePAT1 transcript levels are highly induced after a blood meal (Sanders et al., 2003). To confirm these microarray experiments, we used quantitative PCR (qPCR). Mosquitoes were given a blood meal 4–5 days post emergence and midguts were dissected 3, 24 and 72 h after the blood meal. As controls, midguts were dissected from non-blood-fed mosquitoes of identical age. RNA was isolated as described above and used for reverse transcriptase reaction using SuperScript II (Invitrogen). qPCR was performed using Brilliant SYBR Green (Qiagen). Utilizing 18S RNA as a reference to normalize data, the C_q cycle method was used to determine the fold change between blood-fed and non-blood-fed mosquito midguts (Fig. 5). The qPCR data confirmed that in comparison with non-blood-fed mosquitoes AaePAT1 is upregulated ~16-fold at 3 and 24 h after a blood meal (Fig. 5). Since V-ATPase generates protons that are required for PAT we also monitored changes in V-ATPase B subunit transcript levels. This subunit showed a similar pattern of expression at these time points, although the fold induction is much lower, 2.5- and 1.3-fold, respectively at these time points. At the 72 h time point AaePAT1 transcript levels in the midgut of blood-fed mosquitoes were not statistically different from that in non-blood-fed mosquitoes.

Western blot and immunolocalization of AaePAT1

Western blot using extracts of membranes from larval midgut had a single major band at about 51 kDa, showing the antibody is quite specific for AaePAT1 (Fig. 6), The protein detected is similar to the

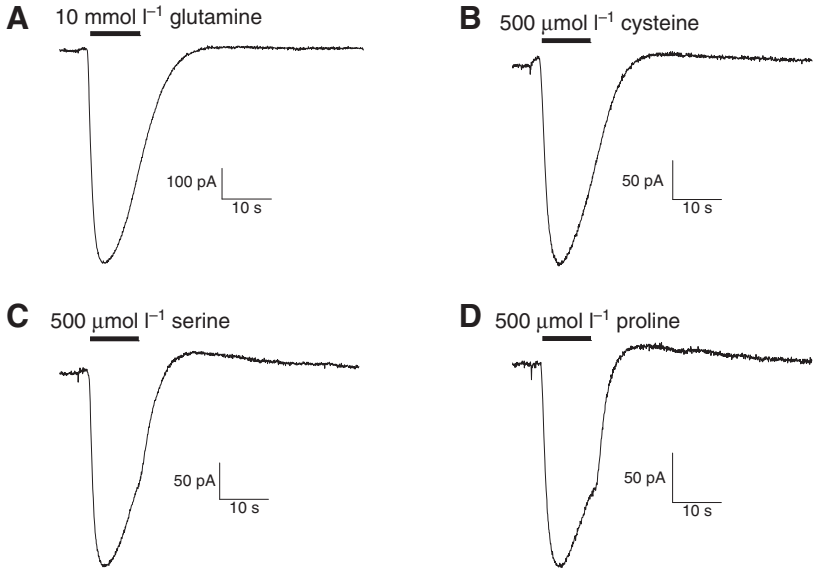

Fig. 2. Amino-acid-induced currents in *Xenopus* oocytes expressing AaePAT1. Using AaePAT-injected oocytes, as described in Materials and methods, current responses were recorded following exposure to an amino acid solution (150 μl) in ND96 at a flow rate of 1 ml min^{-1}. The addition of (A) 10 mmol l^{-1} glutamine, (B) 500 μmol l^{-1} cysteine, (C) 500 μmol l^{-1} serine and (D) 500 μmol l^{-1} proline for the period indicated by the black bars resulted in small inward currents as shown.

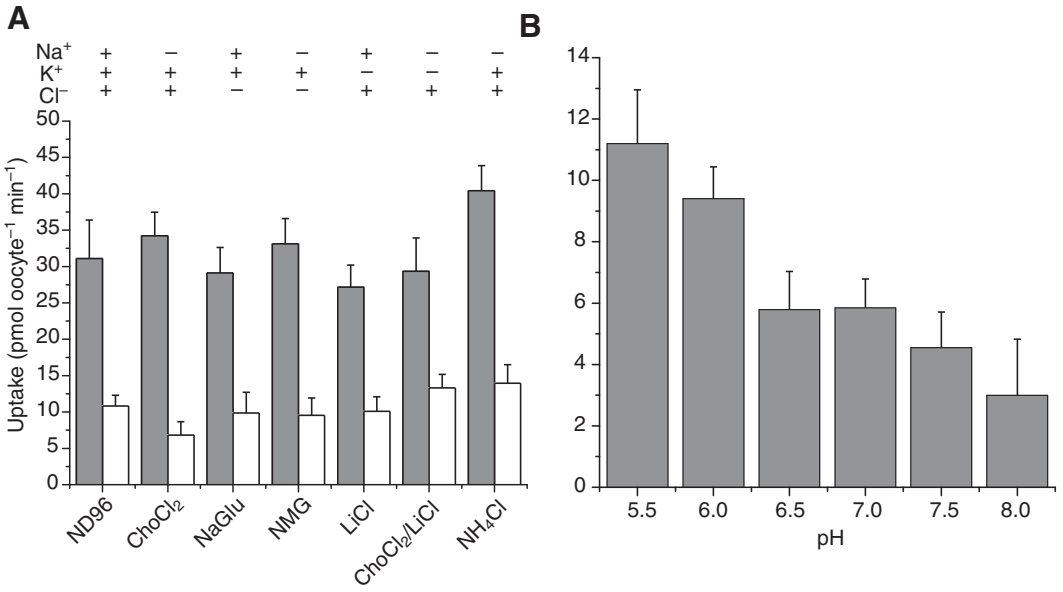

Fig. 3. Ion dependence of glutamine uptake by AaePAT1. (A) Glutamine uptake was measured in ND96 buffer (ND96) and in the following ion-substituted buffers: Na^+-free choline-substituted buffer ($ChoCl_2$), Cl^--free gluconate-substituted buffer (NaGlu), Na^+- and Cl^--free *N*-methylglucamine-substituted buffer (NMG), K^+-free lithium-substituted buffer (LiCl), Na^+- and K^+-free choline and lithium-substituted buffer ($ChoCl_2$/LiCl), and Na^+-free ammonium-substituted buffer (NH_4Cl). Gray bars, AaePAT1-injected oocytes; white bars, uninjected controls. (B) The effect of pH on glutamine transport was tested by conducting uptake assays at pH 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. Data are means ± standard error of three replicates, each consisting of five oocytes.

predicted 52.8 kDa size from the cDNA. Thus the transporter does not appear to be post-translationally glycosylated.

AaePAT1 was expressed in both distal and proximal ends of the gastric caecae (Fig. 7A–D). No expression was observed in larval Malpighian tubules (Fig. 7F) and midgut (Fig. 7G). When the antibody was preabsorbed with peptide antigen significantly less immunoreactivity was observed in the distal gastric caecae (Fig. 7E). In adult female mosquitoes AaePAT1 was expressed in the midgut; specifically in the apical membrane of the midgut (Fig. 8). No immunoreactivity was detected in the rectum, rectal pads or the Malpighian tubules. Also, no reactivity was observed with the preimmune serum (Fig. 8A) as noted with immune serum preabsorbed with the peptide antigen. AaePAT1 immunoreactivity was also observed in male midgut (data not shown).

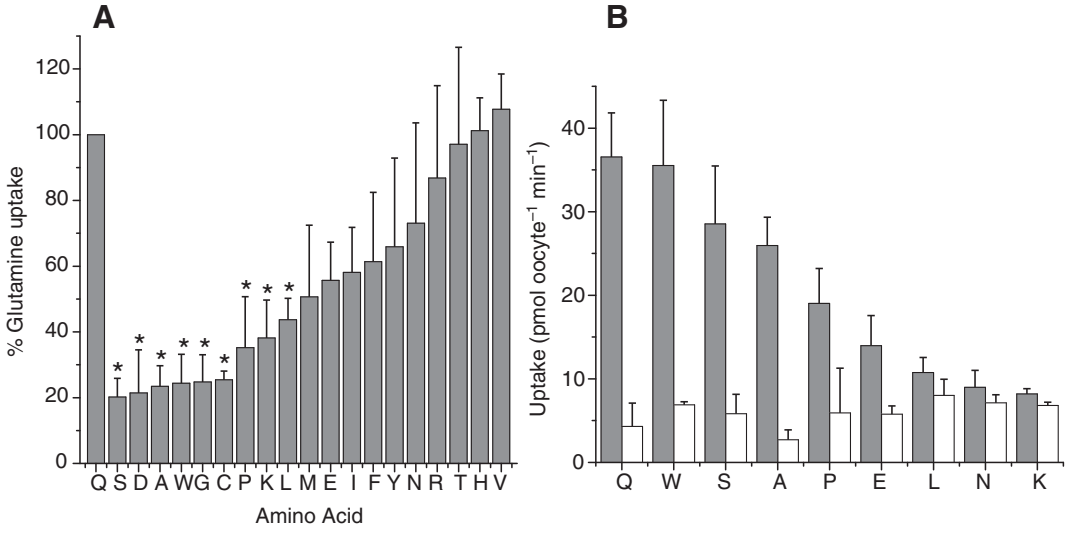

Fig. 4. Selectivity of amino acid uptake by AaePAT1. (A) Competitive inhibition of L-glutamine uptake by the other 19 amino acids. Uptake was examined with 1 mmol l^{-1} glutamine and 50 nmol l^{-1} [^{3}H]-L-glutamine and compared with transport when 50 mmol l^{-1} of another L-amino acid was added. Values are the percentage of control transport (glutamine without the competing amino acids). (B) Radiolabeled uptake was conducted using 1 mmol l^{-1} of L-alanine, asparagine, glutamine, glutamic acid, leucine, lysine, proline, serine and tryptophan with 50 nmol l^{-1} of the corresponding ^{3}H-labeled amino acid. Gray bars, AaePAT1-injected oocytes; white bars, uninjected controls. Data are the means ± standard error of three replicates each consisting of five oocytes. Asterisks indicate values are statistically different from uptake of glutamine alone, using Student's *t*-test.

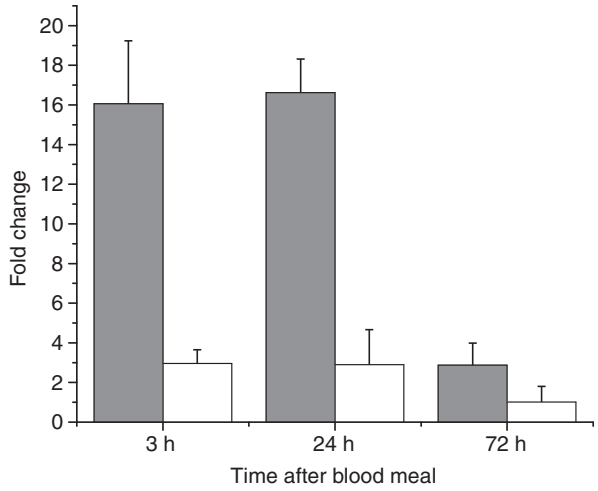

Fig. 5. Analyses of AaePAT1 transcript changes in the midgut of *Aedes aegypti* following ingestion of blood meal. Induction of AaePAT1 expression at 3, 24 and 72 h after a blood meal was analyzed by qPCR. Data are presented as fold induction in AaePAT1 transcript levels (gray bars) in the midgut of blood-fed mosquitoes over that in non-blood-fed mosquitoes. The highest increase in transcript induction is observed at 3 h and 24 h after a blood meal. By contrast there were limited increases in transcript levels of the B subunit of V-type ATPase (white bars). Data are means ± standard error of four replicates. AaePAT1 transcript levels in blood-fed mosquitoes at 3 and 24 h are statistically different (P<0.05) from that in non-blood-fed mosquitoes.

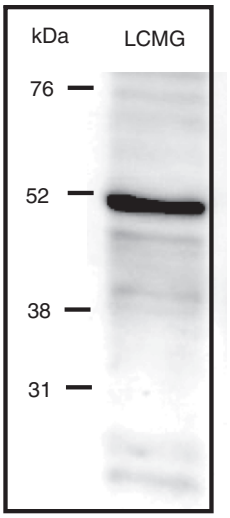

Fig. 6. Western blot of extracts from membranes of the larval caecae and midgut of *A. aegypti*. Proteins from larval caecae and midgut (LCMG) were separated by SDS-PAGE and electrotransferred to PVDF membrane. A protein of about 51 kDa was detected using anti-AaePAT1 antibody.

DISCUSSION

In anautogenous mosquitoes, such as *Ae. aegypti,* blood feeding provides not only the necessary cues for initiation of vitellogenesis, but also nutrients required for egg production and survival of the mosquitoes (Clements, 1992; Telang et al., 2006). Although it is expected that mosquitoes would have the necessary transporters for nutrient uptake (e.g. amino acids and sugars), our knowledge of these transporters in nearly all mosquito species, is poor.

Early studies demonstrated amino acid uptake in the midgut of lepidopteran insects (Giordana et al., 1998; Giordana et al., 1989); however, the identification of specific insect amino acid transporters has been slow, with only a limited number of transporters functionally characterized [for reviews, see Castagna et al. and Boudko et al. (Castagna et al., 1997; Boudko et al., 2005a) and references cited therein]. These studies show that insects, including mosquitoes, have amino acid transporters that are found in most metazoans, such as the glutamate and L-amino acid transporters (Donly et al., 1997; Donly et al., 2000; Jin et al., 2003; Meleshkevitch et al., 2006; Okech et al., 2008; Umesh et al., 2003), or novel insect transporters with no apparent vertebrate orthologues (Boudko et al., 2005b; Castagna et al., 1998; Feldman et al., 2000).

Here we report the characterization of a proton amino acid transporter, AaePAT1, from the disease vector, *Aedes aegypti*, the expression of which is induced following a blood meal. AaePAT1 is a low affinity transporter with an ability to transport a number of different amino acids, including amino acids with small and aromatic side chains. The low affinity and low specificity of transport observed here is similar to that of mammalian PAT1 orthologues (Boll et al., 2003a; Boll et al., 2002; Chen et al., 2003a; Kennedy et al., 2005).

Ingestion of a blood meal activates a number of proteases in the mosquito midgut (Barillas-Mury et al., 1995; Noriega et al., 1994; Noriega et al., 1996), leading to an available pool of amino acids for transport into epithelial cells. The presence of AaePAT1 on the apical membrane of adult female midgut, probably facilitates this absorption of specific amino acids from the lumen into the cells. Furthermore, the increase of AaePAT1 transcript levels shortly after a blood meal supports the physiological importance of this transporter in amino acid uptake. However, expression of AaePAT1 on the apical membrane of larval gastric caecae and adult male midgut suggests this transporter also plays a role in the nutrition of other developmental stages.

Amino acid transport by AaePAT1 is pH dependent, as is transport by mammalian PATs. In previously identified PATs from mice and humans (Boll et al., 2002), it was hypothesized that V-ATPases and sodium–hydrogen exchangers (NHEs) may create a proton gradient (Boll et al., 2003a; Foltz et al., 2004; Foltz et al., 2005). The resulting proton gradient developed can then be used as a driving force for amino acid transport. Interestingly, V-ATPases are also upregulated after the ingestion of a blood meal, and therefore, probably cause an increase in the proton gradient. Since V-ATPases and NHEs are also located on the midgut apical membrane of mosquitoes (Filippova et al., 1998; Kang'ethe et al., 2007; Pullikuth et al., 2006; Rheault et al., 2007; Zhuang et al., 1999), the resulting proton gradient could be the driving force for the amino acid transport by AaePAT1.

Although AaePAT1 transports a number of amino acids, only tryptophan and proline of the amino acids tested here, are considered essential for mosquito development (Dadd, 1978; Singh and Brown, 1957). Without either tryptophan and proline, and other essential amino acids in the blood meal, mosquito egg development is not completed (Clements, 1992). By contrast, although glutamic acid and aspartic acid are not essential, mosquitoes lay significantly fewer eggs with each gonotrophic cycle if these amino acid are absent in the diet (Zhou et al., 2004a; Zhou et al., 2004b). However, suppression of AaePAT1 using dsRNA did not lead to any statistical decrease in the number of eggs laid (data not shown).

In addition to their ability to transport amino acids, PATs have also been shown to regulate growth by interacting with the kinase TOR (target of rapamycin), an ancient regulated system, which is also linked to the insulin receptor (InR) signaling pathway (Jacinto and Hall, 2003). In *Drosophila*, *pathetic*, a mutant of the PAT CG3424 shows reduced growth, whereas overexpression of either *pathetic* or CG1139 resulted in overgrowth as measured by eye size (Goberdhan et al., 2005). However, the wing size responses to

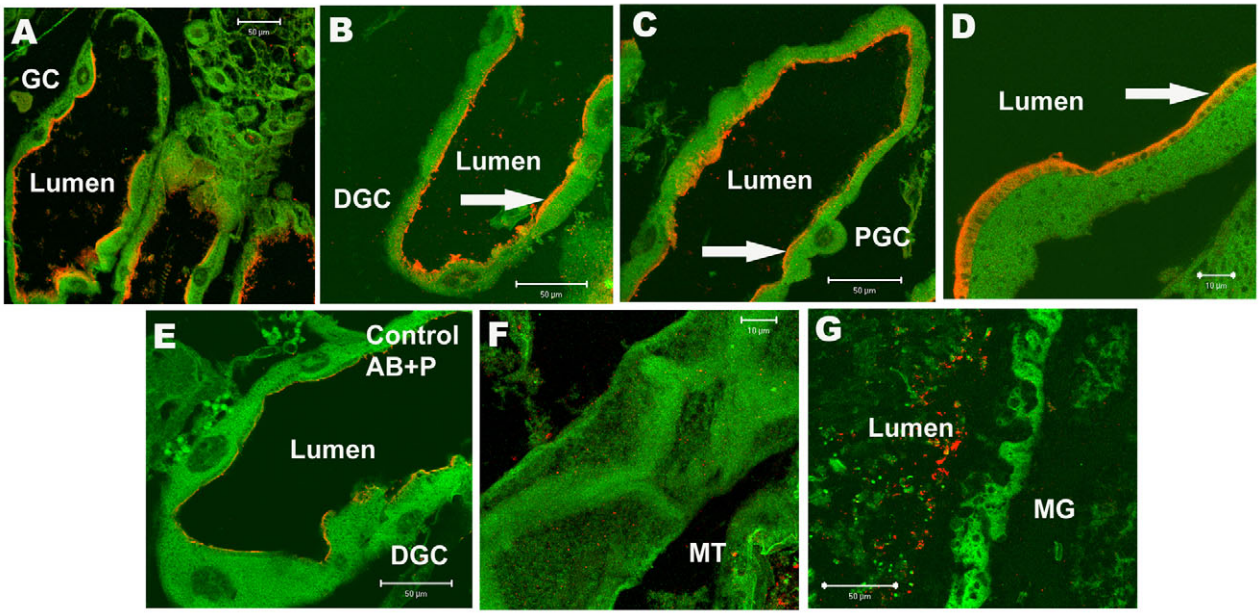

Fig. 7. Immunolocalization of AaePAT1 in *Aedes aegypti* fourth instar larvae. Tissues sections were incubated with anti-AaePAT1 whole serum antibody diluted 1:100 as described in Materials and methods. Immunoreactivities were detected using Cy-3-conjugated goat anti-rabbit secondary antibody (red). Alexa-Fluor-488–Phalloidin secondary antibody (green) was used to stain actin. (A,D) Gastric caeca (GC) cells at different magnifications. (B) Apical membrane of the distal part of the gastric caeca (DGC), and (C) the proximal part of gastric caeca (PGC). A–D show immunoreactivity with the anti-AaePAT1 antibody (red, arrow). (E) Control distal gastric caeca (DGC) section that was incubated with anti-AaePAT1 antibody that had been preabsorbed with the peptide antigen before immunostaining; immunoreactivity is considerably reduced. (F) Malpighian tubules (MT), (G) midgut (MG) showed no immunoreactivity with the antibody. Scale bars, 50 μm in A–C, E and G; 10 μm in D and F.

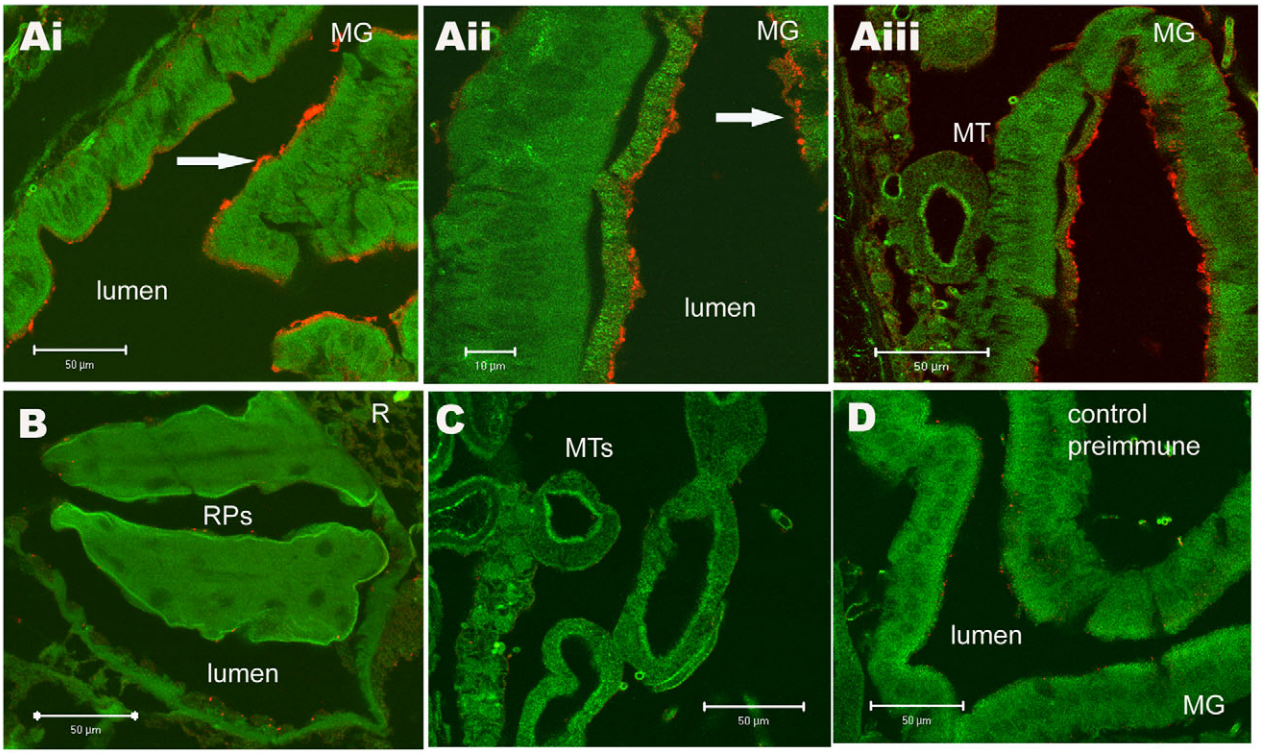

Fig. 8. Immunolocalization of AaePAT1 in non-blood-fed female *Aedes aegypti.* AaePAT1 expression in tissues of non-blood-fed (NBF) female of *Ae. aegypti.* (Ai,Aii,Aiii) Midgut (MG) at different magnifications. Immunoreactivity was detected in the apical membrane (red, arrow) of midgut epithelial cells. (B) Rectum (R) and rectal papilla (RP), and (C) Malpighian tubules showed no immunoreactivity. (D) Control midgut (MG) cells, no immunoreactivity was observed in the control using preimmune serum (compare with Fig. 7E). Scale bars, 50 μm in Ai, Aiii and B–D; 10 μm in Aii.

overexpression of *pathetic* and CG1139 is different (Goberdhan et al., 2005) from that of the eye, suggesting PATs may also have other tissue functions in mosquitoes in addition to amino acid transport.

The presence of a large number of PATs in most organisms suggests that PATs may also function through the TOR–InR signaling pathway (Goberdhan and Wilson, 2003; Jacinto and Hall, 2003). Indeed in mosquito, inhibition of TOR by rapamycin or knock down of TOR by dsRNA inhibited amino acid stimulation of yolk protein precursor gene expression (Hansen et al., 2004; Hansen et al., 2005). Thus amino acid uptake probably affects egg development through the TOR–InR signaling pathway, in addition to having a direct effect on the nutritional state of the mosquito.

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