

# Molecular analysis of the serine/threonine kinase Akt and its expression in the mosquito *Aedes aegypti*

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## Abstract

**A key component of the insulin-signalling pathway, the protein kinase Akt, was identified and cloned as a cDNA from ovaries of the mosquito *Aedes aegypti*. An ortholog gene was found in the *Anopheles gambiae* genome database, and like other Akts, both mosquito Akts possess pleckstrin homology domains for membrane binding and a serine/threonine kinase domain. When *Ae. aegypti* ovaries were treated with bovine insulin *in vitro*, a putative Akt was threonine-phosphorylated, as expected for Akts. *AaegAKT* was only expressed in embryos for the first 6 h after oviposition and in ovaries before and during a gonotrophic cycle.**

**Keywords:** protein kinase B (PKB), insect, ecdysteroids, oogenesis, ovary.

## Introduction

In the female mosquito *Aedes aegypti*, reproductive arrest ends and egg development begins when a blood meal is obtained. Subsequently, neurohormones are released from neurosecretory cells in the brain (Brown *et al.*, 1998) and stimulate the ovaries to secrete ecdysteroids, which in turn initiate production of vitellogenin in the fat body for uptake by the developing oocyte. Two types of steroidogenic hormones, ovary ecdysteroidogenic hormone I (OEH I) and insulin-like peptides (ILPs), are known to exist in the brain neurosecretory cells of mosquitoes (Brown & Cao, 2001; Cao & Brown, 2001). OEH I regulates ecdysteroid production by ovaries *in vitro* and egg development *in vivo* (Brown

*et al.*, 1998). Recently, genes for five ILPs were identified in another mosquito, *Anopheles gambiae* (Riehle *et al.*, 2002), and mosquito ILPs probably regulate ovarian ecdysteroid production through a conserved insulin signalling pathway, as has been demonstrated using bovine insulin (Riehle & Brown, 1999). The insulin receptor activates this pathway, and its expression has been well characterized in the ovaries of female *Ae. aegypti* before and during a gonotrophic cycle (Riehle & Brown, 2002). Genes for other proteins in the pathway, including insulin receptor substrate, phosphatidylinositol 3-kinase and Akt (also known as protein kinase B or Rac), have been identified in *An. gambiae* (Riehle *et al.*, 2002), but their expression has yet to be defined in mosquitoes.

Originally discovered over 10 years ago, Akt is now considered to be a key nexus for the effects of insulin on metabolism, cell proliferation and apoptosis in mammals (Scheid & Woodgett, 2001). Akt is a member of the AGC kinase family named for three of its members, protein kinases A, G and C (Peterson & Schreiber, 1999). Other members of this family include p70<sup>S6K</sup>, phosphoinositide-dependent kinases (PKDs) and PKC-related kinases. Although these kinases display a wide range of physiological functions, all phosphorylate protein substrates on serine/threonine residues and share structural characteristics, such as an activation loop in the kinase domain and a pleckstrin homology domain for binding phosphatidylinositol(3,4,5) $P_3$  in membranes. Mammalian Akts are activated after a specific Thr in the activation loop and Ser at the carboxy-terminus are phosphorylated.

Akt has been shown to be a component of the insulin signalling pathway in only two invertebrate species, the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*. In *D. melanogaster*, a single copy of the Akt gene, *Dakt1* (*DmelAKT*, herein), is translated into two proteins of 66 and 86 kDa (Andjelkovic *et al.*, 1995; Franke *et al.*, 1994). This gene has been shown through mutational analysis to induce apoptosis in the early stages of embryogenesis (Staveley *et al.*, 1998; Scanga *et al.*, 2000), and in later stages, it regulates cell size without affecting apoptosis (Verdu *et al.*, 1999; Scanga *et al.*, 2000). Two Akt genes identified in *C. elegans* (*CeleAKT*, herein) suppress *Daf-16*,

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a forkhead transcription factor ortholog, and expression of both Akts must be reduced to initiate dauer arrest, which is regulated by the insulin signalling pathway (Paradis & Ruvkun, 1998).

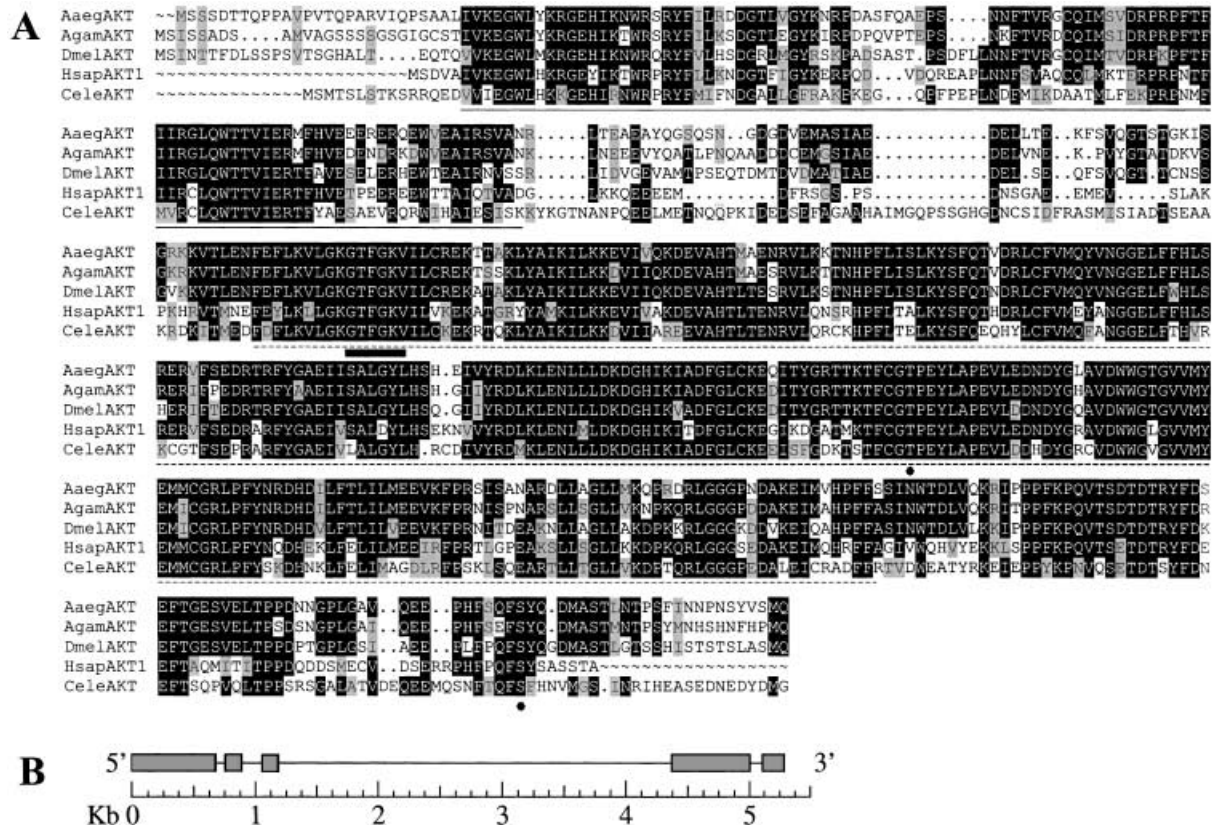
Notably, only for female *Ae. aegypti* has this pathway been shown to directly affect a key reproductive process. Thus, studies of the mosquito Akt are warranted, especially because it plays a role in the activation of ovarian ecdysteroid production as demonstrated with a known activator, okadaic acid, that alone stimulated this process *in vitro* (Riehle & Brown, 1999). In this paper, we describe the identification of an Akt cDNA obtained from the ovaries of *Ae. aegypti* and show its relationship to ortholog genes in *An. gambiae* and other animals. Conservation of protein structure is demonstrated with models of the putative AaegAKT and DmelAKT. Transcript expression of *AaegAKT* is characterized for different life stages and in ovaries of females before and during a gonotrophic cycle. Phosphorylation of a putative Akt in extracts of ovaries incubated with bovine insulin offers additional evidence for its inclusion in the insulin signalling pathway of mosquitoes.

**Results**

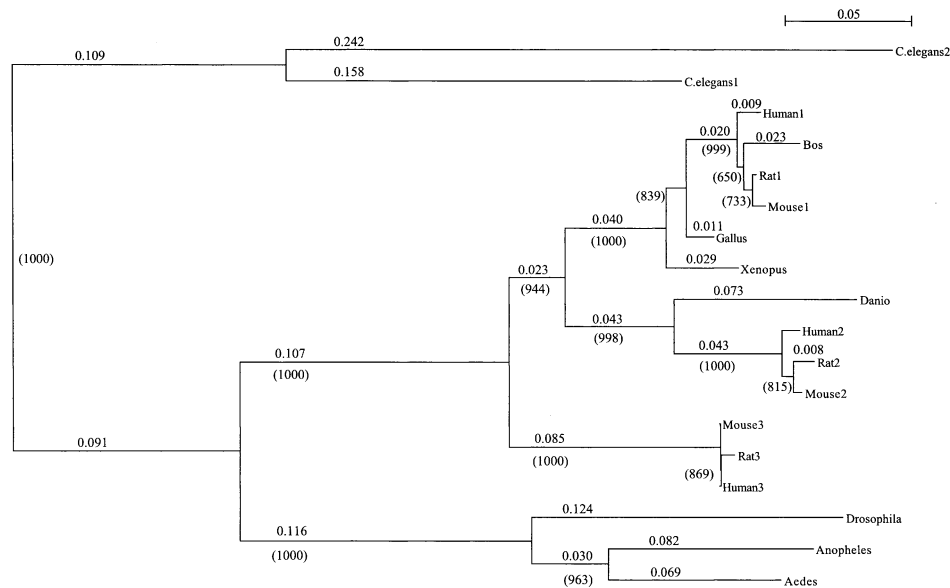
*Identification of mosquito Akt genes*

The complete nucleotide sequence of a cDNA encoding Akt in the ovaries of female *Ae. aegypti*, *AaegAKT* (GenBank accession #AF532204), was assembled from sequences of products obtained through different PCR techniques. *AaegAKT* is a 2239 bp transcript with an open reading frame that encodes a 529 amino acid protein with a predicted molecular weight of 60 kDa. As with other members of the Akt family, AaegAKT possesses a pleckstrin homology (PH) domain (AA 28–105), shown to be important for Akt binding to membranes (Fig. 1A). A Ser/Thr-type protein kinase domain (AA 184–441) is responsible for the phosphorylation of other proteins, and this domain contains an ATP binding motif (AA 191–196). Threonine<sup>345</sup> and Ser<sup>504</sup> in AaegAKT are putative phosphorylation sites.

With the *An. gambiae* genome database recently completed, we were able to elucidate the entire Akt coding region for a second mosquito species (GenBank accession #EAA03708). For AgamAKT, the PH domain (AA 17–120),



**Figure 1.** (A) PILEUP comparison of amino acid sequences for mosquito and other AKTs. Identical residues in at least 50% of the sequences are highlighted in black, and conserved residues in at least 50% of the sequences are shaded grey (Boxshade; GCG Wisconsin package). The PH domain is underlined with a solid line and the ser/thr kinase domain with a dashed line. The Thr and Ser phosphorylation targets have asterisks under them and the ATP binding motif has a black bar beneath it. Protein sequences for AKTs from *D. melanogaster* (GenBank accession no. CAA81204), *C. elegans* (T43232), and human AKT1 (P31749) were obtained from GENBANK/NCBI. (B) The intron/exon structure of the *An. gambiae* Akt is represented with exons represented by shaded boxes and introns by solid lines. The scale underneath indicates the length of the introns and exons in kilobases.



**Figure 2.** Phylogenetic tree of the AKT family was generated using the neighbour joining distance method of the CLUSTALX package and was drawn with NJPLOT. Distances are indicated above the branch and bootstrap values below. Protein sequences for AKTs from selected invertebrate and vertebrate species were obtained from GENBANK/NCBI.

Ser/Thr-type kinase domain (AA 163–409) with a ATP binding motif (AA 190–195), and the PDK phosphorylation sites (Thr<sup>344</sup> and Ser<sup>503</sup>) are similarly conserved (Fig. 1A). The coding region of *AgamAKT* has five exons and four introns. Introns 1, 2 and 4 are similarly positioned in *DmelAKT*, but the third intron, spanning over 3000 bp in *AgamAKT*, is absent (Fig. 1B).

#### Comparison of Akts

Examination of the amino acid sequences of Akts from mosquitoes and representative model organisms revealed that *AeegAKT* has 86% sequence similarity (80% identity) to *AgamAKT*, 79% sequence similarity (74% identity) to *DmelAKT*, 54% and 60% sequence similarity (44% and 49% identity) to *CeleAKT* 1 and 2, respectively, and 67% sequence similarity (58% identity) to the human *Akt2* (Fig. 1A). The greatest degree of sequence identity and similarity is found in the kinase domain of these Akts. Phylogenetic analysis of known vertebrate and invertebrate Akts was performed using the neighbour joining method based on data compiled from a CLUSTALX pileup (Fig. 2). The SWISSMODEL program was used to generate a model of the tertiary structure of the *AeegAKT* kinase domain based on models of kinase domains in other proteins and *DmelAKT* (Fig. 3). The kinase domain structure of *AeegAKT* and *DmelAKT* is similar, as demonstrated by an overlay in Fig. 3, but must be refined with X-ray crystallography or NMR studies of purified protein.

#### *AeegAKT* expression in different life stages

Northern blots of total RNA obtained from pooled eggs and individuals in different life stages were performed to

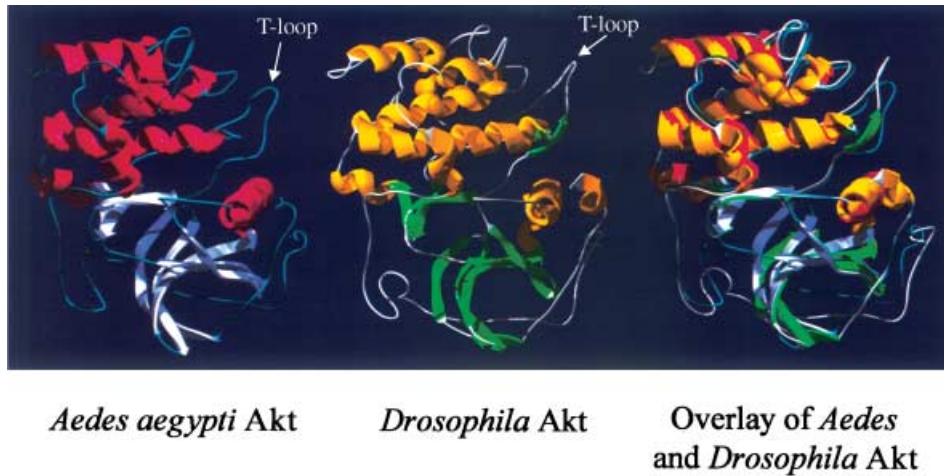
determine when *AeegAKT* is expressed. Two *AeegAKT* transcripts (2.3 and 2.5 kb) were detected in embryos during the first 6 h after oviposition and faintly in whole adult females (Fig. 4A). Transcripts were not detected in embryos > 6 h old, 4th instar larvae, pupae or males. In non-oogenic females, *AeegAKT* transcript was detected in ovaries, but not midgut, body wall, head or thorax (Fig. 4B). More specifically, *AeegAKT* expression in ovaries was not evident in females for the first 24 h after eclosion (0 day), but from 1 to 7 days after eclosion transcripts were evident (Fig. 4C).

#### *AeegAKT* expression during a gonotrophic cycle

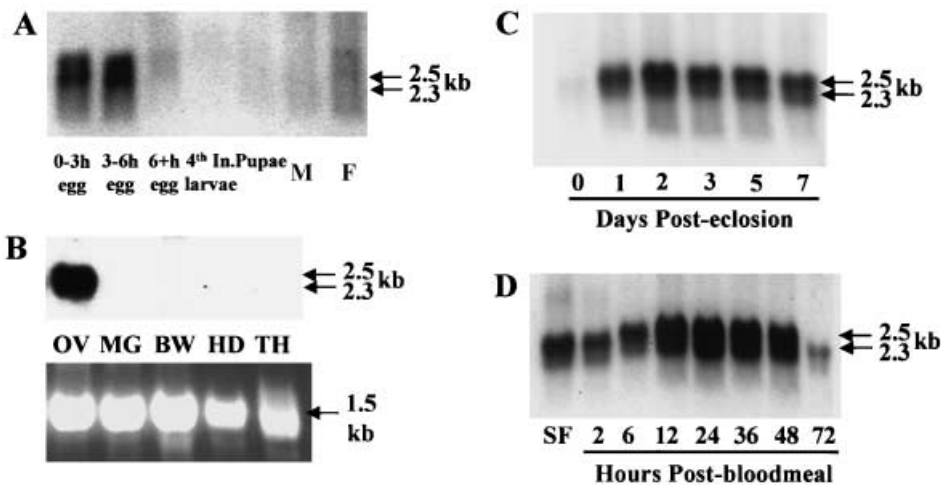
The expression pattern of *AeegAKT* was examined in pooled ovaries taken from females at different times after a blood meal to encompass a gonotrophic cycle (Fig. 4D). Total RNA from ten pairs of ovaries was used to standardize the loading of RNA, because rRNA levels rise and fall dramatically during this cycle. The level of ovary *AeegAKT* transcript in females 2 and 6 h post blood meal (pbm) appeared to be similar to that in ovaries from sucrose-fed, non-oogenic females. From 12 to 48 h pbm, much greater levels of ovary *AeegAKT* transcript were observed, and by 72 h pbm after oviposition, the level of transcript had decreased markedly.

#### Effect of insulin on *AeegAKT* phosphorylation

To determine whether insulin affects Akt phosphorylation, ovaries from non-oogenic females were incubated alone or with bovine insulin at a dose known to stimulate ecdysteroidogenesis and a phosphatase inhibitor to prevent constitutive dephosphorylation. Pooled ovaries treated as such



**Figure 3.** Putative tertiary structure of the AegAkt kinase domain generated with SWISSMODEL to an accuracy of  $\approx 1 \text{ \AA}$  and compared with that of DmelAkt. Note the conserved activation loop that when phosphorylated on a Thr residue exposes the catalytic core allowing substrate and ATP access to the kinase domain.



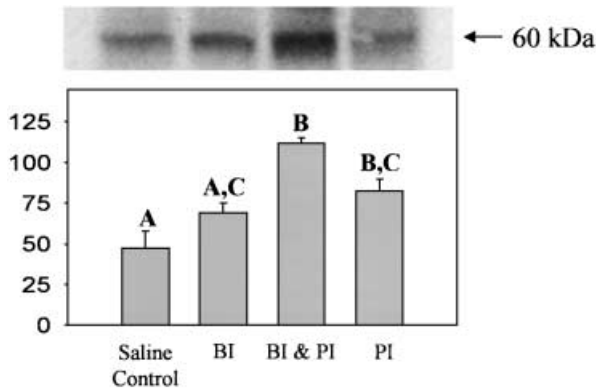
**Figure 4.** Detection of *AegAkt* transcripts in different life stages of *Ae. aegypti* on representative Northern blots. (A) *AegAkt* expression is evident only in embryos at 0–3 h and 3–6 h post-oviposition and adult females (F). Expression of *AegAkt* in whole adult females is faint because expression only occurs in the ovaries. No transcripts were detected in embryos > 6 h post-oviposition, 4th instar larvae (4th L), pupae (P), or males (M). Total RNA (10  $\mu\text{g}$ ) was loaded into each lane. (B) For sugar-fed, non-oogenic females, only ovaries (OV) contain transcripts and not midgut (MG), body wall (BW), head (HD) or thorax (TH). Ethidium bromide staining of ribosomal RNA demonstrates equal loading of total RNA (10  $\mu\text{g}$ ) from pooled tissues of 3–5-day-old females. (C) For non-oogenic females, *AegAkt* expression is typically low in ovaries for up to 24 h post-eclosion (day 0), increases during the next 2 days, and remains unchanged during previtellogenetic arrest (3–7 days). Total RNA from 10 pairs of ovaries was loaded into each lane. (D) For females during a gonotrophic cycle, *AegAkt* expression is low in ovaries from sugar-fed, non-oogenic females (SF) and for up to 6 h post-blood meal (pbm) in comparison to the high levels detected during 12–48 h pbm, and drops after oviposition, 72 h pbm. Total RNA from 10 pairs of ovaries was loaded for each time point.

were processed for electrophoresis and immunoblots that were incubated with antiphosphorylated Thr antibody (Fig. 5). Along with other proteins, a 60 kDa protein – the expected size of AegAkt – was evident most prominently in ovaries incubated with bovine insulin and phosphatase inhibitor. The 60 kDa protein showed a 2.3-fold increase in density above that evident in the saline-control ovaries, as well as a 1.7-fold increase in ovaries incubated with phosphatase inhibitor alone, and a non-significant increase with insulin alone. These same ovary immunoblots were treated

with an antiphosphoserine antibody, but no proteins were detected (data not shown). Antisera to human Akt (Calbiochem and Affinity Bioreagents, Inc.) and DmelAkt were screened on ovary immunoblots, but failed to detect a protein of the expected size.

### Discussion

The existence of an Akt with a key role in the insulin signaling pathway of female *Ae. aegypti* was demonstrated *in*



**Figure 5.** Representative immunoblot of extracted ovaries incubated *in vitro* (30 min) in saline solution (control), 17  $\mu$ M bovine insulin (BI) and phosphatase inhibitor (PI) or phosphatase inhibitor alone. The putative AegAeAkt, 60 kDa, was detected with antibodies to phosphorylated Thr. In the lower panel, the results of immunoblot densitometry on this protein in ovary samples were analysed for statistical significant differences with an ANOVA and using Tukey's test.

*in vitro* for ovaries (Riehle & Brown, 1999). The gene encoding an Akt, *AegAeAkt*, has been identified in this species and its ortholog, *AgamAkt*, in a second mosquito, *An. gambiae*. We examined the expression pattern of *AegAeAkt* in all developmental stages and adult tissues and found that when it is expressed, two transcripts of 2.3 and 2.5 kb were detected. The gene is expressed first in early embryos for up to 6 h after oviposition, where it may have maternal or positional effects. Surprisingly, *AegAeAkt* was expressed later only in females, and specifically only in ovaries and not other tissues, thus substantiating its role in activating ecdysteroid production.

Ovarian ecdysteroid production is presumed to occur in the follicle cells of the developing egg chambers. The haemolymph titre of ecdysteroids starts to rise at 6 h pbm, peaks around 18–24 h pbm, and falls by 30 h pbm (Hagedorn *et al.*, 1975). Between 30 and 48 h pbm, follicle cells switch to chorion formation around yolk-filled oocytes, which are oviposited by 72 h pbm. Before and during a gonotrophic cycle, *AegAeAkt* transcripts are detected in ovaries. Although *AegAeAkt* transcript levels appear relatively unchanged in ovaries from females not given a blood meal and those from females 2 and 6 h pbm, it is likely that *AegAeAkt* is present during this time and involved in the activation of steroidogenesis, especially because this process is activated *in vitro* in ovaries from non-blood females. Surprisingly, the greatest expression of *AegAeAkt* occurs 12–48 h pbm and encompasses the period when follicle cells produce optimal amounts of ecdysteroids and shift to chorion synthesis. Furthermore, *AegAeAkt* expression may be occurring in secondary follicles, which start developing around 24 pbm for the next gonotrophic cycle.

In general, Akts are globular, cytosolic proteins of approximately 56–60 kDa with > 65% amino acid identity

and similarity among the vertebrate and invertebrate forms described to date. Typically, the kinase domain of Akts shares the greatest sequence identity, thus favouring the modelling of its tertiary structure (e.g. Fig. 3). The phylogenetic tree determined for the Akt family (Fig. 2) shows that the dipteran Akts form a separate branch from the three classes of vertebrate Akts, but are more closely related to the vertebrate Akts than to those identified in *C. elegans*.

Other important features of Akts were identified in AegAeAkt, including a conserved Thr in the activation loop of the kinase domain. In human Akt1, Thr<sup>308</sup> lies in the activation loop, and when unphosphorylated, it inhibits kinase activity. Phosphorylation of this Thr by PDK results in a conformational change in the loop allowing ATP and substrate access to the kinase domain (Alessi *et al.*, 1996; 1997). In *Ae. aegypti* ovaries, a putative Akt of 60 kDa was Thr-phosphorylated after incubation with bovine insulin and a phosphatase inhibitor (Fig. 5) and most likely reflects phosphorylation of Thr<sup>345</sup> on AegAeAkt. A conserved Ser is present near the carboxy terminus of vertebrate and dipteran Akts. Phosphorylation of Ser<sup>473</sup> is required for full activation of human Akt1 (Schubert *et al.*, 2000). A different PDK was thought to phosphorylate this Ser, but recent evidence suggests that it may be phosphorylated by Akt itself (Scheid & Woodgett, 2001). Unlike human Akt1, AegAeAkt did not appear to be Ser phosphorylated in extracts of ovaries stimulated with bovine insulin.

Akt is a well-characterized component of the insulin signalling pathway in *D. melanogaster* (Franke *et al.*, 1994; Andjelkovic *et al.*, 1995; Staveley *et al.*, 1998). *DmelAkt* is transcribed in two forms of 2.7 and 4 kb with different expression patterns during development (Andjelkovic *et al.*, 1995). The coding domains of both transcripts are identical, but the larger transcript has a 3' extension with multiple polyadenylation sites. Interestingly, the 2.7 kb *DmelAkt* transcript is similar in size to those transcribed from *AegAeAkt* and has a comparable expression pattern (0–3 h embryos and adult females only). In contrast, the 4 kb *DmelAkt* transcript is strongly expressed during the first 12 h in the embryo, moderately expressed in older embryos and larvae, and weakly expressed in adult females. Translation of both transcripts produces two Akts of 66 kDa and 85 kDa. The larger *DmelAkt* results from a weak translational start site 81 amino acids upstream of the primary start site and is moderately expressed in early embryos, weakly expressed in late embryos, pupae and adult, and not expressed in larvae. The 66 kDa *DmelAkt* protein is more widely expressed, with high levels found throughout embryonic development and in day 1 pupae, and moderate levels found in both larvae and adults. Interestingly, the adult *DmelAkt*, although less abundant than in embryos, has an intrinsic kinase activity approximately 8-fold higher than in embryos (Andjelkovic *et al.*, 1995).

In *C. elegans*, Akts encoded by two genes are part of the insulin signalling pathway known to regulate development, metabolism and possibly lifespan (Kimura *et al.*, 1997). CeleAKT1 with two phosphorylation sites (Thr<sup>308</sup> and Ser<sup>473</sup>) is more biologically active than CeleAKT2 with only Thr<sup>308</sup> (Ser<sup>473</sup> is absent due to a truncated carboxy-terminus; Paradis & Ruvkun, 1998). An activating mutation or overexpression of CeleAKT1 in *age-1* (phosphatidylinositol 3-kinase) and *daf-2* (insulin receptor) mutants results in a shift from dauer formation to resume development to an adult.

In mammals, Akt regulates the effects of insulin on metabolism, apoptosis and cell proliferation. In cell systems, Akt directly affects glucose metabolism through the phosphorylation and inactivation of glycogen synthase kinase 3 (GSK3), resulting in increased protein and glycogen synthesis (Cross *et al.*, 1995), and the translocation of glucose transporters to the cell surface (Hajdуч *et al.*, 2001). Mice deficient in Akt2 have a phenotype similar to Type II diabetes mellitus in humans, including insulin resistance and elevated glucose levels (Cho *et al.*, 2001). Cell survival is promoted by Akt through the direct or indirect phosphorylation and inactivation of several apoptotic genes, including BAD, caspases and possibly GSK3 (Datta *et al.*, 1997; Brunet *et al.*, 1999; Ding *et al.*, 2000; Hajdуч *et al.*, 2001). Originally identified as an oncogene, Akt also promotes cell proliferation by the phosphorylation and inactivation of p21 proteins that reversibly inhibit the cell cycle (Zhou *et al.*, 2001) and by the increased translation of cyclin D proteins, which regulate cell cycle progression (Gille & Downward, 1999).

The pleiotropic effects of the insulin signalling pathway described above for *D. melanogaster*, *C. elegans* and mammals provide leads for future investigations of this pathway in mosquitoes. The annotation of genes encoding ILPs and other proteins in this pathway in *An. gambiae* (Riehle *et al.*, 2002) establishes a foundation for such work. Molecular analysis of these genes and their expression, as described for *AeagAKT* in this report and the *Ae. aegypti* insulin receptor (Riehle & Brown, 2002), probably will offer more evidence that this pathway regulates ovarian ecdysteroid production directly and, possibly, affects the acquisition and transmission of pathogens by blood-feeding female mosquitoes.

## Experimental procedures

### Mosquito colony

Mosquitoes were maintained at 27 °C in a 16 h light/8 h dark photoperiod, and larvae were fed ground rat chow:lactalbumin:brewers yeast (1 : 1 : 1). Adults fed at will on a 10% sucrose solution for 2 days and thereafter only water. Females used for the age-time course had continuous access to 10% sucrose solution, and for the post-blood meal time course, they were engorged on an anaesthetized rat, separated from unfed and partially fed individuals, and given water.

### Akt gene identification

After dissecting and homogenizing ovaries from 3-day-old female *Ae. aegypti*, mRNA was isolated using oligo-dT paramagnetic beads (Dyna). First strand cDNA was synthesized from ovary mRNA using the First-Strand cDNA synthesis kit (Pharmacia-Biotech). Degenerate primers to conserved regions of the Akt family (forward primer 5'-CARTGGACNACNGTATYGA-3', amino acids 79–85 of human Akt1; and reverse primer 5'-CCRCACAT-CATYTCRTACAT-3', amino acids 339–345 of human Akt1) were used to amplify products from ovary cDNA with 'touchdown' PCR (95 °C for 5 s, 60 °C to 50 °C (–2 °C every three cycles) for 7 s, 72 °C for 10 s, 17 cycles followed by 95 °C for 5 s, 48 °C for 7 s, 72 °C for 10 s, 40 cycles) in glass capillary tubes with a Rapidcycler PCR machine (Idaho Technologies). A 900-bp fragment was gel purified, cloned (TOPO TA cloning kit, Invitrogen), sequenced, and found to encode an Akt-like protein sequence. The First Choice RLM-Race kit (Ambion) was used to determine the 5' and 3' ends of *AeagAKT*. With this information, nested, specific primers (5' Outer CGATACGACCCAATAAACC; 5' Inner CCCAATAAACCTCAGACATACC; 3' Outer TCCTTCTGCCTTGCTATTC; 3' Inner CCTTGCTATCAAAACCTC) were used to PCR amplify the entire *AeagAKT* cDNA sequence from ovary cDNA obtained from three different mosquito pools. These PCR products were sequenced in both directions to confirm their nucleotide sequence.

*AgamAKT* was identified from the completed genomic sequence of *An. gambiae sensu stricta* (PEST strain) at the NCBI <<http://www.ncbi.nlm.nih.gov>> using a TBLASTN search against the complete *AeagAKT* sequence. The single sequence obtained was compared to the predicted transcript/protein sequences for *An. gambiae* to obtain putative transcript (agCT44125) and protein (agCP14714) sequences. These were visually verified against known Akts from *D. melanogaster* and *Ae. aegypti*.

### Sequence alignment and phylogenetic tree

Amino acid sequences of Akts from *Ae. aegypti*, *An. gambiae* (Riehle *et al.*, 2002), *D. melanogaster* (XP\_081482), humans (HsapAKT1, AAL55732), and *C. elegans* (CeleAKT1, T43234) were aligned with the CLUSTALX program (Thompson *et al.*, 1997). The pair-wise distances between these Akts and others from selected vertebrate species were determined with the neighbour joining method in CLUSTALX. With these values, the tree was bootstrapped 1000 times and generated using the NJPLOT program (Perriere & Gouy, 1996). Sequence similarity and identity scores between *AeagAKT* and Akts from *An. gambiae*, *D. melanogaster*, *C. elegans* and humans were determined using the BOXSHADE program (GCG Wisconsin package).

### Tertiary structure model

Due to the high percentage of protein sequence identity between *AeagAKT* and *DmelAKT*, a tertiary structure model of the kinase domain of *AeagAKT* was generated. The amino acid sequence of this domain in *AeagAKT* was submitted to the Swiss model <<http://www.expasy.ch/swissmod/>> for model construction, based on those for *DmelAKT* and human protein kinase A (Peitsch, 1996; Guex & Peitsch, 1997). The amino and carboxy termini of *AeagAKT*, including the conserved Ser, were not included due to their low sequence identity.

### Northern blots

To produce the RNA probes, the forward primer for *AeegAKT* (see above) and a M13 (-20) forward primer were used to PCR amplify the 900 bp product from the cloned *AeegAKT* cDNA in the pCR II vector (Invitrogen), with a T7 promoter. This product was used as a template in the MAXIscript *in vitro* transcription kit (Ambion) to generate antisense RNA probes with dUTP-digoxigenin (Dig; Roche).

Ovaries were dissected from female mosquitoes in a saline solution (128 mM NaCl, 4.7 mM KCl and 1.9 mM CaCl<sub>2</sub>), transferred immediately to RNeasy lysis buffer (50 µl, Ambion), and stored at -20 °C until processing for total RNA with the RNeasy mini kit (Qiagen). Loading buffer (5×: 32% formamide, 2.4% formaldehyde, 4× MOPS, 20% glycerol, 4 mM EDTA, 0.1 mg/ml ethidium bromide, and bromophenol blue) was added to total RNA from 10 pairs of ovaries, and RNase-free water was added to bring the final volume to 20 µl. The RNA was denatured at 80 °C for 10 min, loaded on to a pre-cast 1.25% Reliant MOPS gel (BMA), and separated at 3 V/cm for 3 h in 1× MOPS running buffer. After the gel was denatured in 0.05 M NaOH for 30 min and neutralized in 1 M Tris for 30 min, the RNA was vacuum transferred on to a nylon membrane (MSI) for 1 h and crosslinked (120 000 J/cm, Stratilinker® UV crosslinker, Stratagene). The blot was prehybridized in ULTRAhyb solution (7 ml, Ambion) at 65 °C for 1 h, after which labelled probe (denatured for 10 min at 80 °C) was added (1 : 20 000 final concentration) and hybridized overnight at 65 °C. The blot then was washed three times (20 min/wash) in 0.5× SSC/0.1% SDS at 65 °C followed by a 1-h block in 1× blocking reagent (Roche). The blot was incubated with an anti-Dig-alkaline phosphatase antibody (1 : 10 000) and 1× blocking reagent for 30 min followed by three washes (20 min/wash) in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) and 0.3% Tween-20. *AeegAKT* transcripts were detected with CDP-Star (Roche) and visualized with Biomax light X-ray film (Kodak) or a GeneGnome chemiluminescent imager (Syngene). Densitometry was performed with the chemiluminescent imager. Blots were replicated at least three times for each experiment.

### Immunoblots of *in vitro* ovary samples

Ovaries (20 pairs) were incubated in saline solution alone or with bovine insulin (17 µM, Sigma), bovine insulin and phosphatase inhibitor cocktail (Sigma), or phosphatase inhibitor cocktail alone, for 30 min at 30 °C with gentle shaking. After 30 min, the ovaries were homogenized in SDS/reducing loading buffer with 5× protease inhibitor cocktail (Roche) and 1× phosphatase inhibitor. Samples were homogenized, vortexed to shear the genomic DNA and reduce viscosity, boiled for 10 min, and plunged into an ice bath. Ovary samples and molecular weight markers (10–250 kDa, Rainbow Markers, Amersham) were loaded onto a 4–20% Tris-glycine gel (Bio-Rad), separated by electrophoresis (10× Tris/Glycine/SDS running buffer, BioRad), and tank transferred (10 mM Tris, 100 mM Glycine, 10% methanol) to a PVDF membrane (Bio-rad). After the transfer, blots were blocked for 1 h in TBST containing 5% BSA and then incubated with polyclonal antibodies against phosphorylated Ser or Thr (1 : 20 000; Zymed) overnight at 4 °C. Blots were washed in TBST (3×, 20 min), incubated with goat anti-rabbit antibodies conjugated to horseradish peroxidase (1 : 20 000 dilution, Sigma) in TBST (1 h, RT), and washed an additional three times in TBST (20 min/wash). Labelled proteins were detected using the Western lightning kit (NEN) and Kodak Biomax light film, and quantified with a chemiluminescent imager. Experimental immunoblots were replicated three or more times.

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