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Characterization of the Mosquito Insulin Receptor and its Signal Transduction Pathway in the Ovaries of the Mosquito *Aedes aegypti*
(Under the Direction of Mark R. Brown)

The reproductive cycle in female mosquitoes consists of alternating periods of rapid development and arrest. After a blood meal, ovary steroidogenesis, a key switch between arrest and egg development, is regulated by an insulin signaling cascade. The first step in this cascade is activation of the mosquito insulin receptor (MIR). After adult eclosion, from previtellogenesis until the end of vitellogenesis, MIR was observed on follicle cell membranes. During post-vitellogenesis, when follicle cells switch from steroid to chorion production, MIR was not expressed. Bovine insulin and an activator of the insulin receptor, pervanadate, both stimulated ovarian steroid production in vitro, and an inhibitor of the insulin receptor, HNMPA-(AM_3), prevented steroid production. Furthermore, MIR became tyrosine phosphorylated when ovaries were stimulated with bovine insulin or pervanadate. Together, these data strongly suggest that MIR regulates steroidogenesis in the mosquito ovary.

A second signaling component, protein kinase B (PKB), was characterized in mosquito ovaries. The mosquito PKB gene, MPKB, encodes a 60 kDa protein containing a pleckstrin homology and catalytic domain. MPKB transcript was only found in early embryos and ovaries, with increased expression late in egg development. MPKB was detected in follicle cells prior to a bloodmeal, and was threonine phosphorylated after the ovaries were stimulated with insulin. Okidaic acid, an activator of PKB, stimulated ovary steroidogenesis in the absence of a steroidogenic hormone.

Phosphoinositide 3-kinase (PI3K), a third component of the insulin signaling cascade, acts between the insulin receptor and PKB. Partial sequences from two classes of PI3K were isolated from mosquito ovaries and share considerable sequence identity with PI3Ks from *Drosophila*. The catalytic subunit of a Class one PI3K in *Ae. aegypti*, named *Ae*p110, contains catalytic, accessory, and C2 domains. Two inhibitors of PI3K, wortmannin and LY20094, prevented ovarian steroidogenesis in vitro. The *Ae*p110 transcript was found in ovary, head, midgut, and body wall of adult females.

INDEX WORDS: Ecdysteroid, Protein Kinase B, akt, Phosphoinositol, Steroidogenesis, Reproduction, Insect, Culicidae

CHARACTERIZATION OF THE MOSQUITO INSULIN RECEPTOR AND ITS SIGNAL TRANSDUCTION PATHWAY IN THE OVARIES OF THE MOSQUITO *Aedes aegypti*

by

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B.S., The University of Wisconsin, 1993

M.S., The University of Wisconsin, 1996

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy

Athens, Georgia

2002

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May 2002

DEDICATION

This work is dedicated to my wife, Kristin Riehle, whose unconditional support and love helped me complete this dissertation.

ACKNOWLEDGMENTS

First and foremost I must express my heartfelt thanks to my mentor Dr. Mark Brown whose enthusiasm for science has inspired me and whose support and guidance has led to this dissertation. I would also like to express my gratitude to the members of my committee, Dr. Donald Champagne, Dr. Joe Crim, Dr. Prema Narayan, and Dr. Judy Willis, who all provided instruction and support whenever it was needed. I would like to thank Steve Garczynski for being there to brainstorm new ideas with and Chun Cao and Genelle Grossman for teaching me a variety of new techniques. Special thanks goes to Dawn Stanek, Anna Majzlic, Stacie Thompson, Doug Sieglaff, and Dudley Thomas who supplied a constant stream of mosquitoes to meet their untimely fate and kept the lab running smoothly. Many thanks to Danny Fendley, whose skill with a pair of forceps is unsurpassed, for performing countless dissections and keeping everything running smoothly.

I would finally like to express my thanks to my friends and family who supported me throughout all these years of education. And most importantly I would like to thank Kristin Riehle, my wife, who has stood beside me, supported me, and kept me sane throughout this project.

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CHAPTER 1

INTRODUCTION

Mosquitoes, in addition to their nuisance factor, are efficient vectors of a wide range of pathogens that cause malaria, dengue, filariasis, encephalitis, and yellow fever, and result in millions of deaths each year. Traditional control methods consist of pesticide application and the removal or treatment of reproductive sites. Unfortunately, pesticide resistance is increasing and new formulations have not proven as effective. Furthermore, removing or treating the breeding sites of container breeding mosquitoes such as *Anopheles* is logistically impossible. Thus, it is becoming increasingly important to generate new and novel means of controlling mosquitoes. One promising avenue of vector control research is the manipulation or disruption of the reproductive cycle through hormonal analogs or compounds that interfere with normal reproduction. The work in this dissertation focuses on an insulin signaling cascade that regulates gonad steroid production that in turn, initiates the reproductive cycle of mosquitoes.

MOSQUITO REPRODUCTION

Previtellogenesis - Ovary and fat body development

Reproduction in the mosquito *Aedes aegypti* consists of alternating periods of development and arrest after the completion of oogenesis (74). This reproductive cycle begins immediately after adult emergence when the pair of undifferentiated, polytrophic ovaries, located in the posterior end of the abdomen, develops a set of 75 primary and secondary follicles that separate from the germarium. Each follicle consists of seven nurse cells and one oocyte surrounded by a monolayer of follicular epithelial cells (Fig.

1.1). During the first two to three days after adult emergence the primary follicle increases in size from approximately 50 μm to 100 μm in length.

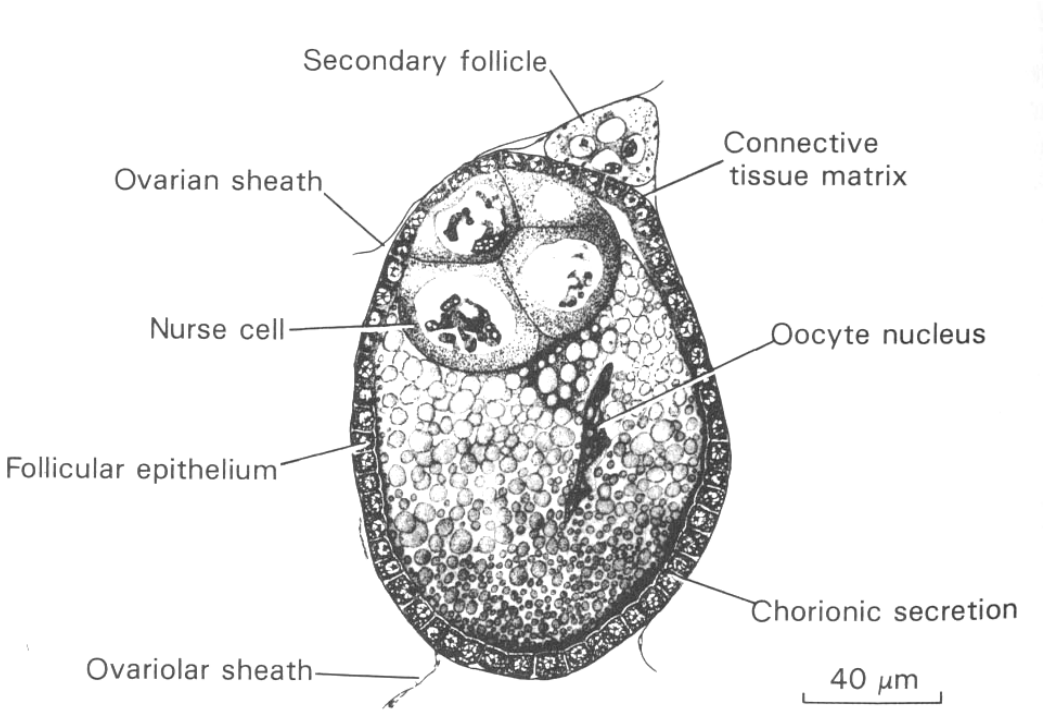

Figure 1.1: Single ovariole in the mosquito. Note the layer of cuboidal follicular epithelial cells surrounding the large primary follicle, the nurse cells attached to the developing oocyte, and the much smaller secondary follicle. Reprinted from The Biology of Mosquitoes Vol 1, 1992, page 330, A. N. Clements, Figure 17.3, with kind permission of Kluwer Academic Publishers.

The undiffentiated follicle cells increase in size from 3 to 5.5 μm in diameter, and increase in number from approximately 20 at emergence to over 250 when mitosis ends by the third day. At the same time, microvilli, clathrin coated pits, and endosomes form on the surface of the oocyte rendering it competent for yolk protein uptake.

The fat body found throughout the adult mosquito, but concentrated in the abdomen, is the primary metabolic tissue in the mosquito and during a reproductive cycle is responsible for yolk protein production. After adult eclosion, trophocytes in the fat body become polyploid in preparation for increased gene transcription during vitellogenesis. Total RNA levels in the fat body increase 50% during the first three days after emergence with the highest level of previtellogenic transcription occurring during the first two hours. The fat body also becomes responsive to 20-hydroxyecdysone during the first three days post-emergence. By three days after eclosion, the previtellogenic period of rapid ovary and fat body development ends, and the mosquito enters a state of reproductive arrest until a blood meal is consumed.

Vitellogenesis and Post-vitellogenesis

Consumption of a blood meal initiates a series of metabolically rigorous events leading to the production of an egg batch. Stretch receptors in the abdomen or components in the bloodmeal stimulate the release of hormones that initiate yolk protein synthesis or vitellogenesis (Fig 1.2). The ovaries are thought to produce a releasing factor that signals medial neurosecretory cells in the brain to produce and release ovary ecdysteroidogenic hormone I (OEH1) (11). This latter hormone is released from axons of medial neurosecretory cells extending out of the brain to the corpora cadiaca, a neurohemal organ associated with the aorta, for up to 12 h post blood meal (pbm). OEH1

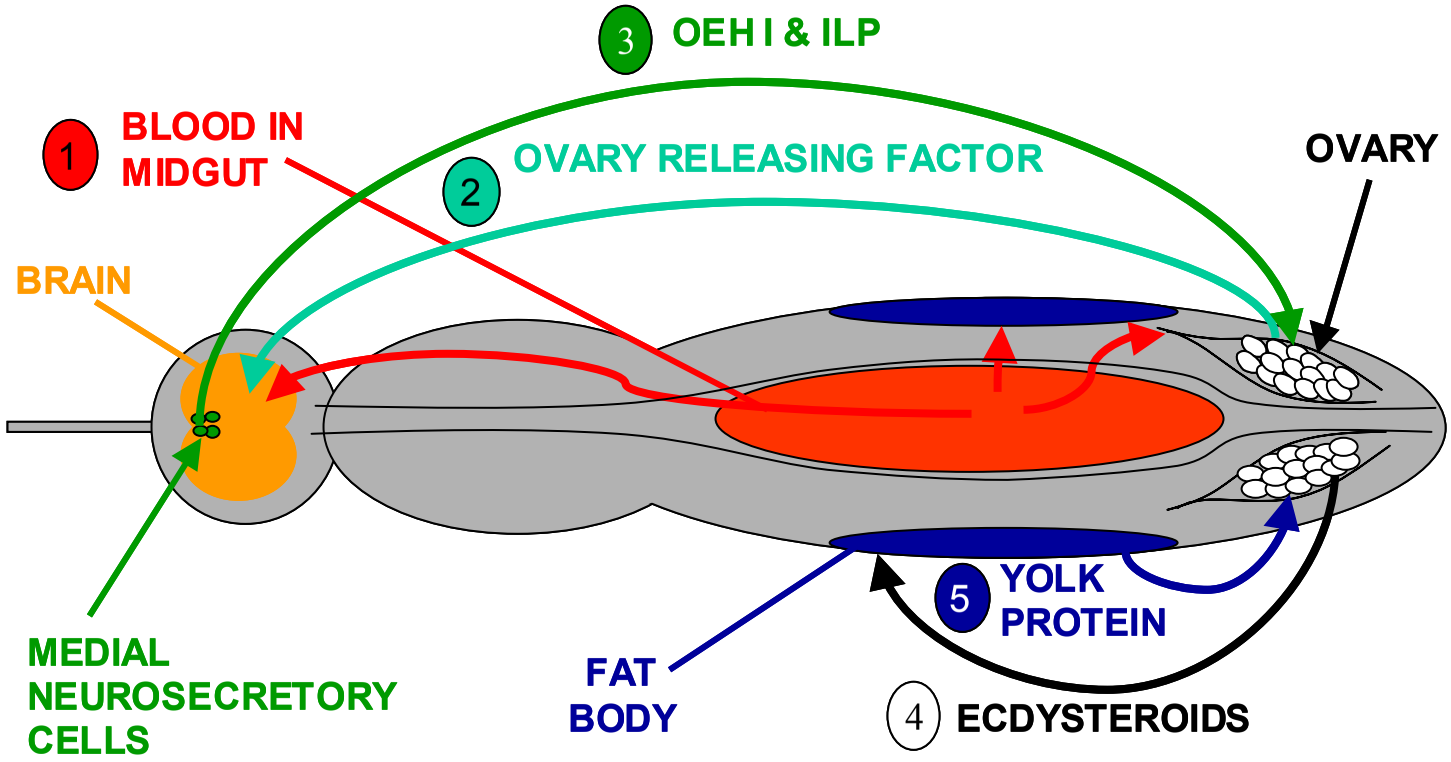

Figure 1.2: Sequence of hormonal regulation of egg production in the mosquito *Aedes aegypti*. (1) Signals from the ingestion of a bloodmeal prepare the brain, fat body, and ovary for egg production. (2) Ovaries produce a releasing factor, that stimulates (3) neurosecretory cells in the brain to release OEH and possibly an insulin-like peptide. OEH and ILP bind to MIR on the follicle cells initiating a signaling cascade leading to the production of ecdysteroids (4), which stimulate vitellogenin production in the fat body. The vitellogenin is then incorporated into the developing oocyte (5).

then initiates steroidogenesis in the follicle cells surrounding the oocyte (11, 32). Neither the OEH I receptor nor its downstream signaling components have been identified in these cells. Furthermore, the enzymes involved in the conversion of cholesterol to ecdysteroids by ovary follicle cells are largely unknown, although new genes in *Drosophila* termed *dare*, *disembodied*, and *spook* may be involved (14, 27). After a blood meal, mitosis resumes in the follicle cells surrounding the primary follicle, and their number increases from 200 to approximately 500 by ten hours pbm, after which mitosis again stops.

During this same period, the fat body undergoes numerous changes to prepare for the rigors of yolk protein synthesis (74). Ribosomal RNA production rapidly increases, and the rough endoplasmic reticulum and Golgi complexes proliferate to accommodate the synthesis of yolk protein precursor (YPP) synthesis within 30 min pbm. Ecdysteroids secreted by the ovary, bind to ecdysteroid receptors in the nucleus of the fat body cells and stimulate the secretion of YPP into the hemolymph. Hemolymph YPP titers rise sharply from 6 to 8 h pbm and remain high until approximately 32 h pbm. The YPPs pass through the basal lamina surrounding the egg chamber of the primary follicle, enter the perioocytic space formed as the follicle cells separate from the developing oocyte, and bind to the oocyte surface. Uptake of YPPs into the oocyte is accomplished through receptor-mediated endocytosis via clathrin coated pits. YPPs are taken into the developing oocyte for up to 30 h pbm resulting in an eightfold increase in oocyte length and a several hundred-fold increase in volume. By 36 h pbm, YPP uptake ends, and postvitellogenesis begins. During postvitellogenesis the follicle cells switch from ecdysteroid production to chorion deposition. Once chorion secretion around the oocyte

is completed, eggs are oviposited between 60 and 72 h pbm. In addition, ecdysteroids regulate the maturation of secondary follicles to the previtellogenic arrest stage (5). During the same time, the biosynthetic organelles in the fat body are degraded, and fat body cells return to previtellogenic arrest, preparing the mosquito for the next reproductive cycle.

As stated above, little is known about the OEH I receptor or its downstream signaling components. In two other invertebrate species, *Drosophila melanogaster* and *Caenorhabditis elegans*, orthologues of the insulin receptor and its signaling protein have been shown to have a wide range of physiological effects on reproduction and the regulation of brood size and follicle growth (95). Furthermore, an insulin receptor orthologue has been described in the follicle cells of mosquito ovaries and both bovine insulin and the distantly related endogenous hormone OEH I are capable of stimulating steroidogenesis (30). Thus, we hypothesized that steroid production in the mosquito ovary is regulated through an insulin signaling cascade.

THE INSULIN SIGNALING CASCADE

The insulin signaling cascade is one of the most studied hormone transduction systems in mammals and more recently in invertebrates, but many questions remain about its pleotropic effects, multiple branches, and cross talk with other pathways. In mammals, the insulin-signaling cascade is separated into two branches (Fig 1.3). The mitogenic Ras/mitogen-activated protein kinase (MAP kinase) cascade, branching from insulin receptor substrate (IRS), regulates many of insulin's mitogenic effects, including cell proliferation and growth (100). However it is not a major focus of this dissertation.

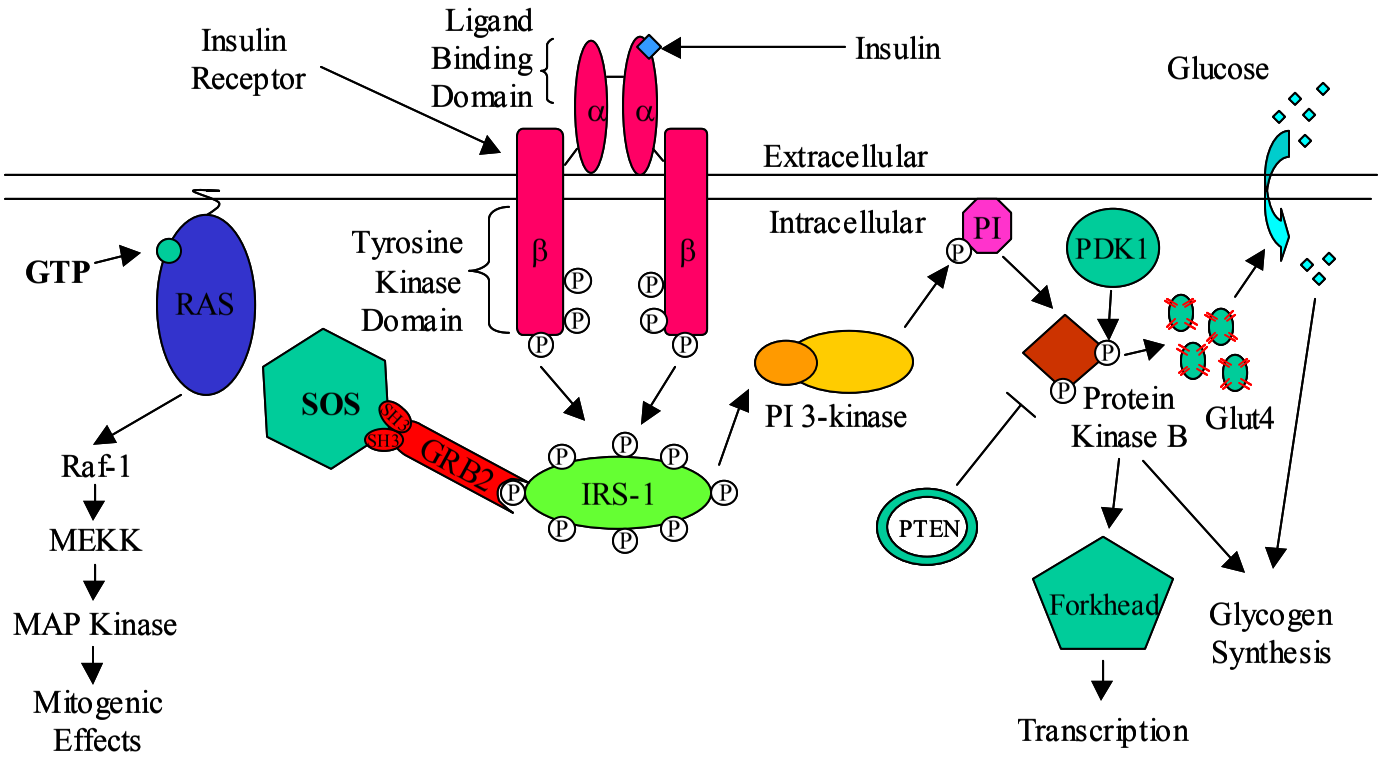

Figure 1.3: **The insulin signaling cascade in vertebrates.** The insulin signaling pathway forks at IRS-1 into a mitogenic Ras signaling cascade and a metabolic PI3K signaling cascade. Either cascade is activated by the binding of insulin to the extracellular α-subunit of the insulin receptor resulting in the autophosphorylation and activation of its β-subunit. The insulin receptor phosphorylates IRS-1 providing a binding site for PI3K and GRB2 that lead to the separate signaling cascades.

The individual components of the metabolic branch, including phosphoinositide 3-kinase (PI3-kinase), protein kinase B (PKB), phosphoinositide-dependant kinase 1 (PDK1), PTEN, and forkead transcription factors, in both mammals and invertebrates are examined in more detail below. Stimulation of this cascade in both mammals and invertebrates is initiated by the release of insulin or an insulin-like peptide.

The Insulin Family

Originally discovered by Banting and Best in 1922, insulin is a small, 6 kDa, peptide hormone that has been shown to regulate a wide range of physiological events in vertebrates. The production and release of insulin in most vertebrates occurs in the β cells of the islets of Langerhans found in the pancreas, although in some primitive vertebrates, such as hagfish, insulin is produced in the gut itself (86, 87). The insulin gene transcript in humans is translated as a prepropeptide consisting of a signal peptide, B-chain, C-peptide, and A-chain. The A and B chains are highly conserved among the vertebrates, differing only at amino acids 8 thru 10 of the A chain and the terminal amino acid of the B chain. In contrast the C peptide is highly variable even among closely related species. Processing of the prepropeptide begins with the proteolytic cleavage of the signal peptide in the rough endoplasmic reticulum resulting in proinsulin. Proinsulin is transported to the Golgi complex and packaged with endopeptidases in secretory granules where it is properly folded and the C-peptide is removed proteolitically. The mature insulin is stored in the secretory granules until released by the proper stimuli. In vertebrates, insulin can form dimers and hexamers with the help of a zinc molecule, but this is not thought to occur in invertebrates (9, 23). The C-peptide, previously thought to be biologically inert, has been shown to prevent or attenuate neural and vascular

dysfunction (38). However, the C-peptide has no known role in invertebrates. Other members of the insulin family in mammals include relaxin and the insulin-like growth factors (37, 73). Interestingly, the insulin-like growth factors in mammals do not possess a C peptide but do possess a C-terminal extension (Fig 1.4). However, the A and B chains of the IGFs are proteolytically cleaved.

Numerous insulin-like peptides have been described in invertebrates, often with multiple insulin-like genes and psuedogenes present. The first insulin-like peptide identified in an invertebrate was bombyxin in the silkmoth *Bombyx mori*, originally isolated for its prothoracicotropic activity in the moth *Samia cynthia ricini*, including stimulation of adult development via ecdysone production (59, 60). Preprobombyxin, like preproinsulin, consists of A and B chains connected by two disulfide bonds and a signal peptide and C-peptide that are proteolytically removed to generate the mature bombyxin. One difference between bombyxin and the vertebrate insulins is that bombyxin forms an alpha helix at the carboxy terminus of the B chain instead of a beta sheet making it unlikely to form dimers or hexamers (1). More than 32 bombyxin genes have since been described in *B. mori*, including some with in-frame stop codons that most likely are psuedogenes (44). Although a clear role for these peptides in *B. mori* has not been determined, some researchers suggest that these peptides may play a role in nutrient metabolism, including a hypotrehalosemic role in larvae in response to glucose (54, 75). In addition, adult males have significantly higher levels of bombyxin after eclosion compared to females suggesting a physiological role in male specific activities (76). Two other lepidopteran species, the sweet potato hornworm, *Agrius convoluvuli*, and the saturniid silkworm, *Samia cynthia ricini,* have also had insulin-like peptides described

Figure 1.4: An amino acid sequence comparison of several mammalian and invertebrate insulin like peptides. OEH I and the closely related protein neuroparsin, although not members of the the insulin superfamily, have many of the same cysteine residues in the correct position that are critical for insulin's tertiary structure. Furthermore, they share the basic Signal peptide, B-chain, C-peptide, A-chain structure as preproinsulin. However, the protease that removes the C-peptide using only a single Lys is unknown.

for them. In *Samia*, six copies of the insulin-like gene have been described, while *Agrius* has three (39, 43). In these three lepidoteran species, the insulin-like mRNA and mature peptide is produced and stored in medial neurosecretory cells in the brain as determined with *in situ* hybridization and immunocytochemistry respectively. None of these insulin-like genes in Lepidoptera contain introns.

In the mollusc *Lymnaea stagnalis*, Smit et al (82) identified a cDNA encoding a preproinsulin and termed it molluscan insulin-related peptide (MIP). Seven different MIPs were subsequently described (MIP I thru VII), five of which have the standard arrangement (MIP I, II, III, V, and VII) while the other two (MIP IV and VI) lack the A chain and may be psuedogenes (82). Interestingly, sequence identity between the 5 transcribed MIP genes is as low as 38% (B chain of III and VII), a sharp contrast with the vertebrate ILPs that vary by only a few amino acids. The mature MIPs are localized in the approximately 200 giant neuroendocrine cells or light green cells (LGCs), located in the cerebral ganglia of *Lymnaea*. The LGCs have a role in growth, glycogen breakdown, and shell development. The MIP genes all contain introns in contrast to lepidopteran ILPs.

In the locust *Locusta migratoria*, the first cDNA encoding an insulin like peptide from a non-lepidoteran insect was termed locust insulin related peptide (LIRP) (36, 46). Two LIRP mRNA transcripts were identified with differences in the 5' UTR, but they translate an identical peptide. The most striking feature of LIRP is the 48 amino acid C-peptide, approximately twice as long as the C-peptide of most invertebrates insulin-like peptides, which possesses a unique FRRR processing site at the C-terminus. As in other

invertebrates, LIRP is produced, processed, and stored in medial neurosecretory cells. Evidence suggests that the LIRP has a hypolipaemic role in *Locusta* (6).

The genome sequencing projects for both *C. elegans* and *D. melanogaster* have been completed, and numerous ILPs have been identified in both species. In *C. elegans*, ten peptides distantly related to the insulin superfamily have been described (41), however none of these peptides conform to the standard ILP structure. The *C. elegans* ILPs either do not possess a C-peptide or lack the dibasic processing sites for cleavage of the C-peptide, much like IGF in mammals. The ten peptides are separated into 3 families, α, β, and γ, based on their sequence similarities. The γ-family, with only a single member, has the most sequence identity to other invertebrate ILPs, including the expected number of cysteine residues with the proper spacing. However, a number of hydrophobic amino acids thought to be critical to the tertiary structure of the A and B chain are different. The six members of the β-family maintain more of the core hydrophobic residues, but possess one extra cysteine residue at the C-terminus of both the putative A and B chains, believed to form a third interchain disulfide bridge. The three members of the α-family share the least amount of sequence similarity to known ILPs. Like members of the β-family, they possess an extra cysteine residue at the carboxy teminus of both the A and B chain. However, a key cysteine residue, responsible for forming an intrachain disulfide bond, is missing in the putative A chain. While it is difficult to predict the disulfide bridge arrangement in this family, it is not likely to form a tertiary structure similar to other ILPs.

The *Drosophila* genome contains at least seven conserved ILPs, named *dilp*1 thru 7 (10). Unlike the ILPs in *C. elegans*, the *Drosophila* ILPs maintain most of the

structural characteristics of the insulin superfamily. All *Drosophila* ILPs possess the correct number of cysteine residues with the correct spacing and follow the standard structure. Furthermore, the peptides all possess putative processing sites for the cleavage and removal of the C-peptide. Three of the peptides (*dilp*2, 4, and 7) were expressed in the embryo and *dilp*2 thru 7 were expressed in the larvae (10). Larval expression of these peptides occurred in the brain (*dilp*2, 3, and 5), gut (*dilp*4, 5, and 6), imaginal discs (*dilp*2), salivary glands (*dilp*2), and ventral nerve cord (*dilp*7). An insulin-like peptide has not been described in *Aedes aegypti*, however the steroidogenic hormone OEH1 shares some sequence similarity, and a cysteine residue arrangement that is strikingly similar (Fig. 1.4)

Regardless of the organism they are produced in or the site of synthesis, members of the insulin family transduce their pleotropic effects across the membrane of the target cell by binding to a receptor.

Insulin Receptors

The insulin receptor is a transmembrane protein whose primary role is to transfer the insulin signal to the intracellular signaling cascade (Fig. 1.3). As a member of the receptor tyrosine kinase (RTK) family, the insulin receptor is able to phosphorylate tyrosine residues on itself and other proteins after activation by insulin binding. The insulin receptor consists of four subunits, two α subunits and two β subunits (96). The α and β subunits are translated as a single proreceptor and remain bound by disulfide bonds after cleavage at a tetrabasic processing site. After insertion into the membrane, two processed proreceptors dimerize through a disulfide bridge between the α subunits resulting in the mature tetrameric receptor. The α subunits possess a ligand binding

domain and are completely extracellular. The β subunits have an extracellular domain, a hydrophobic transmembrane domain, and an intracellular domain consisting of a kinase domain, regulatory domain, and seven tyrosines capable of being phosphorylated (TYPs). When insulin binds to the extracellular α subunits, the intracellular domains of the β subunits are autophosphorylated and the tyrosine kinase domain activated (47). Three closely spaced TYPs in the regulatory domain are critical for autophosphorylation (66). Phosphorylation of these three tyrosine residues results in a stable conformational change exposing the catalytic pocket and activating the receptor's kinase activity.

An insulin receptor-like receptor has been identified in the mosquito *Aedes aegypti* and was termed mosquito insulin receptor (MIR) (30). Like other members of the insulin receptor family it is transcribed as a proreceptor consisting of putative α and β subunits and a tetrabasic processing site. However the processing and dimerization events that occur after translation of MIR are unknown. The α subunit has a putative ligand binding domain from amino acids 61 to 160, and the β subunit's kinase domain extends from amino acids 1005 to 1287. Northern blot analysis detected two MIR transcripts approximately 7.5 and 8.5 kb in the ovary and, after long exposures, in the head and abdomen (30). Helbling and Graf, (35) localized the MIR transcript and protein to the follicle cells and nurse cells of the developing oocyte using in situ hybridization and immunohistochemisty.

Insulin receptor orthologues have been described in several other invertebrates. The insulin receptor in *C. elegans*, termed *daf-2*, was found through mutational analysis to regulate dauer arrest, longevity, and nutrient storage (22, 29, 42). As with other members of this family, *daf-2* is translated as a proreceptor with both an α and β subunit.

The proreceptor is processed, inserted into the membrane, and dimerizes to form the mature *daf-2* receptor. In contrast to the MIR and vertebrate insulin receptors, *daf-2* has a carboxy terminus extension of approximately 300 amino acids. The role of this extension is unknown but may be important for downstream signaling due to the presence of numerous phosphorylated tyrosine residues with SH2 binding motifs.

An insulin receptor orthologue named *Drosophila* insulin receptor (DIR) has been well characterized in *D. melanogaster* (15, 72). The DIR, like *daf-2*, possesses a carboxy terminus extension of approximately 400 amino acid residues with no known function. The C-terminus extension of DIR possesses three YXXM consensus binding sites to bind proteins with SH2 domains, and four NPXY phosphotyrosine-binding (PTB) sites. Earlier work suggested that the extension might be proteolytically removed and act as an insulin receptor substrate, however subsequent work has shown DIR to have an intact extension yet act through the IRS1 orthologue *Chico* (72, 101). Mutational and overexpression studies have demonstrated a role for DIR in growth, DNA synthesis, and apoptosis. Furthermore, like the daf-2 receptor, DIR has been shown to regulate longevity (17, 92).

Two other arthropods, the tobacco hornworm *Manduca sexta* (83) and the shrimp *Penaeus japonicus* (16), have had a putative insulin receptor orthologue identified. In *Manduca sexta*, a 178-kDa membrane protein was immunoreactive to a human insulin receptor antibody. When assayed for kinase activity, this putative β subunit became phosphorylated on tyrosine residues (83). The shrimp *P. japonicus* posesses a 79-kDa protein immunoreactive to an insulin receptor antibody that in the presence of bovine insulin becomes phosphorylated on tyrosine residues (16). This protein is also believed

to be the β subunit of an insulin receptor orthologue. However, the sequence of both proteins will need to be elucidated for final confirmation.

Insulin receptor orthologues in other invertebrates are not as well characterized, and their physiological roles have not been determined. The mollusk *Aplysia californica* possesses an insulin receptor with a structure similar to the mosquito and human insulin receptor (40), whereas the insulin receptor in the pond snail *Lymnaea stagnalis* possesses a carboxy terminus extension similar to that of *Drosophila* and *C. elegans* (70). Small orthologues to the insulin receptor were identified in four species of sponge and demonstrate the presence of these receptors in the earliest metazoans (81). With the insulin receptor activated by the appropriate insulin-like ligand, it can act on the next downstream signaling component insulin receptor substrate.

Insulin Receptor Substrates

Activation of the insulin receptor in mammals results in the phosphorylation of the insulin receptor substrate (IRS) family, with four members identified in humans (IRS 1-4) (58). These closely related 130 kDa proteins are highly phosphorylated, act as a major branching point for the signaling cascade, and amplify the insulin signal in the cell. The insulin receptor phosphorylates members of the IRS family on some or all of the 18 to 21 potential tyrosine phosphorylation sites. Eight confirmed tyrosine phosphorylation sites consist of a motif (YXXM and in particular YMXM) capable of binding proteins with an SH2 domain. In addition to the tyrosine phosphorylation sites, the IRS proteins possess over 30 putative Ser/Thr phosphorylation sites (88, 89). The reason for this heavy Ser/Thr phosphorylation is unknown, but it may prevent efficient binding of IRS to the insulin receptor and interfere with SH2 binding of downstream signaling components

(57). In fact, evidence suggests that protein kinase B (PKB), a downstream signaling protein in the insulin signaling cascade, can phosphorylate IRS resulting in a negative feedback loop after prolonged insulin stimulation (51).

It is not known whether the four IRS proteins have redundant or unique roles in insulin signaling although all four can be phosphorylated by the insulin receptor. The subcellular localization and tissue distribution of the IRS proteins suggest specific physiological functions. IRS 1 and 2 appear to be localized near organelles, and are widely distributed in various tissues. IRS 1 appears to have a dominant role in muscle and adipose tissue, whereas IRS 2 is dominant in the liver. In contrast, IRS 3 and 4 tend to be located primarily at the plasma membrane with IRS 3 playing an important role in adipocytes and IRS 4 playing an important role in the kidney. Targeted disruption of IRS 1 or 2 in mice results in retarded growth and insulin resistance, but is not lethal suggesting partial compensation by another IRS. Interestingly, targeted disruption of either IRS 3 or 4 results in either no phenotype or only mild defects.

An IRS orthologue has been identified in only two invertebrates and is characterized best in *D. melanogaster*. The *Drosophila* IRS, named *Chico*, has been shown to have both mitogenic and metabolic roles in *Drosophila*, consistent with the idea that IRS is a key branching point in the insulin-signaling cascade. Loss of *Chico* function results in a reduction of overall body size. This reduction was shown to affect body parts autonomously through a series of elegant experiments by Bohni, et al. (8). Deletion of the *Chico* gene in the eye imaginal disc, which gives rise to the compound eyes and head but not the proboscis, resulted in adults with strongly reduced heads and eyes, but a normal proboscis. The *Chico* gene is also important in regulating lipid levels in adult

male flies (8). Although of a smaller size than wild type males, *Chico*$^{-}$ males stored nearly twice as much lipid per milligram of fresh weight. This increase in lipid levels was not observed in *Chico*$^{-}$ females, and there were no apparent differences in protein or glycogen levels between *Chico*$^{-}$ and wild type flies. The loss of *Chico* also extended adult lifespan by up to 48% in homozygotes and 36% in heterozygotes, again indicating that the insulin signaling cascade plays an important role in regulating lifespan (17).

The IRS orthologue in *C. elegans* was elucidated from the genome but has not been well studied (Wolkow, C., Hu, P., Tewari, M., Vidal, M. and Ruvkun, G., unpublished). The putative *C. elegans* IRS is not closely related to other members of this family, with 36% sequence similarity in the SH2 and PTB binding domains and no significant similarity outside of these regions. Surprisingly, while the *C. elegans* IRS possesses numerous tyrosine residues, they do not appear to follow the consensus SH2 or PTB binding motifs.

Phosphorylation of mammalian IRS, and most likely invertebrate IRS orthologues, on tyrosine residues provides a binding site for phosphoinositide 3-kinase, the next important signaling protein in the insulin-signaling cascade.

Phosphoinositide 3-kinase

Phosphoinositide 3-kinase (PI3K) is the first protein in the metabolic branch of the insulin signaling cascade. The role of the PI3K family is to phosphorylate insositol lipids at the D3 position of the inositol ring (49, 67, 97). Three classes of PI3K, varying in their physiological role and substrate specificity, have been described in mammals.

Class 1 PI3Ks in mammals regulate many of insulin's metabolic effects and are composed of two subunits, an 85 kDa regulatory subunit named p85 and a 110 kDa

catalytic subunit named p110. Phosphorylated tyrosine residues on IRS act as docking sites for the SH2 domain on the regulatory subunit. Binding of the p85 regulatory subunit releases the p110 catalytic subunit, activating its kinase activity and allowing it to translocate to the cell membrane. At the cell membrane it phosphorylates phosphatidylinositol (PtdIns), PtdIns(4)*P*, or PtdIns(4,5)P_2 at the third position creating PtdIns(3)*P*, PtdIns(3,4)P_2, or PtdIns(3,4,5)P_3 respectively and provides a binding site for numerous downstream components of the metabolic signaling pathway.

Class 1 PI3Ks have been described for a number of non-vertebrate organisms including *C. elegans*, *D. melanogaster*, *Dictystelium*, yeast, and soybean. As with other components of this signaling cascade they have been best characterized in the two model invertebrates *D. melanogaster* and *C. elegans*. The *C. elegans* p110 subunit, *age*-1, was the first gene found to regulate longevity. One mutation in *age*-1 results in a two-fold increase in *C. elegans* lifespan, dauer arrest, and fat accumulation (63). The a*ge*-1 protein contains the expected domains of the PI3K catalytic subunit, including a kinase domain and a p85 binding domain. Ruvkun et al. have submitted the sequence of a putative regulatory subunit named aap-1 (unpublished Genbank submission, AAF28335), a protein similar to a previously described regulatory subunit named p55 (20).

In *Drosophila*, the catalytic subunit of the Class 1 PI3K has been termed Dp110 (50, 52, 56), and the regulatory subunit is a 60 kDa protein that immunoprecipitates with Dp110 antibodies (99). As with DIR and *chico*, Dp110 is involved in the regulation of growth and body size. Over expression of Dp110 in eye and wing imaginal discs results in enlargement of the respective tissues (50). In contrast, expression of a nonfunctional Dp110 results in reduced wings and eyes. Although it is believed the insulin signaling

cascade, and thus PI3K, is involved in regulating adult lifespan in *Drosophila*, no evidence yet exists for the involvement of Dp110 in this role.

Members of the second class of PI3Ks, including PI3K_68D in humans, have not been well characterized. They have an N-terminal extension with several novel motifs but interactions with other proteins are unknown. They are capable of phosphorylating both PtdIns and PtdIns(4)*P* in vitro. Members of the final class of PI3Ks, class 3, are orthologues of the *S. cerevisiae* protein Vps34. Vps34 was originally identified through defective sorting and delivery of hydrolases between the Golgi complex and vacuoles. Thus the primary role of the class appears to be the regulation of protein trafficking events. The Class 3 PI3Ks have been shown to specifically phosphorylate PtdIns only, creating PtdIns(3)*P*. Both Class 2 and 3 PI3Ks have been described in *D. melanogaster* and *C. elegans* (53, 93).

Phosphorylation of PtdIns(4,5)P_2 creates a new binding site at the cell membrane for several proteins with pleckstrin homology domains including the next signaling component of the metabolic branch, protein kinase B.

Protein Kinase B

Protein kinase B (PKB), also termed akt or Rac, is a downstream target of PI3K. The phosphorylation of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 by PI3K provides a membrane binding site for the pleckstrin homology (PH) domain of PKBα (24, 26). Phosphatidylinositol dependent kinase 1 (PDK1), also associated with the cell membrane through binding of its PH domain to PtdIns(3,4)P_2, PtdIns(3,4,5)P_3, or PtdIns(4,5)P_2, phosphorylates the Thr^{308} residue of PKB (77). This results in a conformational change thought to allow ATP and substrate access to the kinase domain (2, 3). However,

phosphorylation of a second amino acid residue, Ser^{473}, is required for full activation of mammalian PKBs (78). Ser^{473} may be phosphorylated by the kinase activity of PKB or by a different protein tentatively called Phosphatidylinositol dependent kinase 2 (77).

The activated PKB is a nexus for the metabolic branch of the insulin signaling cascade and regulates numerous downstream components (Fig. 1.3). It is known to phosphorylate and inactivate glycogen synthase kinase 3 (GSK3) resulting in increased glycogen synthesis (18). It is also thought to regulate the translocation of the GLUT4 glucose transporter from intracellular vesicles to the cell surface for transport of glucose into the cell (33). PKB has been shown to phosphorylate members of the forkhead transcription family, including FKHR and AFX, preventing their translocation into the nucleus and thus their transcriptional activity (7, 12, 31, 68, 90). Another regulator of transcription, $p70^{s6k}$ has been shown to be negatively regulated by PKB (65). Furthermore, PKB has been shown to promote cell survival in vertebrates and *C. elegans* by inhibiting the apoptotic effects of BAD, caspase-9, and forkhead transcription factors (13, 19, 48). Finally, PKB may be able to down regulate the insulin signaling cascade during periods of prolonged stimulation by phosphorylating IRS on ser/thr residues (51).

PKB is known for only two invertebrate species, *D. melanogaster* and *C. elegans*. In *Drosophila* a single copy of the PKB gene, named Dakt1, has been found, however two proteins of 66 kDa and 86 kDa are translated from the gene due to multiple transcriptional start sites (4, 25). Dakt1, like other members of the insulin signaling cascade in *Drosophila*, regulates cell and organ size in an autonomous manner (98). In flies with a mutation in Dakt1, cells improperly undergo apoptosis during embryogenesis

(85). However, the regulation of apoptosis by Dakt1 is independent of the known apoptotic genes, *reaper*, *grim*, and *hid*.

In the genome of *C. elegans*, two PKB genes, termed Akt-1 and Akt-2, have been identified (63). An activating mutation or overexpression of Akt-1 in *age*-1 and *daf-2* mutants results in a shift from dauer formation to L4 and adult development. However, this shift is only partial in *daf-2* mutants suggesting a second signaling cascade for dauer formation (63). Both Akt-1 and 2 suppress the forkhead transcription factor *daf*-16 and prevent dauer arrest (63). However Akt-1 is more biologically active due to the presence of two phosphorylation sites (Thr-308 and Ser-473) compared to only one site (Thr-308) on Akt-2 (63).

Insulin, the insulin receptor, IRS, PI3K, and PKB are the best characterized components of the metabolic branch of the insulin signaling cascade. However, numerous proteins have been identified that act on or are acted upon by this cascade, and it is likely that more will be found. Several additional regulators and targets of this cascade are described below.

Other important molecules: forkhead, target of rapamycin, and PTEN.

Numerous proteins regulate insulin signaling in mammals at various points in the cascade. Some of these proteins have been identified in both *Drosophila* and *C. elegans*, including members of the forkhead family of transcription factors, target of rapamycin (TOR), and the Ser/Thr phosphatase PTEN. Although studies have shown that all of these proteins are critical components of the insulin signaling cascade in these two invertebrates, they have yet to be identified in *Ae. aegypti* or any other insect.

Downstream of PKB, the forkhead family of transcription factors is thought to be involved in many of insulin's metabolic effects. In mammals, the insulin signaling cascade regulates members of the forkhead family by inhibiting nuclear import or initiating nuclear export, preventing their transcriptional activity (45). This is accomplished through the phosphorylation of one or more residues on the transcription factor by PKB. On the human forkhead transcription factor FKHR, phosphorylation occurs on three phosphoacceptor sites in vitro. Replacement of these phosphorylated amino acids with alanine results in increased transcriptional activity and resistance to PKB inactivation (91). A second forkhead transcription factor in humans, AFX, has also been shown to be directly inhibited by PKB phosphorylation (45).

The *C. elegans* forkhead transcription factor, *daf*-16, also appears to be negatively regulated by the insulin signaling cascade and PKB in particular (48). Mutations in *daf*-16 can completely suppress dauer formation in stressed larvae. Furthermore, *daf*-16 mutations can prevent the metabolic shift, dauer formation, and increased longevity of worms with mutations or deletions of *daf*-2, *age*-1, and akt 1 and 2. *daf*-16 appears to be constitutively active and is only suppressed by akt phosphorylation. Although forkhead transcription factors have been identified in *Drosophila* and shown to regulate a variety of developmental aspects, a forkhead transcription factor regulated by the insulin signaling pathway has not yet been identified.

A second protein involved in the insulin signaling cascade of both mammals and invertebrates is target of rapamycin (TOR). TOR proteins, also known as mTOR in mammals, respond to growth factors and amino acids and are key regulators of growth in yeast, mammals, and *Drosophila*. mTOR stimulates protein translation through the

activation of p70 S6 kinase ($p70^{S6k}$) and the inactivation of the translational inhibitor 4E-BP1 (34, 94). Some mammalian studies have demonstrated that insulin stimulates the phosphorylation and activation of mTOR, and even that mTOR is a direct substrate of PKB (79). However, mutations in the putative PKB phosphorylation site on mTOR failed to inhibit its kinase activity, and overexpression of PKB resulted in only minor or no stimulation of mTOR (64, 80). New studies suggest that *Drosophila* TOR (dTOR) works in parallel with the PI3K branch of the insulin signaling cascade to regulate cell growth, and both are required for the phosphorylation and activation of $p70^{S6k}$ (62). Mutations in dTOR resulted in smaller larvae and reduced growth in the wing (10, 62, 102), phenotypes similar to those observed in flies with mutations in other components of the insulin signaling cascade. Furthermore, overexpression of $p70^{S6k}$ can rescue dTOR mutants and restore normal growth (102).

A third important protein regulating insulin signaling in both mammals and invertebrates is PTEN. PTEN is a phospholipid phosphatase whose primary role is the dephosphorylation of PtdIns. Originally identified as a tumor suppressor, it was found to regulate cell cycle arrest and apoptosis. Mice homozygously deficient for PTEN did not survive beyond day 9.5 of embryogenesis due to incorrect patterning and overexpression of various cell types, and heterozygous mice were prone to tumor development in multiple tissues and reduced apoptosis in B cells, macrophages, and T lymphocytes (21, 84).

The role of PTEN in the insulin signaling cascade was first elucidated in *C. elegans* (55). Mutations inactivating *daf*-18, the *C. elegans* PTEN orthologue, were found to prevent dauer formation under nutrient limiting conditions, and suppress the

age-1 and *daf*-2 phenotypes. It was also shown that inhibition of *akt* 1 and 2 by RNAi still resulted in dauer formation in both wild type and *daf* -18 mutants demonstrating that *daf* -18 operates upstream of Akt 1 and 2 in the insulin signaling pathway (61, 71). The *Drosophila* PTEN orthologue, dPTEN, was also found to have a role in the insulin signaling cascade (28). Flies with a homozygous mutation in PTEN displayed the same phenotype as flies over expressing dp110, specifically excessive growth in both the eye and wing. Over expression of dPTEN can completely suppress the increased growth seen in flies over expressing dp110. Furthermore, mutations of dPTEN in *Chico*$^{-}$ mutants results in a rescue of the reduced eye phenotype. As in *C. elegans*, overexpression of Dakt1, was able to suppress the dPTEN overexpression phenotype, again suggesting that PTEN acts upstream of PKB.

Objectives

As described above, much is known about the reproductive cycle or oogenesis in the mosquito *Aedes aegypti*. However, large gaps remain, including our understanding of steroid production in the ovary and the hormones that regulate this process. Understanding this complex pathway may provide novel targets for control not only for mosquitoes but for other insects as well. In another fly, *Drosophila melanogaster*, reproduction, metabolism, and longevity appear to be governed by an insulin signaling pathway. In *Ae. aegypti*, an insulin receptor orthologue was identified in the ovary and the exogenous hormone, bovine insulin, was capable of stimulating steroid production in vitro. This suggests that steroidogenesis in the mosquito may be regulated by an insulin signaling cascade. It was my interest to elucidate the signaling cascade regulating

steroidogenesis in the mosquito ovary and to identify and characterize the individual signaling components. To this end seven objectives were set.

1. Elucidation of the steroidogenic signaling cascade in the ovaries of the mosquito *Ae. aegypti*. Inhibitors and activators of key components in the insulin signaling cascade, including the insulin receptor, MAPK, PI3K, and PKB were tested for their ability to stimulate or inhibit steroid production in the mosquito ovaries in vitro. This work demonstrated that steroid production in the mosquito ovary was regulated through the metabolic branch of the insulin signaling cascade.

   The results of studies from Objective 1 are presented in Chapter Two of this dissertation in manuscript format and were published in Insect Biochemistry and Molecular Biology (69).

2. Structural characterization of the mosquito insulin receptor. The primary structure of an insulin proreceptor orthologue in *Ae. aegypti* had previously been described. However, it could not be determined if the proreceptor was proteolytically cleaved into an α and β subunit or whether the subunits dimerized to form the typical tetrameric structure of the mature insulin receptor. Protein extracts from mosquito were size fractionated under reducing and non-reducing conditions to determine the size and subunit configuration of the native receptor. It was determined that the MIR proreceptor was processed into an α and β subunit that subsequently dimerized with a second processed proreceptor to form the mature MIR.

3. Characterization of MIR expression during development and a reproduction cycle. The expression pattern of the MIR transcript and protein during immature development, previtellogenesis, vitellogenesis, and postvitellogenesis was examined using northern blot analysis, immunoblot analysis, and immunocytochemistry. The MIR is expressed in the adult mosquito from the beginning of previtellogenesis shortly after eclosion, until the end of vitellogensis. During postvitellogenesis, when the follicle cells switch from steroid production to chorion production, the MIR protein is not expressed. The MIR transcript and protein were not detected in the embryo, larval, or pupal stages.
4. Tyrosine phosphorylation of MIR by bovine insulin. Protein extracts from ovaries incubated with bovine insulin or pervanadate were immunoblotted and incubated with antiphosphotyrosine antibodies to determine if the MIR became tyrosine phosphorylated. Bovine insulin stimulation resulted in a 4- to 5-fold increase in MIR tyrosine phosphorylation compared with the saline control, and pervanadate resulted in a 2-fold increase, suggesting that the MIR is the receptor regulating steroidogenesis.

The results of studies from Objectives 2 to 4 are presented in Chapter Three of this dissertation in manuscript format and will be published in Cell and Tissue Research

5. Identification of the cDNA encoding a protein kinase B in *Ae. aegypti.* Degenerate primers were designed against conserved regions of the PKB family and used to amplify a fragment by PCR of the mosquito PKB.

6. Characterization of MPKB expression during development and a reproduction cycle. Northern blot analysis was used to determine the presence of the MPKB transcript during early embryogenesis and in the adult ovary throughout a reproductive cycle. Immunocytochemistry was used to localize the MPKB protein in the cytosol of the follicle cells and immunoblot analysis was used to determine the presence of MPKB protein late in the reproductive cycle.

   The results of studies from Objective 5 and 6 are presented in Chapter Four of this dissertation and will be submitted to Insect Molecular Biology.

7. Identification and partial characterization of two PI3K's in *Ae. aegypti.* Degenerate primers were designed against conserved regions of the PI3K family and used in PCR to amplify a fragment of a Class 1 and 3 PI3K in the mosquito. A whole body cDNA library was screened and RACE on the 5' and 3' performed to isolate full length clones of each gene, but the 5' end of each PI3K has not yet been determined. Northern blot analysis identified the Class 1 PI3K in a wide range of adult tissues including ovary throughout a reproductive cycle, the head, and the abdominal body wall.

   The results of studies from Objective 7 are presented in Chapter Five of this dissertation.

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CHAPTER 2

INSULIN STIMULATES ECDYSTEROID PRODUCTION THROUGH A CONSERVED SIGNALING CASCADE IN THE MOSQUITO *Aedes aegypti*[1]

[1]Riehle, M.A. and Brown, M.R. 1999. Insect Biochemistry and Molecular Biology. 29:855-860. Reprinted here with permission of the publisher.

Abstract

Selective activators and inhibitors of signaling cascades for insulin action in mammalian cells were tested for effects on insulin stimulation of ecdysteroid production by ovaries of the mosquito *Aedes aegypti*. Bovine insulin in the concentration range of 1.7 μM to 85 μM stimulated ecdysteroidogenesis *in vitro*. Pervanadate, an inhibitor of tyrosine kinase phosphatase, stimulated ecdysteroid production at concentrations of 250 μM to 1 mM. Okidaic acid, a serine/threonine phosphatase inhibitor, stimulated steroidogenesis with an ED_{50} of 77.39 nM. A selective inhibitor of tyrosine kinase activity, HNMPA-(AM_3), inhibited ecdysteroid production with an IC_{50} of 14.2 μM. Two selective inhibitors of phosphatidylinositol 3-kinase, wortmannin and LY294002, inhibited ecdysteroid production at low concentrations (IC_{50} = 1.6 nM and 30 nM, respectively). These concentrations are similar to those inhibiting insulin action in mammalian cells. A selective inhibitor of mitogen-activated protein kinase, PD098059, had no effect on ecdysteroid production even up to 100 μM. Thus, insulin stimulation of ecdysteroid production by ovaries *in vitro* appears to be controlled by the tyrosine kinase activity of the mosquito insulin receptor and the signaling cascade involving phosphatidylinositol 3-kinase and protein kinase B.

Keywords: Insect, Diptera, Phosphatidylinositol 3-kinase, Protein kinase B, Insulin receptor, Tyrosine kinase.

1. Introduction

Homologues of vertebrate insulin receptors have been characterized from the fruit fly, *Drosophila melanogaster* (Fernandez *et al*., 1995), the silkworm, *Bombyx mori* (Fullbright *et al*., 1997), and the mosquito, *Aedes aegypti* (Graf *et al*., 1997). The mosquito insulin receptor cDNA was cloned, expressed, and characterized as a proreceptor (200 kDa) consisting of an α subunit with a putative insulin binding domain, a β subunit with a tyrosine kinase domain, and a putative processing site (Graf *et al*., 1997). Similar domains exist in the insulin proreceptor of vertebrates (200 kDa), which is proteolytically cleaved into α and β subunits (135 kDa and 95 kDa, respectively). Two α subunits and two β subunits are covalently bound through disulfide bridges to form the mature receptor. The mosquito insulin receptor has been localized in follicle cells surrounding the ovaries and in the nurse cells by immunocytochemistry and *in situ* hybridization (Helbling and Graf, 1998).

The proreceptor of the *Drosophila* insulin receptor is proteolytically cleaved into an α subunit (120 kDa) and a β subunit (170 kDa), with a 300 amino acid extension on the carboxy terminus. This extension may play a role in signal transduction similar to that of insulin receptor substrate 1 (IRS1) in vertebrates (Fernandez *et al*., 1995). Insulin receptors of vertebrates and mosquitoes do not have comparable extensions on the carboxy terminus, but other members of the receptor tyrosine kinase family, which bind epidermal growth factor and platelet derived growth factor, possess similar extensions. Although the *Drosophila* insulin receptor has been shown to bind mammalian insulin specifically (Fernandez-Almonacid and Rosen, 1987), endogenous ligands for the insulin receptors in either *Drosophila* or *Aedes aegypti* have not been identified to date.

An insulin-like peptide, bombyxin, was originally isolated from the silkworm, *Bombyx mori*, based on its "molting hormone"-like activity, and it stimulates ecdysone production by the prothoracic glands of another silkworm, *Samia cynthia ricini* (Yoshida *et al*., 1998). Bombyxin also has been shown to affect the metabolism of carbohydrate stores in silkworms (Satake *et al*., 1997). At least 32 bombyxin genes, classified into seven families (A-G), have been identified from *Bombyx mori* (Yoshida *et al*., 1998). Binding of bombyxin to ovarian receptors has been demonstrated for three different species of Lepidoptera (Fullbright *et al*., 1997). The bombyxin receptor has a mass of approximately 300 kDa which, when reduced, forms two distinct bands of 90 kDa and 116 kDa (Fullbright *et al*., 1997). In the tobacco hornworm, *Manduca sexta*, a 178 kDa protein immunoreactive to anti-phosphotyrosine and human insulin receptor antisera has been identified in the prothoracic glands, fat body, and muscle of larvae (Smith *et al*., 1997).

In vertebrates, the pleiotropic effects of insulin are controlled, in part, by the phosphatidylinositol (PI) 3-kinase and Ras/mitogen activated protein (MAP) kinase cascades (Avruch, 1998). The tyrosine kinase activity of the insulin receptor and the subsequent phosphorylation of IRS-1 stimulate these two pathways. A variety of agonists and antagonists have known effects on each of these pathways. Vanadium compounds and okidaic acid have been shown to stimulate the insulin receptor and protein kinase B, respectively, and mimic many of the effects of insulin (Molero *et al*., 1998; Tanti *et al*., 1997). The molecule, (hydroxy-2-naphthalenyl-methyl) phosphonic acid trisacetoxymethyl ester (HNMPA-$(AM)_3$), inhibits the tyrosine kinase activity of the insulin receptor (Saperstein *et al*., 1989). The PI 3-kinase pathway is inhibited by the

fungal metabolite, wortmannin (Okada *et al*., 1994), and the compound 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) (Vlahos *et al*., 1995). An inhibitor of MAP kinase kinase (MEK), PD098059, is capable of inhibiting the Ras/MAP kinase cascade (Avruch, 1998).

A direct stimulatory effect of bovine insulin on ecdysteroid production and protein synthesis of ovaries has been demonstrated *in vitro* for *Aedes aegypti* (Graf *et al*., 1997). Using activators and inhibitors of critical steps in the signaling pathways of the model insulin receptor for vertebrates, we investigated the signaling pathways stimulated by bovine insulin to initiate ecdysteroid production by the ovaries of *Aedes aegypti*.

2. Materials and Methods

Mosquitoes were maintained at 27° C in a 16L/8D photoperiod, and larvae were fed ground rat chow/lactalbumin/brewers yeast (1:1:1). Adults fed at will on a 10% sucrose solution via wick for 2 days, and then, were given only distilled water. For the following assays, three to five day old female mosquitoes were immobilized on ice, and ovaries were dissected in a saline solution (128 mM NaCl, 4.7 mM KCl, and 1.9 mM $CaCl_2$).

The bioassay for ecdysteroid production by ovaries *in vitro* (Brown *et al*., 1998) was modified as follows. Four pairs of ovaries were incubated in 60 µl of medium (139 mM NaCl, 4.05 mM KCl, 1.85 mM $CaCl_2$, 12.5 mM HEPES, 2.5 mM trehalose, 0.3 mM $MgCl_2$, and 0.9 mM $NaHCO_3$; pH 6.5, adjusted with NaOH) for 6 hours in the caps of 1.5 ml polypropylene microcentrifuge tubes. The caps were placed in moistened, 24 well microtitre plates and gently shaken in a water bath at 30^{o} C. Fifty µl of the medium were

analyzed for ecdysteroid content using a radioimmunoassay (RIA) with an ecdysteroid antiserum at a 1:9000 final concentration.

Bovine insulin (Sigma) was prepared as a 1 mM stock solution in medium and serially diluted concentrations (170 μM - 0.17 μM) were tested, as described above. Stock solutions of inhibitors were prepared in ethanol: PD098059 (20 mM, Calbiochem), Wortmannin (2 mM, Sigma), LY294002 (2 mM, Sigma), and HNMPA-(AM_3) (50 nM, Calbiochem). Inhibitors were stored at -80° C and diluted serially in medium immediately before use. Ovaries were tested for ecdysteroid production in the presence of bovine insulin (17 μM) and various concentrations of the four inhibitors. Each dose of the test substance was performed in triplicate and replicated three to seven times. For negative controls, ovaries were incubated in medium alone or in 1.6% EtOH both with and without bovine insulin. Stock solutions of 20 μM okidaic acid (Sigma) and 50 mM sodium ortho-vanadate (Sigma) were prepared in medium. Pervanadate was synthesized by incubating sodium ortho-vanadate with equimolar amounts of H_2O_2 at room temperature for 15 minutes. A serial dilution of pervanadate (2 mM to 125 μM) and okidaic acid (250 nM to 19nM) in medium only were tested for ecdysteroid production.

Statistical analysis, using the SAS statistical software package (SAS institute), was performed on the treatments using the general linear model with analysis of variance (ANOVA). Treatment means were compared using least square means, Bonferroni, and Tukey's studentized range test. Means were considered significantly different if $p < 0.05$. The ID_{50} values and dose-response curves of okidaic acid, HNMPA-(AM_3), wortmannin, and LY294002 were determined by nonlinear regression using a sigmoidal dose-response equation. These values were calculated using the Prism program (GraphPad).

3. Results and Discussion

Bovine insulin stimulates ecdysteroid production by the ovaries of *Aedes aegypti* in a dose dependant manner *in vitro*. A dose-response curve comparing insulin concentration to ecdysteroid production in ovaries demonstrated that a concentration of bovine insulin as low as 1.7 μM could significantly increase ecdysteroid production compared to control ovaries in medium only (Fig. 2.1). Maximum ecdysteroid production was achieved in the presence of 17 μM insulin, the concentration used for subsequent experiments with inhibitors. The highest concentration of insulin tested (170 μM) showed no stimulation of ecdysteroid production. Studies with mammalian cells typically use insulin concentrations between 10 nM and 100 nM for optimal activity. Considering bovine insulin is a distantly heterologous ligand, it is surprising that the concentration required to stimulate ecdysteroid production in *Aedes aegypti* is less than twenty fold higher than those used to stimulate physiological responses in mammalian cells.

In mammalian cells, the binding of insulin to the vertebrate insulin receptor triggers a cascade of events beginning with the autophosphorylation of the β subunit and the subsequent activation of the tyrosine kinase domain. Pervanadate, a tyrosine phosphatase inhibitor, stimulates the insulin signaling cascade in vertebrates by preventing the constitutive dephosphorylation of the insulin receptor. Pervanadate stimulated steroidogenesis in *A. aegypti* ovaries at concentrations as low as 250 μM with the greatest stimulation occurring at 500 μM (Fig. 2.2A). However, ecdysteroid production was approximately 50% less than the insulin control. HNMPA-$(AM)_3$ is a

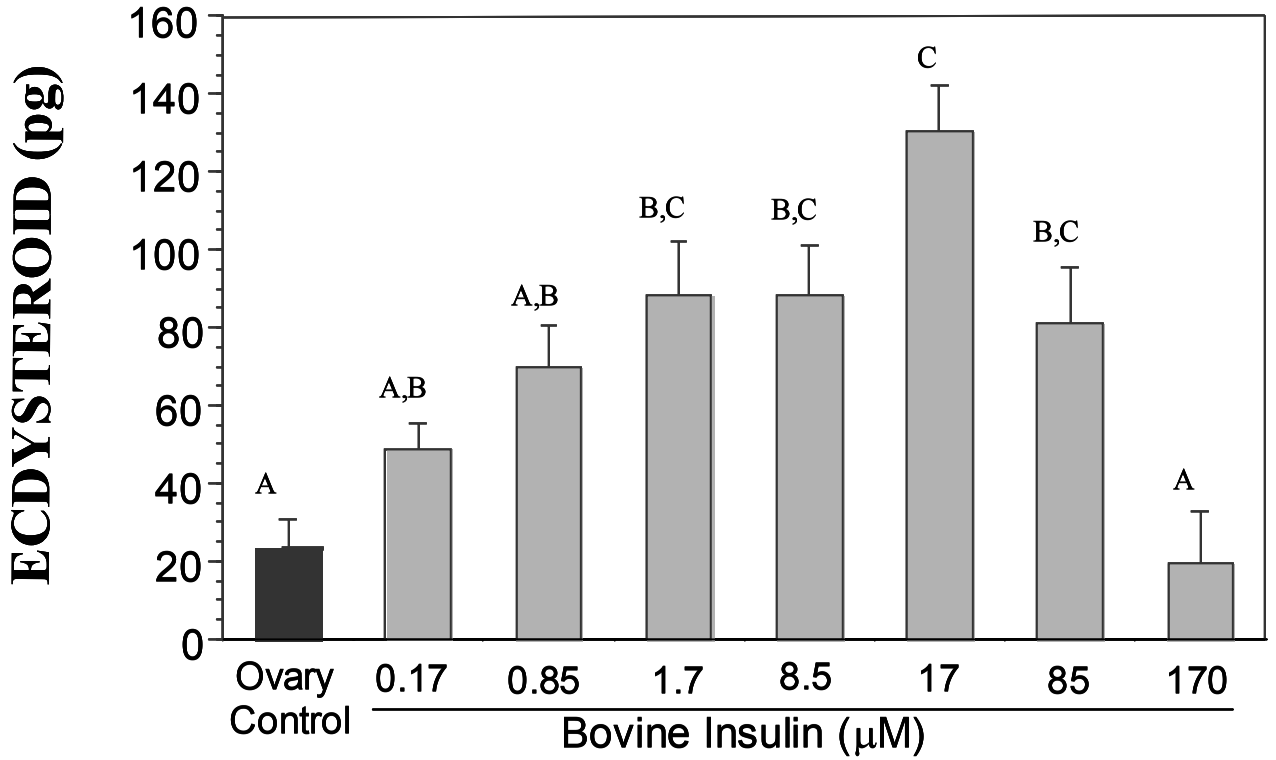


Figure 2.1: Effects of different concentrations of bovine insulin on ecdysteroid production by ovaries *in vitro*. Error bars represent standard errors. Tukey's studentized range (HSD) test was performed between the treatment means. Treatments with different letters are significantly different. ($P < 0.05$; $n = 4$ sets of triplicates/dose).

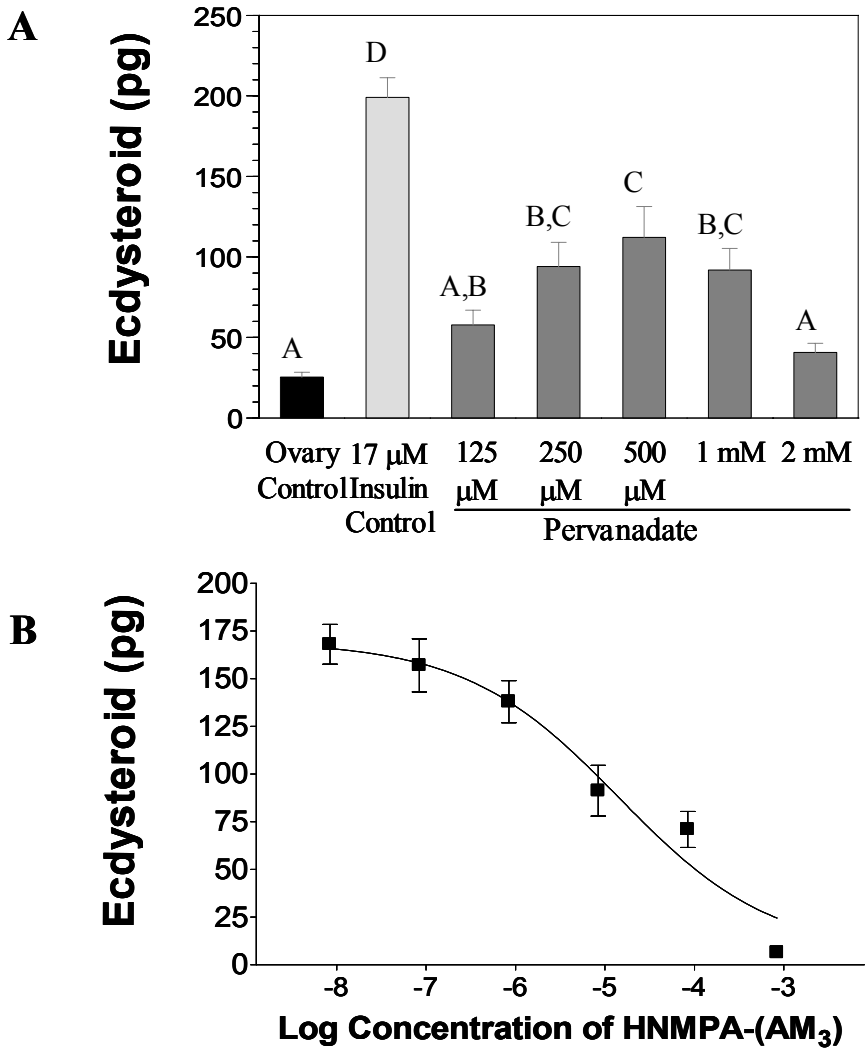


Figure 2.2: Effects of a tyrosine kinase activator and inhibitor on the mosquito insulin receptor. A: Effects of pervanadate, a tyrosine kinase activator, on ecdysteroid production. Error bars represent standard errors. Treatments with different letters are significantly different. ($P < 0.05$; n = 8 sets of triplicates/dose). B: Dose-response curve for HNMPA-(AM-$_3$), a tyrosine kinase inhibitor. ID_{50} = 14.2 µM. Ovaries were incubated *in vitro* with HNMPA-(AM-$_3$) in the presence of 17 µM bovine insulin (n = 4 experiments in triplicate/dose of each inhibitor).

potent inhibitor of tyrosine kinase activity that completely blocks the action of insulin. HNMPA-$(AM)_3$ inhibited ecdysteroid production in the presence of 17 μM insulin at an IC_{50} of 14.2 μM (Fig. 2.2B). Complete inhibition was achieved at 840 μM. Thus, HNMPA-$(AM)_3$ is a more potent inhibitor of the tyrosine kinase activity of the mosquito insulin receptor (IC_{50} = 14.2 μM) than that of the insulin receptor in mammalian cells (IC_{50} = 200 μM) (Saperstein *et al*., 1989). These results strongly suggest that a tyrosine kinase receptor, most likely the mosquito insulin receptor, binds bovine insulin and that phosphorylation of the receptor is a critical step leading to the stimulation of ecdysteroidogenesis by mosquito ovaries.

Our studies demonstrate the involvement of a PI 3-kinase and protein kinase B (PKB) homologue in stimulating ecdysteroid production. In mammalian cells, the PI 3-kinase/PKB pathway regulates the metabolic effects of insulin, including glucose transport and glycogen synthesis, as demonstrated by the use of cell permeable, relatively selective agonists and antagonists of PI 3-kinase and PKB. PKB is activated when phosphorylated on several serine and threonine residues. Okidaic acid, a serine/threonine phosphatase inhibitor, has been shown to activate PKB and GLUT 4 translocation independent of insulin and PI 3-kinase (Tanti *et al*., 1997). Okidaic acid stimulated ovarian steroidogenesis *in vitro* at an ED_{50} of 77.39 nM, and at 125 nM was capable of stimulation comparable to bovine insulin (Fig. 2.3A). Concentrations of okidaic acid greater than 125 nM were less stimulatory.

PI 3-kinase phosphorylates phosphotidylinsolitol, creating a critical membrane binding site for the pleckstrin homology domain of PKB. The fungal metabolite, wortmannin, was found to completely inhibit the activity of PI 3-kinase in rat adipocytes

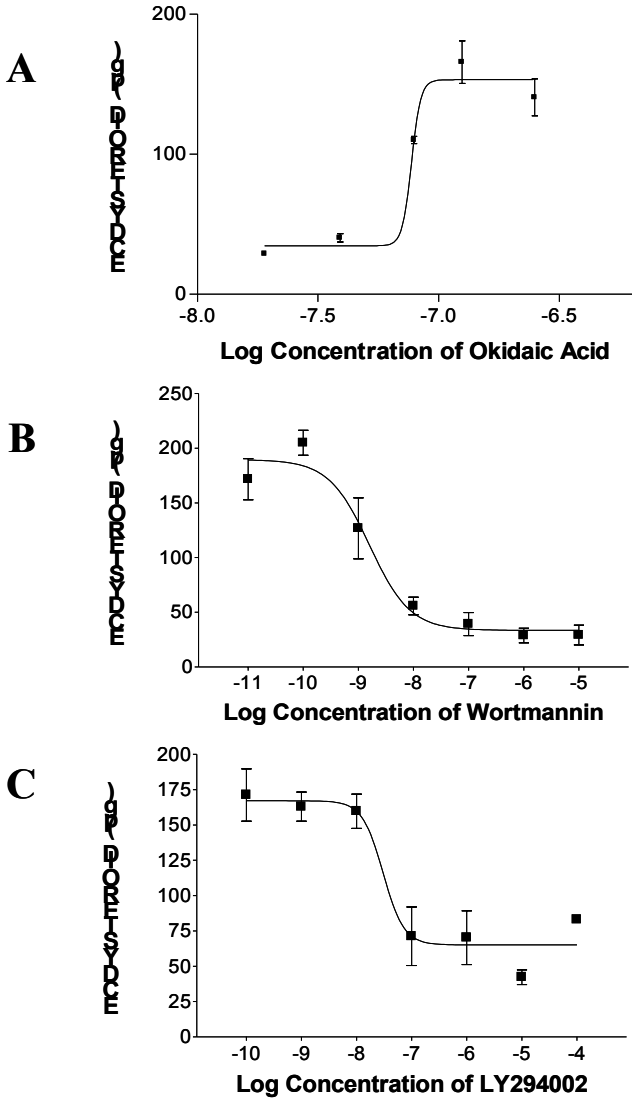


Figure 2.3: Dose-response curves for the effects of a PKB activator and PI 3-kinase inhibitors on ecdysteroid production. Ovaries were incubated *in vitro* with PI 3-kinase inhibitors in the presence of 17 μM bovine insulin (n = 4 experiments in triplicate/dose of each inhibitor) and with okidaic acid (n= 3 experiments in triplicate/dose). A: Dose-response curve for the PKB activator, okidaic acid. ED_{50} = 77.39 nM. B: Dose-response curve for the PI 3-kinase inhibitor, wortmannin. ID_{50} = 1.59 nM. C: Dose-response curve for the PI 3-kinase inhibitor, LY294002. ID_{50} = 30.25 nM.

(IC_{50} = 5 nM) (Okada *et al*., 1994). A more selective compound, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), was developed to inhibit PI 3-kinase (IC_{50} = 1.4 μM) (Vlahos *et al*., 1995). These inhibitors of PI 3-kinase completely inhibited ecdysteroidogenesis at concentrations comparable to those inhibiting the action of insulin in mammalian cells. Wortmannin inhibited ecdysteroid production with an IC_{50} of 1.6 nM (Fig. 2.3B), and complete inhibition was observed at 10 nM. LY294002 is approximately 20 fold less effective than wortmannin at inhibiting ecdysteroid production by ovaries (IC_{50} = 30 nM, Fig. 2.3C).

Our results suggest that a MAP kinase cascade does not control subsequent steps in the activation of ecdysteroid production. In mammalian cells, PD098059 is capable of inhibiting MEK activity at an IC_{50} of 2 μM (Avruch, 1998). Concentrations of PD098059 up to 100 μM displayed no inhibitory effect on ecdysteroid production (data not shown). The MAP kinase cascade stimulated by insulin in vertebrates is responsible for controlling mitogenesis and cell differentiation and does not appear to be involved in the metabolic functions of insulin (Avruch, 1998). In mammalian cells expressing a dominant negative Ras, both MAP and $pp90^{S6}$ kinase activities were markedly reduced, but activation of glycogen synthase was unaffected. Furthermore, PD098059 had no effect on the activation of glycogen synthase (Avruch, 1998). Thus, it is not surprising that a PD098059 had no effect on ecdysteroid production. In *Drosophila*, it has been demonstrated that the insulin receptor is critical for normal growth (Chen *et al*., 1996). Thus, the *Drosophila* insulin receptor, with a β subunit extension homologous to growth factor receptors in vertebrates, may utilize a MAP kinase cascade to regulate development.

Based on these results, we hypothesize that the insulin receptor in mosquito ovaries acts through a PI 3-kinase/PKB cascade that activates ecdysteroid biosynthesis. Three classes of PI 3-kinase have been described for vertebrate cells. In vertebrates, a class 1 PI 3-kinase has been shown to phosphorylate phosphatidylinositol, providing a binding site for PKB that, when constitutively active in 3T3-L1 adipocytes, stimulates the translocation of glucose transporters and glucose uptake (Kohn *et al*., 1996). PI 3-kinases and PKB have been described from *Drosophila* (Franke *et al*., 1994; Leevers *et al*., 1996). This information, coupled with the above studies, provides strong evidence for the involvement of a PI 3-kinase and PKB homologue in ecdysteroid production by mosquito ovaries.

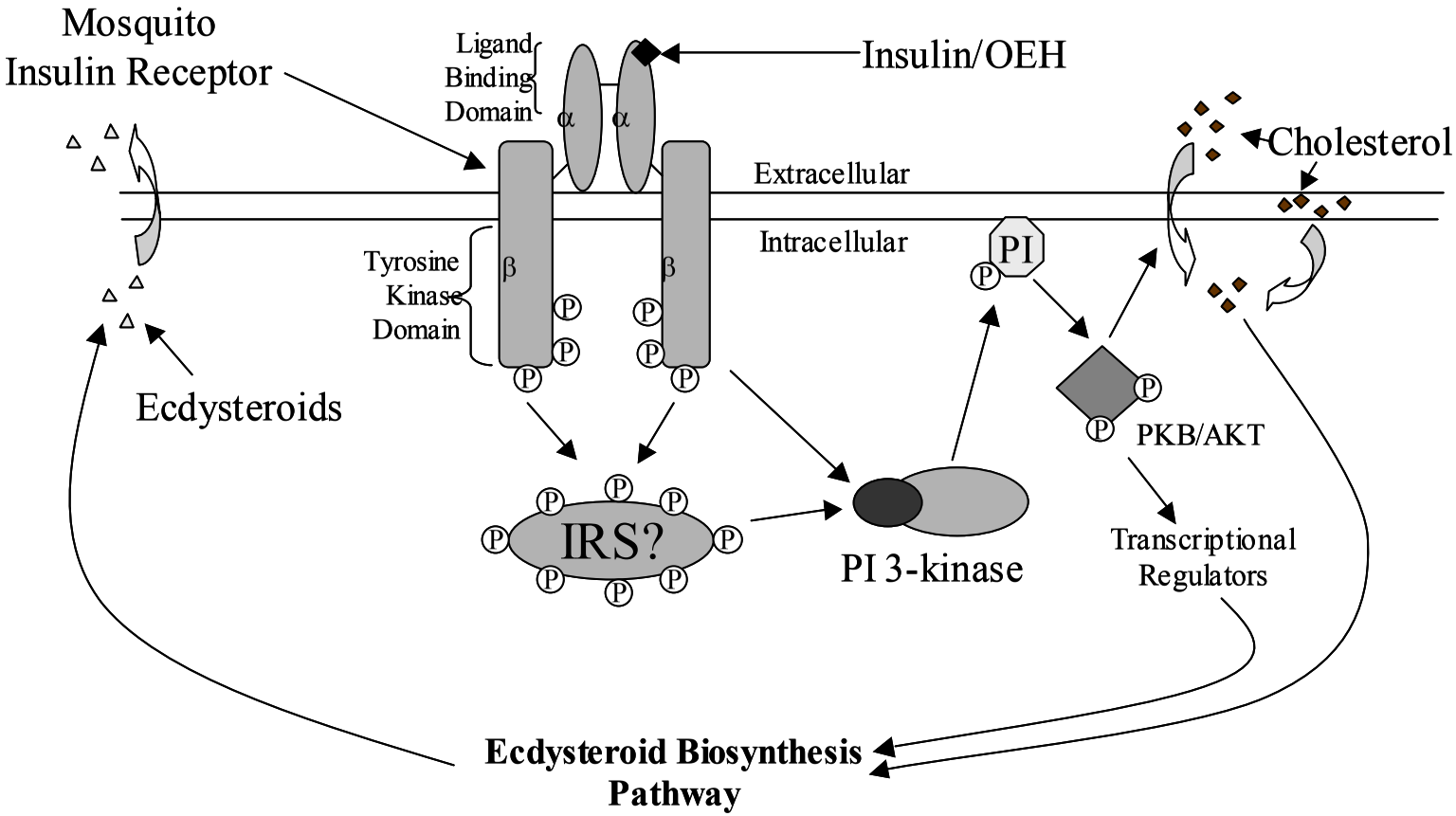
This study has identified a likely signaling pathway for the putative insulin receptor in *Aedes aegypti* (Fig. 2.4). This pathway regulates ecdysteroidogenesis, an essential endocrine process in female mosquitoes. The mosquito insulin receptor is localized on follicle cells surrounding the oocytes (Helbling and Graf, 1998), and these cells in female locusts, and most likely other insects, are the primary source of ecdysteroids (Kappler *et al*., 1986). Bovine insulin was effective but is a distantly heterologous ligand for the receptor. Presumably, the corresponding insulin of mosquitoes may act through the proposed pathway to stimulate cholesterol uptake into the cell and to activate biosynthetic pathways, resulting in ecdysteroidogenesis. Interestingly, ovary ecdysteroidogenic hormone I (OEHI), a neuropeptide, is already known for *Aedes aegypti* (Brown *et al*., 1998). The amino acid sequence of OEHI exhibits no apparent similarity to invertebrate or vertebrate insulins, and the mode of action of OEHI is unknown. Characterization of a mosquito insulin and key proteins in

the pathway identified by the present study are required for comprehensive understanding of the regulation of reproduction in female mosquitoes, including possible interactions between a putative insulin and OEHI.

Acknowledgements

This work was supported by a NIH grant (AI33108). Special thanks to Don Champagne, Joe Crim, Prema Narayan, Judy Willis (University of Georgia), and to Rolf Graf (University Hospital of Zurich) for their critical review of the manuscript.

Figure 2.4: Proposed signal transduction pathway for the mosquito insulin receptor based on a signaling cascade characterized for mammalian insulin receptors (Avruch, 1998). Insulin binds to the receptor on the surface of the follicle cells surrounding the oocytes, resulting in the autophosphorylation of the tyrosine kinase domain of the β-subunit as suggested by experiments with HNMPA-(AM_3). The activated tyrosine kinase domain, in turn, phosphorylates an IRS1 homologue. A PI 3-kinase homologue binds to phosphorylated tyrosine residues on IRS1, thus activating the catalytic subunit. The involvement of PI 3-kinase has been suggested by the use of the inhibitors, wortmannin and LY294002. The catalytic subunit of PI 3-kinase phosphorylates phosphotidyl inositols (PI) in the inner cell membrane creating a binding site for PKB. When phosphorylated by unknown kinases, PKB may activate intracellular membrane movement, recruitment of cholesterol into the cell, and biosynthetic pathways for the conversion of cholesterol to ecdysteroids.


Mosquito
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Pathway

References

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CHAPTER 3

INSULIN RECEPTOR EXPRESSION DURING DEVELOPMENT AND A REPRODUCTIVE CYCLE IN THE OVARY OF THE MOSQUITO *Aedes aegypti*[1]

[1]Riehle, M.A. and Brown, M.R. 2002. Accepted by Cell and Tissue Research, 2/20/02.

ABSTRACT

A key tyrosine kinase receptor regulates steroidogenesis during egg maturation in the mosquito *Aedes aegypti*. This study examined expression patterns and phosphorylation states of the mosquito insulin receptor (MIR) in ovaries during the previtellogenic stage and a reproductive cycle. Little or no MIR protein was present until 24 h after adult eclosion, when the mature MIR appeared as a ~400-kDa tetrameric protein composed of two 116-kDa α subunits and two 95-kDa β subunits. Immunocytochemistry showed that MIR was localized in the cell membranes of follicle cells surrounding the oocyte and nurse cells. Protein and mRNA transcript levels gradually increased in ovaries during the first few days after eclosion, and remained constant during previtellogenic arrest for up to 21 days. During a reproductive cycle, MIR protein levels remained constant up to 12 h pbm. However, from 24 to 48 h pbm, during chorion deposition, the MIR protein was not detected. By 72 h pbm, after oviposition, the level of MIR protein returned to pre-bloodmeal levels. Two peaks of MIR transcript occurred in ovaries after a blood meal, immediately following a bloodmeal and after oviposition. MIR protein was constitutively phosphorylated on tyrosine residues at low levels during the previtellogenic arrest stage. Tyrosine phosphorylation increased 3- to 4-fold when ovaries were incubated with bovine insulin in vitro, and 2-fold when incubated with sodium orthovanadate, thus demonstrating a role for MIR in activating steroidogenesis. Notably, MIR protein and transcript were not detected in eggs, larvae, pupae, or pharate adults.

INTRODUCTION

Reproduction in the female yellow fever mosquito, *Aedes aegypti,* is a cyclical process of ovary development and egg maturation separated by periods of arrest (Sappington and Raikhel 1999). Each of the paired ovaries is comprised of approximately 75 ovarioles. The ovarioles possess a germarium and primary follicle consisting of one oocyte and seven nurse cells surrounded by a layer of epithelial or follicle cells. Ovary development begins immediately after eclosion: the follicles double in size, follicle cells differentiate and increase in number from fewer then 20 to over 200, and the ovary becomes competent for vitellogenin uptake. At the same time the fat body becomes competent for vitellogenesis. By 72 h after eclosion the mosquito enters a state of previtellogenic arrest and remains so until a bloodmeal is consumed. After a bloodmeal, the vitellogenic phase, consisting of an initiation phase and a trophic phase, begins. During the initiation phase, follicle cells separate from the oocyte, mitosis resumes in the follicle cells, and low levels of yolk proteins are produced by the fat body. The trophic phase, beginning 4 to 6 h post-bloodmeal (pbm), is characterized by a large increase in yolk protein secretion by the fat body and the rapid uptake of yolk proteins into the developing oocyte. By 36 h pbm, vitellogenin uptake ends, and postvitellogenesis begins. During postvitellogenesis follicle cells deposit the chorion, eggs are oviposited 60 to 72 h pbm, and the secondary follicles mature to the previtellogenic arrest stage. During the same time, the biosynthetic organelles in the fat body are degraded, and fat body cells return to previtellogenic arrest, thus preparing the mosquito for the next reproductive cycle.

During the initiation phase, gonadotropins, including ovary ecdysteroidogenic hormone I (OEH I), are released from neurosecretory cells in the brain and stimulate ecdysteroid secretion by the ovary (Brown et al. 1998). Ovary ecdysteroid production in vitro is known to be regulated through a receptor tyrosine kinase (RTK) and a phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (PKB) signaling pathway (Riehle and Brown 1999). An RTK with a high degree of sequence identity to the human and *Drosophila* insulin receptors was identified in ovaries of *Ae. aegypti* and termed mosquito insulin receptor (MIR) (Graf et al. 1997). The MIR cDNA encodes a proreceptor that likely is cleaved proteolytically into α and β subunits at a processing site composed of four basic amino acids. The α subunit has a conserved domain involved in ligand binding, and the β subunit has a hydrophobic transmembrane domain and a characteristic tyrosine kinase domain. Protein and transcripts of MIR are found primarily in the ovaries (Graf et al. 1997; Helbling and Graf 1998), but transcripts are present in the head and body wall of females (Graf et al. 1997).

In other organisms, the RTK/PI3K/PKB signal transduction cascade is a nexus for the transcriptional and translational regulation of a number of physiological responses, including carbohydrate metabolism, apoptosis, growth, immunity, and longevity (Kimura et al. 1997; Satake et al. 1997; Tatar and Yin 2001; Yenush et al. 1996). An insulin receptor orthologue has been characterized in another dipteran species, the fruit fly *Drosophila melanogaster*. The *Drosophila* insulin receptor (DIR) and its signaling cascade has been shown through mutational analysis and overexpression studies to regulate several mitogenic effects including growth, DNA synthesis, and apoptosis (Marin-Hincapie and Garofalo 1999; Yenush et al. 1996) and affect adult lifespan

(Clancy et al. 2001; Tatar et al. 2001). Like the vertebrate insulin receptors, the DIR is a tetrameric transmembrane protein consisting of two α subunits and two β subunits (Fernandez-Almonacid and Rosen 1987). In contrast to the MIR and vertebrate insulin receptors, the DIR possesses a 300 amino acid extension at the carboxy terminus of the β subunit that may act as a binding site for signaling proteins (Ruan et al. 1995). Expression of the DIR transcript has been studied primarily in the developing embryo, although expression occurs in the larval imaginal discs, larval and adult nervous system, and adult ovaries (Garofalo and Rosen 1988). A specific function for the DIR in these tissues is not known.

Insulin receptor orthologues have been identified in two other arthropods, the tobacco hornworm *Manduca sexta* (Smith et al. 1997) and the shrimp *Penaeus japonicus* (Chuang and Wang 1994), and in a few other invertebrates. In *Manduca sexta*, a 178-kDa membrane protein was immunoreactive to a human insulin receptor antibody and was phosphorylated on tyrosine residues when assayed for kinase activity (Smith et al. 1997). Although the amino acid sequence of this protein has not been determined, it is thought to be the β subunit of the *M. sexta* insulin receptor. In the shrimp *P. japonicus*, a 79-kDa protein immunoreactive to an insulin receptor antibody was detected on immunoblots (Chuang and Wang 1994). In the presence of bovine insulin, this protein becomes phosphorylated on tyrosine residues and is also thought to be the β subunit of an insulin receptor orthologue.

Besides *D. melanogaster*, an RTK/PI3K/PKB signaling cascade is best characterized in the nematode *Caenorhabditis elegans* by mutational analysis (Kimura et al. 1997). The insulin receptor homologue, *daf*-2, has a carboxy terminus extension

similar to the DIR that may play a role in cell signaling (Kimura et al. 1997). Mutations in the daf-2 receptor affect dauer formation and increase adult longevity (Ailion et al. 1999). Like the MIR, *daf*-2 also appears to have a role in egg development as demonstrated by a temperature sensitive *daf-2* mutant that has a 4-fold decrease in brood size at nonpermissive temperatures compared with wild type individuals and mutants reared at permissive temperatures (Tissenbaum and Ruvkun 1998). Insulin receptor orthologues in other invertebrates are not as well characterized, and their physiological roles have not been determined. The mollusk *Aplysia californica* possesses an insulin receptor with a structure similar to the mosquito and human insulin receptor (Jonas et al. 1996), whereas the insulin receptor in the pond snail *Lymnaea stagnalis* possesses a carboxy terminus extension similar to that of *Drosophila* and *C. elegans* (Roovers et al. 1995). Orthologues to the insulin receptor identified in four species of sponge are the smallest known RTKs, thus demonstrating that these receptors are found in the earliest metazoans (Skorokhod et al. 1999).

Surprisingly, even though MIR is most likely the key receptor regulating steroidogenesis, little is known about its structure and expression pattern in the ovary during oogenesis. Therefore, this paper characterizes the mRNA and protein expression patterns of the MIR in the ovary after adult eclosion, including the previtellogenic phase and subsequent period of arrest prior to a bloodmeal. It examines the expression patterns of the MIR transcript and protein in the mosquito ovary during the initiation and trophic phases of vitellogenesis when steroid production is high, and during postvitellogenesis when steroid production is minimal. The steroidogenic signaling cascade in mosquito ovaries is initiated by the autophosphorylation of tyrosine on the intracellular portion of

an RTK. This paper demonstrates for the first time tyrosine phosphorylation of the MIR when stimulated with known activators of ovary steroidogenesis in vitro. Finally, although insulin receptor orthologues are critical regulators of growth and development in immature *Drosophila* and *C. elegans*, this study has demonstrated that the MIR is expressed only in the adult mosquito.

MATERIALS and METHODS

Insects

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 two days, and subsequently were given only distilled water, whereas those used in the age time course were given continual access to 10% sucrose solution. To initiate a reproductive cycle, adults were fed on an anesthetized rat until engorged, separated from unfed and partially fed individuals, and maintained on water until needed.

Antiserum production and affinity purification

Antisera to the α and β subunits of the MIR were produced in rabbits from synthetic peptides to the amino (α-subunit AA 35-52: GCVGTVDVRNSPAHLDRL) and carboxy (β-subunit AA 1327-1346: DEATTPLRPGDDHDEEPGED) termini of the MIR (Graf et al. 1997). The synthetic peptides were conjugated to thyroglobulin using glutaraldahyde as previously described (Harlow and Lane 1988). Two rabbits were used per antigen, and prior to injection, a pre-immune blood sample was obtained from each. One primary injection (2 mg of antigen/animal in 0.5 ml of both Freund's complete adjuvant and phosphate-buffered saline solution) followed by four antigen boosts (1 mg

antigen/animal as before but with incomplete adjuvant) were performed with four to five week intervals between injections. Sera were collected two weeks after each boost and tested on immunoblots. Sera lots 377E (α subunit antibody) and 376E (β subunit antibody) had the highest affinity for the MIR. These lots were affinity purified after coupling the antigenic synthetic peptides to an Aminolink Plus column (Pierce, per kit instructions), aliquoted, and stored at -80°C.

Immunoblot analysis

Ovaries were dissected from mosquitoes in medium (125 mM NaCl, 5 mM KCl, 1.85 mM $CaCl_2$; pH 6.5, adjusted with NaOH) and immediately transferred to a solution of 2X protease inhibitor cocktail (Roche) and medium. Equal volumes of reducing (2X: 0.125 M Tris-Cl, 4% SDS, 20% glycerol, 0.2 M DTT, 0.02% bromophenol blue, pH 6.8) or non-reducing sample buffer (2X: 0.125 M Tris-Cl, 4% SDS, 50% glycerol, 0.02% bromophenol blue, pH 6.8) were added to the samples. The samples were homogenized, frozen for 15 min, vortexed (to shear genomic DNA and reduce viscosity), boiled for 5 min, and plunged into an ice bath. Treated samples (3 ovary equivalent) and molecular weight markers (10 to 250-kDa, Rainbow Markers, Amersham) were loaded onto a 7.5% Tris-glycine gel (BioRad), fractionated by SDS polyacrylamide gel electrophoresis (10x Tris/Glycine/SDS running buffer, Biorad), and tank transferred (Transfer buffer: 10 mM Tris, 100 mM Glycine, 10% methanol) to a nitrocellulose (0.1 μM, Schleicher and Schulle) or PVDF (0.2 μM, Biorad) membrane. Nitrocellulose blots were air dried (> 1 h), wetted with Tris buffered saline solution (pH 7.5) and 0.1% Tween-20 (TBST), and blocked for 1 h in TBST containing 5% BSA and 2% goat serum (GS). PVDF blots were placed directly into blocking buffer. Affinity purified antibodies to the α and β subunits

of MIR were added to the blocking buffer at a 1/5,000 dilution and incubated overnight at 4°C with gentle rocking. Blots were washed in TBST (3X, 20 min), incubated with an anti-rabbit antibody conjugated to horseradish peroxidase (1/20,000 dilution, Sigma) in TBST (1 h, RT), and washed three times in TBST (20 min/wash). Receptor subunits were detected with the Renaissance chemiluminecent detection kit (NEN) on Kodak Biomax light film. Densitometry of individual bands was performed with a Genegnome chemiluminescent imager and the Genetools program (Syngene). Experimental immunoblots were replicated three or more times, and as a control, the primary antibody was not added to an ovary sample from 5- to 7-day ovaries to identify secondary antibody artifacts.

In vitro phosphotyrosine stimulation and immunoblot analysis

Ovaries were dissected from 6 to 7 day old non-oogenic female mosquitoes in medium (as above) and transferred to a 1.5 ml microtube cap containing 120 μl of medium (10 pairs per cap; 2 caps per sample). When dissections were completed, medium was removed and replaced with 120 μl of fresh medium, medium with the phosphatase inhibitor vanadate (Na_3VO_4, 1 mM), or medium with bovine insulin (17 μM) to stimulate autophosphorylation of the MIR. Ovaries were incubated for varying lengths of time (1 to 30 min) at 30°C in a water bath chamber with slow agitation. The medium was subsequently removed, and 120 μl of 2X homogenization buffer (50 mM Tris-HCl (pH 7), 250 mM sucrose, 2 mM Na_3VO_4, and 2X protease inhibitor cocktail) was added. After the caps were replaced onto the microtube, ovaries were centrifuged to the bottom of the tube (14,000 RPM for 1 min), and homogenized with a plastic pestle. Identical samples were combined into a single tube and centrifuged for 5 min at 5,000 RPM (4°C).

The lysate, containing cytoplasm and organelles, was transferred to a Centricon concentrator (100-kDa MW cutoff) and centrifuged for 12 min at 14,000 RPM. The retentate was collected and prepared for immunoblotting as described above. Lysis buffer (200 µl; 50 mM Tris pH 6.8, 1% SDS, 2 mM Na_3VO_4, 2X protease inhibitor cocktail) was added to the pellet containing the cell membranes. The pellet was homogenized, vortexed, and centrifuged for 5 min at 5,000 RPM (4°C). The cleared lysate containing the solublized MIR was concentrated in a Centricon column (100-kDa MW cutoff) and prepared for immunoblotting. Immunoblotting was performed as described above. Phosphorylated tyrosine residues on the MIR were detected with a polyclonal antiphosphotyrosine antibody generated in rabbits (Zymed, 1:30,000 dilution). Visualization and densitometry were performed on an imager as above.

Northern Blot Analysis

RNA probes labeled with digoxigenin (Dig) were synthesized from the MIR and *Aedes* actin genes as follows. A 700 bp MIR PCR product (sense primer 5'-GCTGACGGAGATCACCGAGTACCTGCTGCT-3', antisense primer 5'-CCGGATTCGAGTCCAGCGAGATCGGTTTCT-3': 95°C, 15 sec: 55°C, 15 sec: 72°C, 60 sec: 35 cycles) and 200 bp *Aedes* actin PCR product (sense primer 5'-GCGATCTGACCGACTACCTGATGA-3', antisense primer 5'-CCAGATTCATCGTACTCCTGC-3': 95°C, 15 sec: 55°C, 15 sec: 72°C, 60 sec: 35 cycles) were ligated into the pCRII-TOPO vector using the TOPO TA cloning kit (Invitrogen). Plasmid DNA was isolated from the resulting colonies and sequenced to verify the correct orientation. Gene specific sense primers for MIR and actin (see above) and a M13 (-20) forward primer were used to generate PCR products for MIR and actin

from the plasmids with a T7 promoter. These products were used as templates in the MAXIscript i*n vitro* transcription kit (Ambion) to generate antisense RNA probes containing dUTP-Dig (Roche).

Ovaries were dissected from female mosquitoes in *Aedes* saline solution (128 mM NaCl, 4.7 mM KCl, and 1.9 mM $CaCl_2$), immediately transferred to RNAlater (50 µl, Ambion) and stored at -20°C until processed. Total RNA was isolated from the ovaries using the RNAeasy mini kit (Qiagen). Loading buffer (5X: 32% formamide, 2.4% formaldehyde, 4X MOPS, 20% glycerol, 4 mM EDTA, and bromophenol blue) was added to 10 µg of total RNA, along with RNAse free water to bring the final volume to 20 µl. RNA was denatured at 80°C for 10 min and loaded onto a precast 1.25% Reliant MOPS gel (BMA) for electrophoresis at 3 volts/cm for 3 h in 1X MOPS running buffer. Afterwards, the gel was stained in ethidium bromide (2 mg/200 ml) for 2 min then destained in DEPC water for 30 min. The gel was denatured in 0.05M NaOH for 30 min followed by neutralization in 1 M Tris for 30 min. The RNA was then vacuum transferred onto nylon membrane (MSI) for 1 h and autocrosslinked (120,000 j/cm Stratagene). The blot was prehybridized in ULTRAhyb (7 ml, Ambion) at 65°C for 1 h. MIR and actin Dig labeled probes were denatured for 10 min at 80°C, added to the prehybridization solution (1:20,000 final concentration for both MIR and actin probes), and hybridized overnight at 65°C. After hybridization, the blot was washed 3 times (20 min/wash) in 0.5X SSC/0.1% SDS at 65°C followed by a 1 h block in 1X blocking reagent (Roche). The blot was incubated with an antiDig-alkaline phosphatase antibody (1:10,000) and 1X blocking reagent for 30 min followed by 3 washes (20 min/wash) in maleic acid buffer (0.1M maleic acid, 0.15 M NaCl, pH 7.5) and 0.3% Tween 20. The

MIR transcript was detected with CDP-Star (Roche) and visualized with Biomax light X-ray film (Kodak) or an imager (as above) for densitometry.

Immunocytochemistry and confocal microscopy

Ovaries were dissected from mosquitoes at various times pbm in fixative solution (4% paraformaldehyde in PBS: 2.5 mM NaH_2PO_4, 8.5 mM Na_2PO_4, and 175 mM NaCl, pH 7.4), transferred into fresh fixative solution for 1 h at room temperature, and frozen at -80° C until needed. The ovaries were subjected to two freeze/thaw cycles followed by two 15 min washes in PBS and 0.5% Triton 100 (PBST). Ovaries were incubated in blocking solution (5% BSA and 2% GS in PBST) for 2 h at 4°C, followed by incubation with the diluted β subunit antibody (1:100 dilution) in blocking buffer overnight at 4° C. Tissues were washed three times in PBST (1 h/wash, 4°C) and incubated with Alexa^{Tm} 488 goat anti-rabbit IgG (H+L) conjugate (1:4000 dilution, Molecular Probes, Inc) for 2 h at room temperature, followed by three additional 20 min washes in PBST. Tissues were mounted on a slide in media (PBS:Glycerol 1:1) and examined with a Leica TCS/SP2 confocal microscope. For consistency, all confocal setting remained unchanged between the samples with the exception of a 50% reduction in magnification at the 24, 36, and 48 h pbm time points due to the size of the oocyte.

RESULTS

MIR expression in ovaries increased during the previtellogenic phase and remained constant during previtellogenic arrest

After eclosion, the primary follicles separate from the germarium, follicle cells undergo cell differentiation and growth, and ultimately the ovary enters an arrested state. Immunoblots and northern blots of ovary extracts were analyzed to determine the

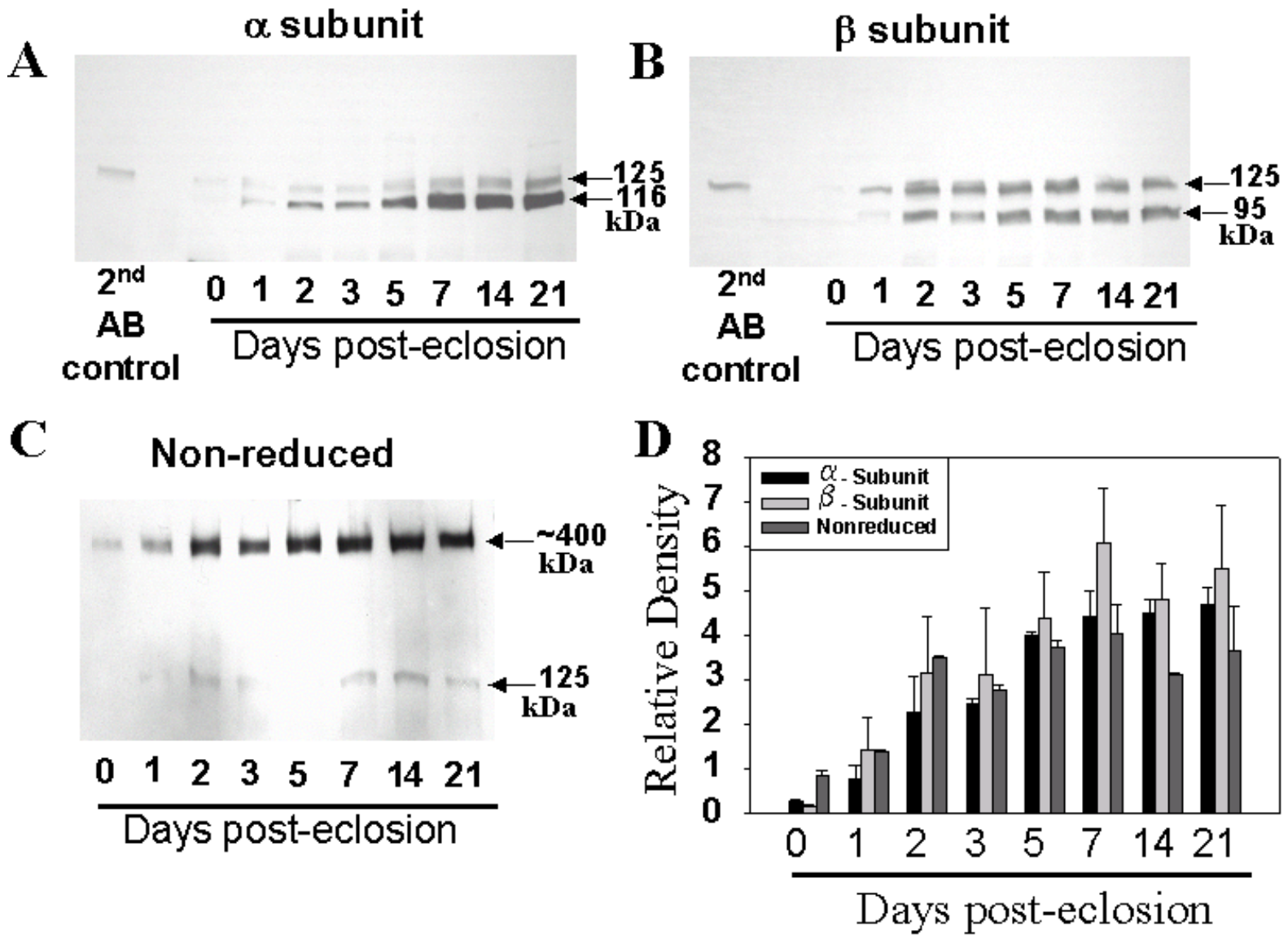
expression of MIR during follicle development and arrest. During the first 24 h after eclosion, little or no MIR protein was detected on immunoblots of ovary extracts (0 day, Fig 3.1A, B, and C). By day 1, a 116-kDa and 95-kDa protein were detected with antibodies to α and β subunits of MIR, respectively, on immunoblots of reduced ovary extracts (Fig 3.1A and B). In non-reduced ovary extracts, a protein of ~400-kDa was detected with the β subunit antibody (Fig 3.1C). The size of the non-reduced protein was consistent with a receptor consisting of two α and two β subunits (Fig 3.1C). The 125-kDa band detected in all blots is a secondary antibody artifact. Qualitative densitometry of each age point from the above immunoblots showed a four to five fold relative increase in the levels of MIR during the first three days after eclosion (Fig 3.1D). During previtellogenic arrest (days 5 to 21) the relative expression of MIR remained unchanged.

Expression of MIR transcript in ovaries was similar to that of the protein. Two bands, of approximately 8 and 9.5 kb, were consistently detected on northern blots with the MIR probe (Fig 3.2A). Sufficient RNA could not be obtained from the undeveloped ovaries of 0-day-old mosquitoes for inclusion on this blot. *Aedes* actin, seen as a 2 kb band, was used as a loading control (Fig 3.2A). The ratio of MIR and actin transcript for each age point was determined from the representative northern blot by densitometry (Fig. 3.2B). MIR/actin ratios increased 2 to 3 fold from 1 to 3 days post-eclosion relative to *Aedes* actin, and remained constant for up to 21 days post-eclosion (Fig 3.2B).

MIR Expression Changes During a Reproductive Cycle

During a reproductive cycle, MIR protein and transcript levels varied in the ovaries. MIR protein levels in the ovaries remained constant during the first 12 h pbm as

Figure 3.1: Expression of MIR protein in ovaries of ageing mosquitoes. A. Detection of a 116-kDa band from ovary extracts under reducing conditions when treated with the MIR α-subunit antibody at various days post-emergence. A secondary antibody artifact (125-kDa) can be seen in the 2nd AB control lane (5- to 7-day ovaries). **B.** Detection of a 95-kDa band from ovary extracts under reducing conditions when treated with the MIR β-subunit antibody post-emergence (MW: 95-kDa under reducing conditions). **C.** Detection of a ~400-kDa band from ovary extracts under non-reducing conditions when treated with the MIR β-subunit antibody at various times post-emergence. **D.** Densitometry analysis of three immunoblots, each with three replicates, described above. Protein extracts for each sample was obtained from three pairs of ovaries.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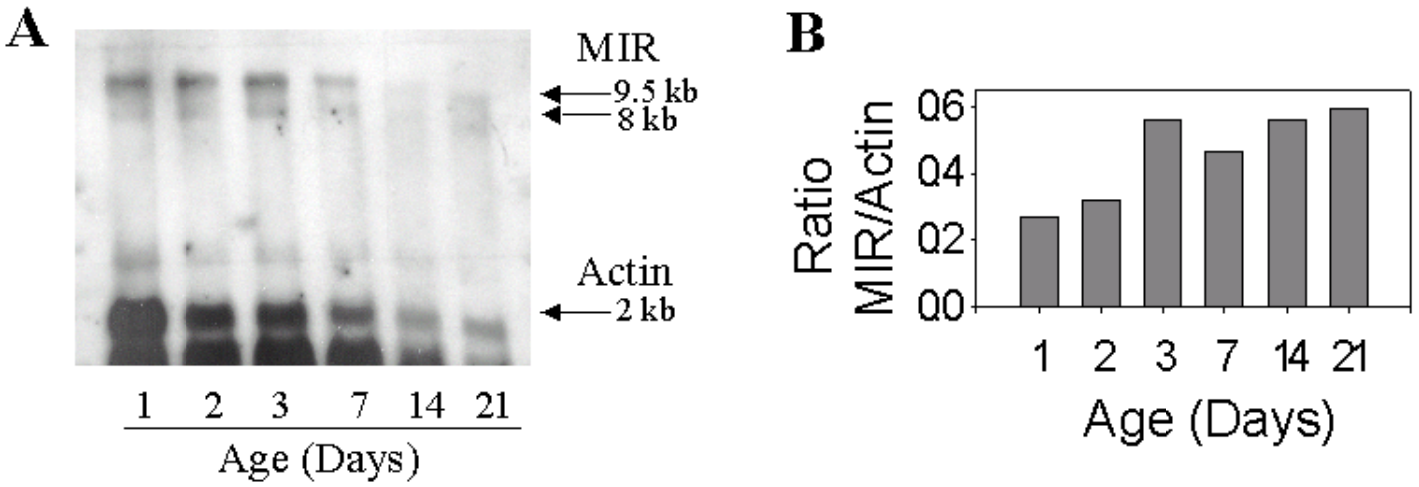


Figure 3.2: Expression of MIR mRNA in ovaries of ageing mosquitoes. A. Northern blot showing two MIR transcripts of 9.5 and 8 kb, at various days after eclosion. Actin was used as a loading control and can be seen at 2 kb. Total RNA (10 µg) was loaded into each lane. **B.** Densitometry analysis of northern blot in A. The MIR/actin transcript ratio was used to illustrate the relative increase in MIR mRNA transcript as the mosquito ages.

shown on immunoblots under both reducing and non-reducing conditions (Fig 3.3A and B). Expression during this time was similar to expression during previtellogenic arrest. From 24 to 48 h pbm, the MIR was not detected in ovary extracts. By 72 h pbm, after oviposition, the MIR protein was again detectable.

To confirm the localization and expression of MIR protein in the ovary during a reproductive cycle, immunocytochemistry was performed at the same times pbm as the above immunoblots. Prior to a bloodmeal, MIR immunostaining with the β-subunit antibody was specifically located in the membrane of the follicle cells (FC) surrounding the primary follicle (Fig 3.4: NBM-a). In a cross-section of the same ovariole, MIR was distributed around the membrane in the cuboidal follicle cells surrounding the primary follicle, as well as on the membranes surrounding the nurse cells in both the primary and secondary follicles and the germarium (Fig 3.4: NBM-b). From 2 to 12 h pbm, the follicle cells and nurse cells were strongly immunostained to the β subunit antibody (Fig 3.4: 2h-a and b, 6h-a and b, and 12h-a and b). At 24 h pbm, when the band observed on the immunoblots could no longer be detected (Fig 3.3), immunostaining of the follicle cells (arrows) dropped markedly but was still detectable (Fig 3.4: 24h-a and b). By 36 h pbm, the immunostaining specific to the follicle cell membranes on the primary follicle was lost (Fig 3.4: 36h-a and b). Diffuse immunostaining was seen on the surface of the primary follicle (arrows), and staining on the secondary follicle and germarium remained strong. By 48 h pbm, all immunostaining was lost on the primary follicle (Fig 3.4: 48h-a and b), and only the secondary follicle and the germarium were immunostained. After egg deposition, approximately 72 h pbm, immunostaining of the new ovarioles was nearly identical to non-bloodfed ovarioles (Fig 3.4: 72h-a and b). Thus, the

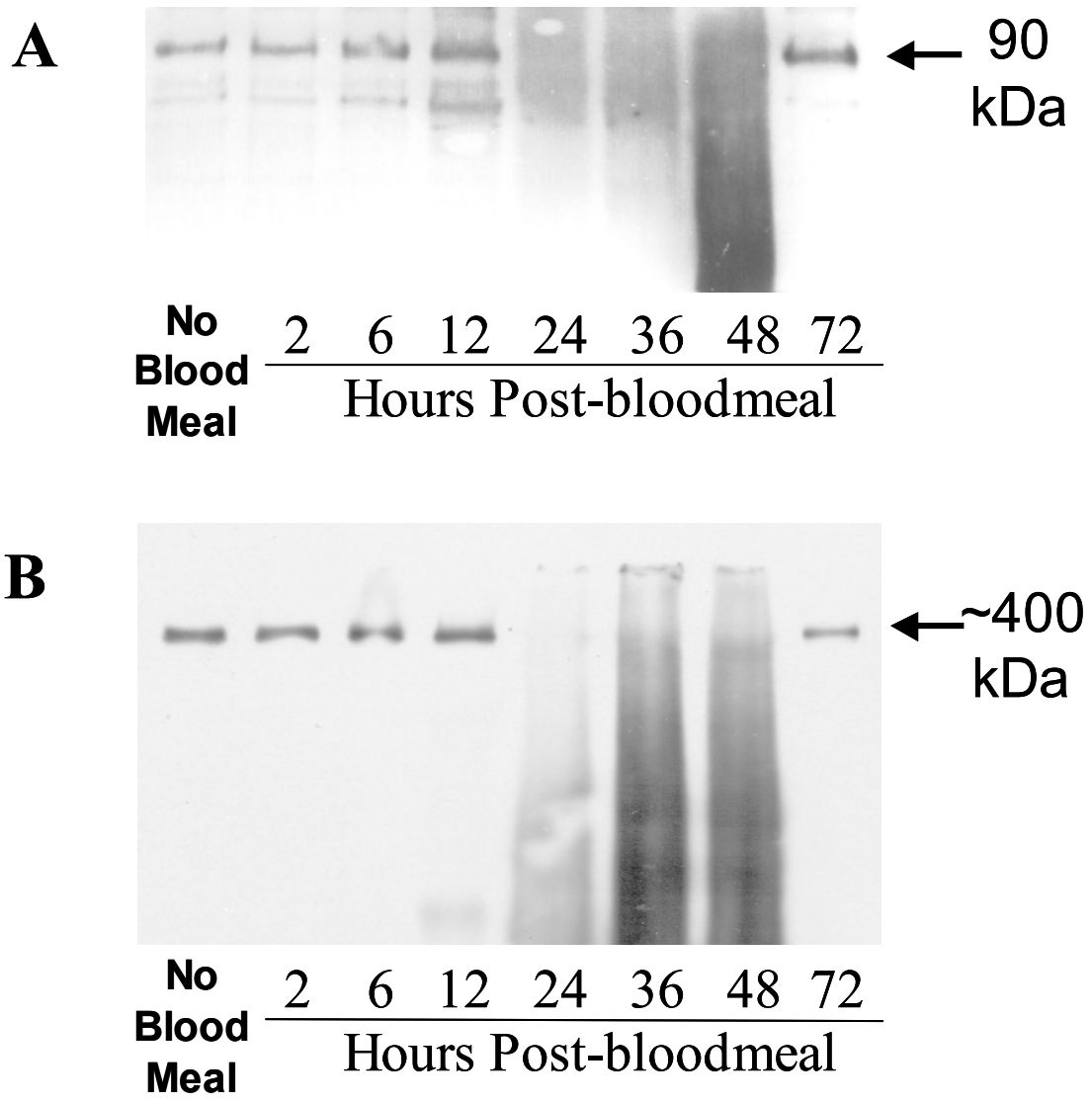
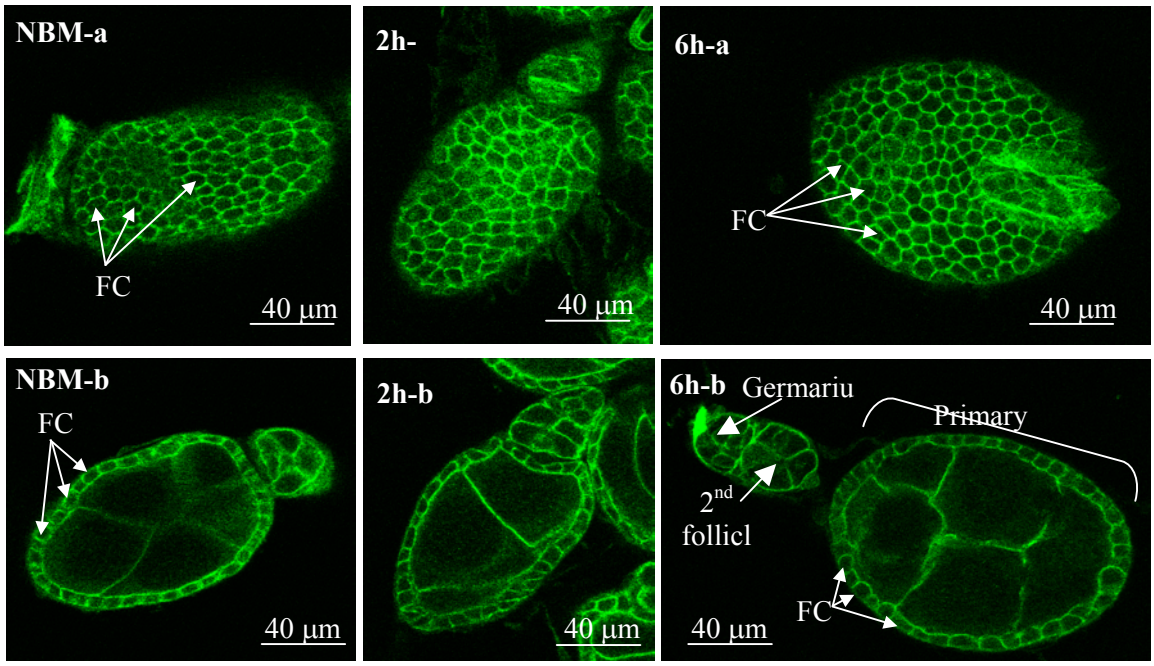
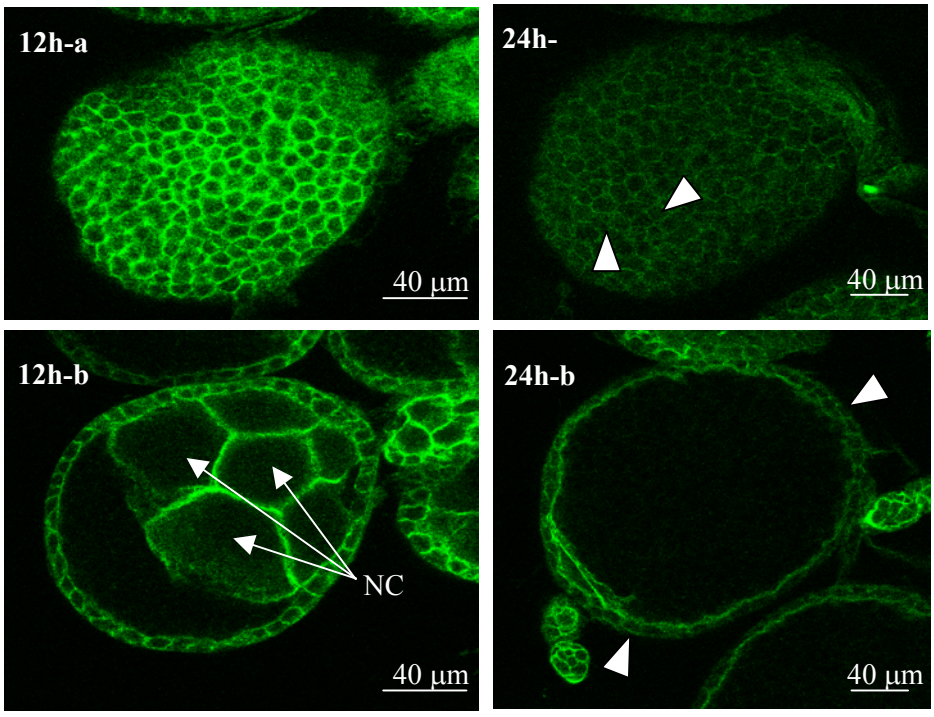
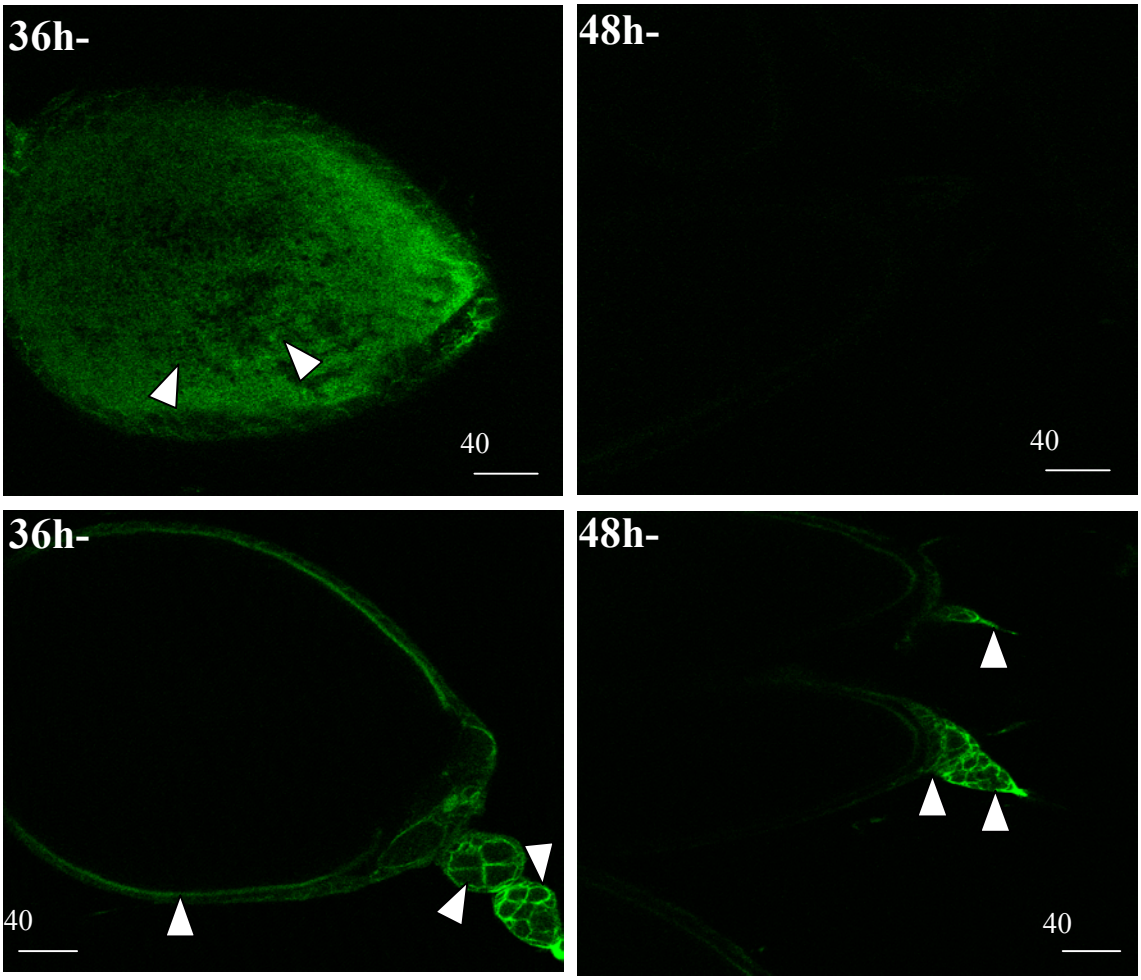


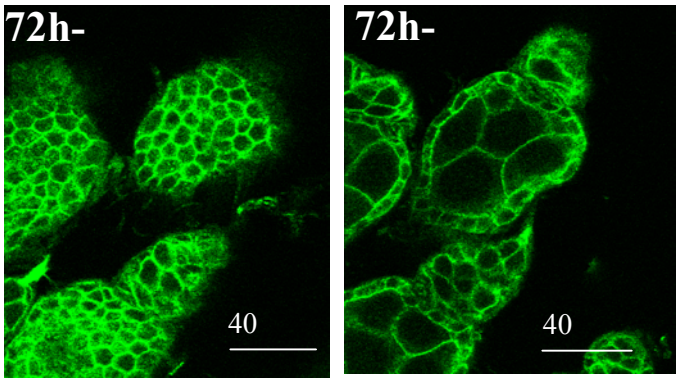
Figure 3.3: Immunoblots of MIR in ovaries of bloodfed mosquitoes. Representative immunoblots of ovaries (3 pairs) treated with MIR β-subunit antibody at various times pbm under **A.** reducing (MW: 90-kDa) and **B.** non-reducing (MW: ~400-kDa) conditions. The MIR is seen at all time points except 24 to 48 h pbm. Ovaries at the 72 h pbm time point were obtained after oviposition.

Figure 3.4: Immunocytochemistry of MIR in ovaries of bloodfed mosquitoes. Ovaries at different times pbm were treated with MIR β subunit antibody and detected with an Alexa Fluor® 488 $F(ab')_2$ fragment of goat anti-rabbit IgG (H + L) secondary antibody. Cross section (**a**) and surface (**b**) of an ovariole shown prior to a blood meal (NBM), and 2, 6, 12, 24, 36, 48 and 72 h after a blood meal. Position of primary follicle, secondary follicle, and germarium indicated in fig. 6h-b. Follicle cells (FC) can be seen on the surface of the ovariole (6h-a) and in cross section (6h-b). Cell membranes of nurse cells can be seen in ovariole cross sections (12h-b). Arrows show the reduced immunostaining at 24 h pbm (24h-a) and the diffuse staining at 36 h pbm (36h-a). By 48 h immunostaining in the primary follicle is gone and only the secondary follicle and the germarium remain immunostained (48h-a and 48h-b). At 72 h pbm, after oviposition, the secondary follicle has matured to the previtellogenic arrest stage and has the same degree of immunostaining as NBM-a and NBM-b.


NBM-a
FC
40 µm
2h-
40 µm
6h-a
FC
40 µm
NBM-b
FC
40 µm
2h-b
40 µm
6h-b
Germariu
Primary
2nd
follicl
FC
40 µm


12h-a
40 µm
24h-
40 µm
12h-b
NC
40 µm
24h-b
40 µm


36h-
40
48h-
40
36h-
40
48h-
40


72h-
40
72h-
40

immunocytochemical results closely correlated with the results from reduced and non-reduced immunoblots (Fig 3.3). Although not visible in every confocal section, immunostaining in the secondary follicles and germarium, including the intense staining at the terminus of the germarium, remained unchanged throughout the reproductive cycle. Ovaries incubated only with the secondary antibody had no detectable staining (data not shown).

A representative northern blot shows MIR and actin transcripts in total RNA from ovaries (10 μg) from 2 to 72 h after a blood meal (Fig 3.5A). Densitometry analysis of the blot gave ratios of MIR and actin transcripts for each time point, and thus determine actual changes in MIR transcript levels during a reproductive cycle (Fig 3.5B). Expression of the MIR transcript was greatest approximately 2 h after a blood meal, and from 6 to 48 h pbm its levels decreased relative to the actin transcript. By 72 h pbm the amount of MIR transcript increased approximately 2 fold over the 6 to 48 h time points and was comparable with the level of expression 2 h pbm.

MIR is phosphorylated in response to activators of steroidogenesis

To determine if stimulators of ovary steroidogenesis activated phosphorylation of tyrosine residues on MIR, 5 to 7 day old ovaries were incubated in vitro with bovine insulin or the phosphatase inhibitor, sodium orthovanadate, at concentrations determined to be optimal for ecdysteroid production in vitro (Riehle and Brown 1999). Immunoblots of the same ovary extracts were treated with an antiphosphotyrosine antibody to examine the level of tyrosine phosphorylation on the MIR and with the MIR β subunit antibody to determine the abundance of the receptor. A representative immunoblot shows an increase in tyrosine phosphorylation on the ~400-kDa MIR when treated with either

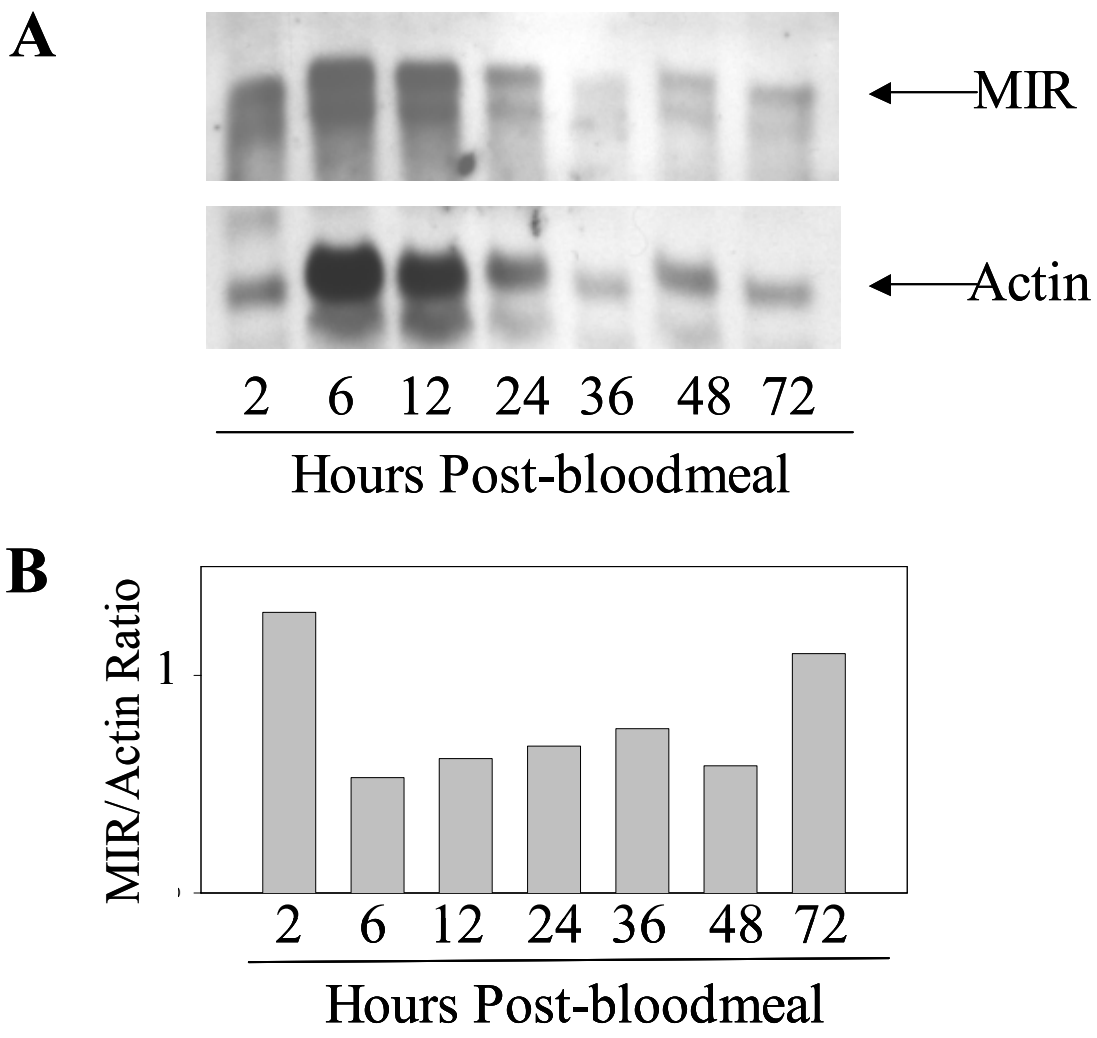


Figure 3.5: Northern blot analysis of MIR in ovaries of bloodfed mosquitoes.

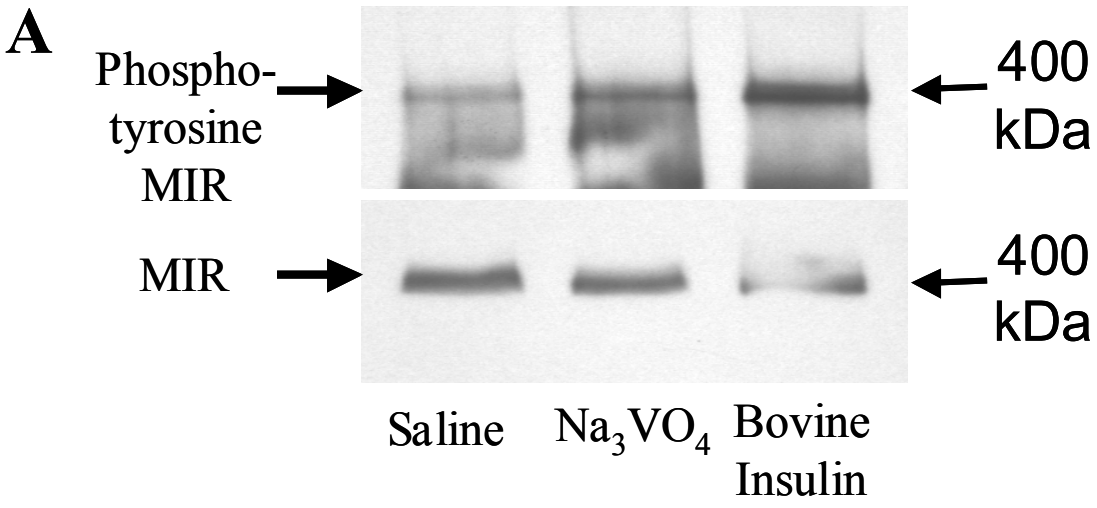
A. Northern blot analysis showing MIR transcript at various time points pbm. Actin was used as a loading control and can be seen at 2.5 KB. Total RNA (10 µg) was loaded into each lane.

B. Densitometry analysis of the northern blot in B. The MIR/actin ratio was used to illustrate the relative differences in MIR transcript at each time point.

bovine insulin or sodium orthovanadate (Fig 3.6A). The ratio of tyrosine phosphorylated MIR (MIR-P) to MIR was determined by qualitative densitometry of the above blots (Fig 3.6B). When treated with the phosphatase inhibitor sodium orthovanadate (1 mM) for 30 min, the MIR-P/MIR ratio in the sample of ovary membranes increased 2 fold over the saline control (Fig 3.6B). Stimulation with bovine insulin (17 μM) resulted in a 4- to 5-fold increase in the MIR-P/MIR ratio compared to the saline control (Fig 3.6B).

Phosphorylated MIR was detected in both cell membranes and the cytoplasm/organelle extract after ovaries were stimulated with bovine insulin (Fig 3.7). A representative immunoblot shows a ~400-kDa band immunoreactive to the antiphosphotyrosine antibody in both the membrane and cytosol/organelle samples (Fig 3.7A, Phosphotyrosine MIR). The same ~400-kDa band was immunoreactive when the representative immunoblot was stripped and incubated with the MIR β subunit antibody (Fig 3.7A, MIR). Considerably less receptor was detected in the cytosol/organelle samples, suggesting that the majority of MIR is present on the cell membrane (Fig 3.7A). The MIR-P/MIR ratio in the membrane samples, as determined by densitometry, doubled during the first five min of incubation compared to the saline control, indicating a two-fold increase in receptor phosphorylation (Fig 3.7B). This high level of receptor phosphorylation was maintained during bovine insulin incubations for up to 30 min. The MIR-P/MIR ratio in the cytosol/organelle sample increased 1.6-fold between 1 and 5 min of insulin stimulation (Fig 3.7B). As in the membrane, high levels of receptor phosphorylation were maintained in the cytosol/orgenelle samples during incubations of up to 30 min with bovine insulin (Fig 3.7B).

Figure 3.6: Stimulation of MIR phosphorylation with sodium orthovanadate or bovine insulin. A. Immunoblot of the phosphorylated MIR after a 30 min incubation in saline solution, sodium orthovanadate (1 mM), or bovine insulin (17 μM). Ten ovary equivalents were loaded per lane and the blots were probed with either antiphosphotyrosine antibody (1:30,000) or the MIR β-subunit antibody (1:5000). The 400-kDa band corresponding to the MIR was quantified and the ratio of MIR to antiphosphotyrosine determined. **B.** Histogram of densitometry showing the degree of receptor phosphorylation relative to the amount of MIR per lane.


A
Phospho-
tyrosine
MIR
MIR
400
kDa
400
kDa
Saline
Na3VO4
Bovine
Insulin

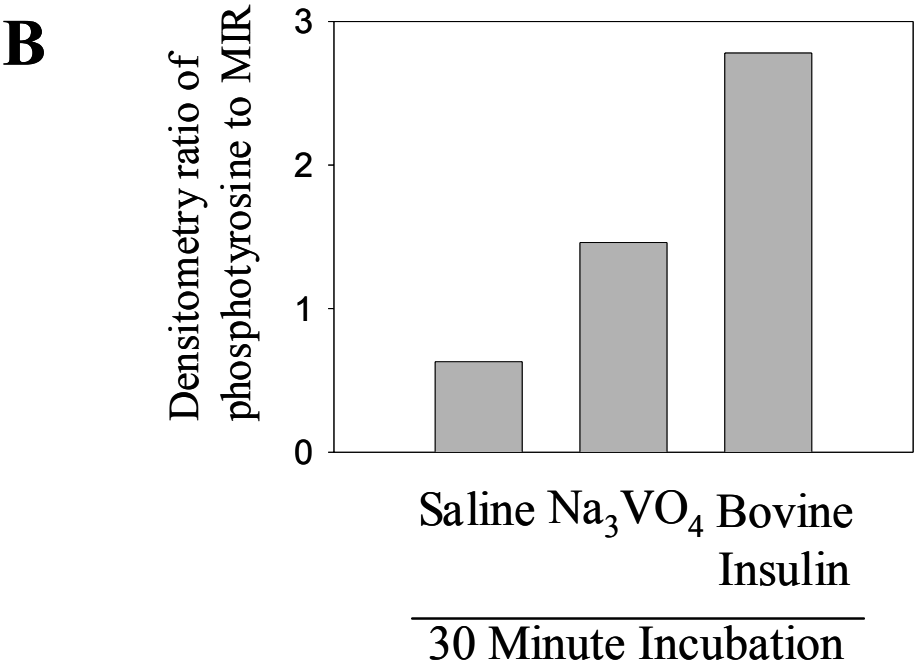
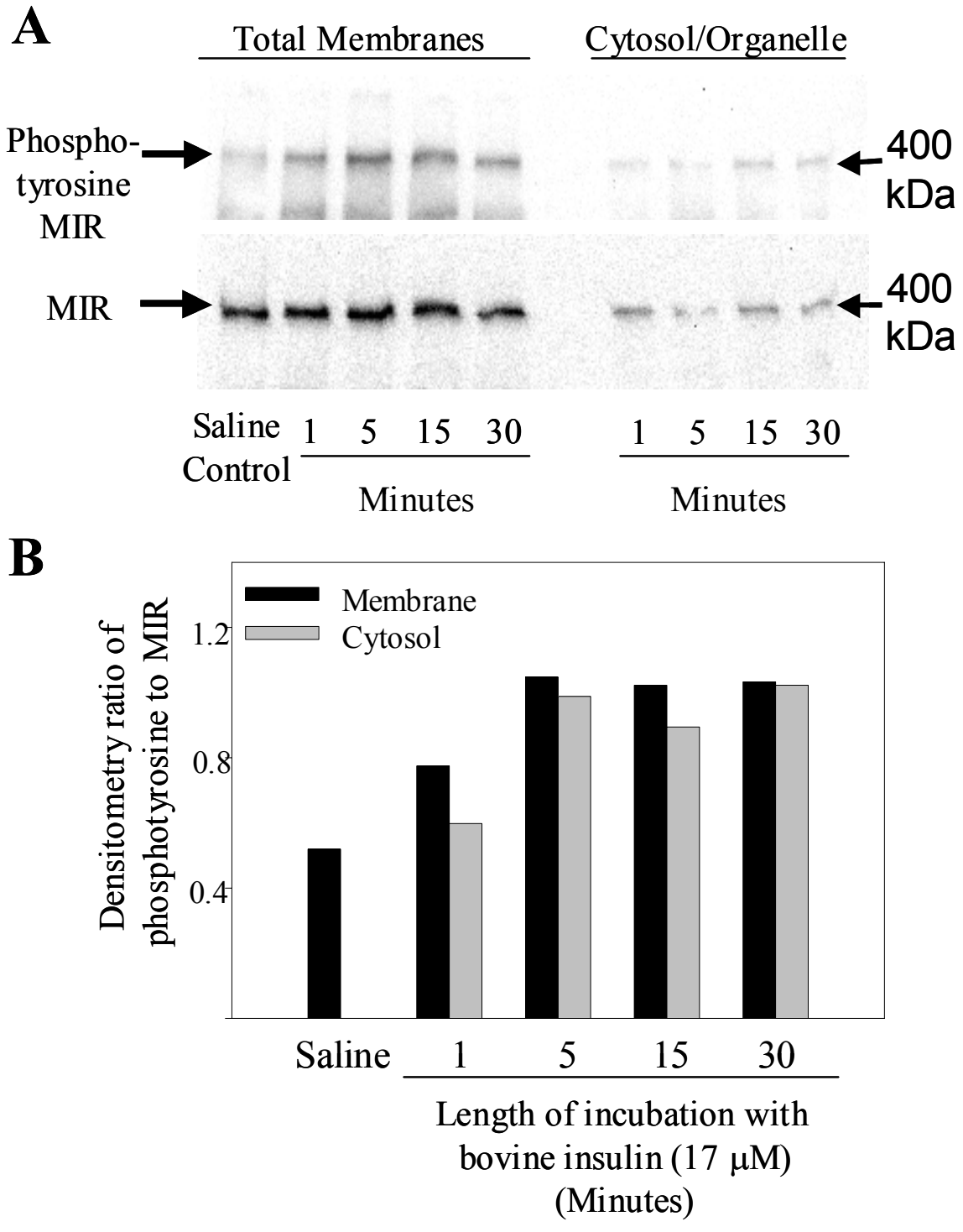

B
Densitometry ratio of
phosphotyrosine to MIR
3
2
1
0
Saline
Na3VO4
Bovine
Insulin
30 Minute Incubation

Figure 3.7: Antiphosphotyrosine time course. A. Immunoblot of phosphorylated MIR in ovaries after incubation with 17 μM bovine insulin for different times. Ten ovary equivalents were loaded per lane. Blots were first probed with an antiphosphotyrosine antibody (1:30,000), stripped, and reprobed with the MIR β-subunit antibody (1:5000). **B.** Histogram of densitometry showing the degree of MIR phosphorylation relative to the amount of MIR per lane for both the membrane and cytosol/organelle fractions.


A
Total Membranes
Cytosol/Organelle
Phospho-
tyrosine
MIR
MIR
400
kDa
400
kDa
Saline
Control
1 5 15 30
1 5 15 30
Minutes
Minutes
B
Membrane
Cytosol
Densitometry ratio of
phosphotyrosine to MIR
1.2
0.8
0.4
Saline 1 5 15 30
Length of incubation with
bovine insulin (17 μM)
(Minutes)

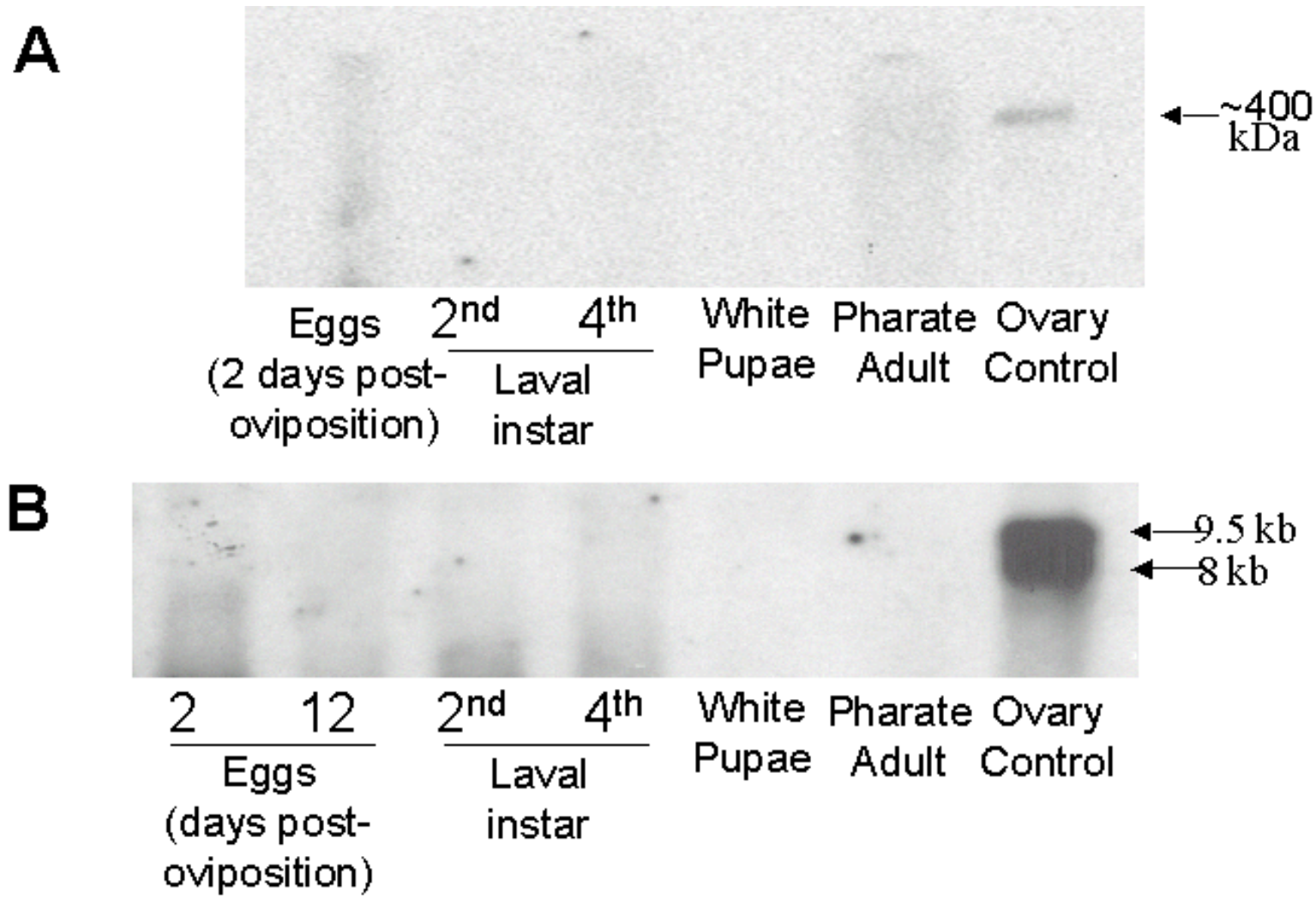
Absence of the MIR in other mosquito life stages

Although MIR transcript and protein is present in adult ovaries, its presence had not been examined in other life stages. Most surprisingly, MIR protein was not detected in immunoblots of eggs, 2nd and 4th instar larvae, white pupae (less then 2 h after pupation), and pharate adults (48 h after pupation), but MIR was present in the 5-day ovary extract included as a control (Fig 3.8A). Furthermore, MIR transcript was not detected on northern blots of mosquito eggs, larvae, pupae, or pharate adults, confirming the above results, but was present in the ovary control (Fig 3.8B).

DISCUSSION

Expression of MIR in the ovaries of *Aedes aegypti* is closely tied to the arrest and vitellogenic cycles of the ovaries. The mature MIR is a ~400-kDa tetrameric RTK consisting of two 116-kDa extracellular subunits, and two 95-kDa transmembrane β subunits. After adult eclosion, MIR expression increases dramatically during the first three days of the previtellogenic phase. When the ovaries enter previtellogenic arrest 2 to 3 days after eclosion, MIR expression remains constant in preparation for a bloodmeal. Once a host is found and a bloodmeal is ingested, vitellogenesis begins, and the follicle cells produce ecdysteroids for the next 24 to 27 h. Throughout vitellogenesis the level of MIR in the follicles cells remains constant. During this time MIR activates steroidogenesis through phosphorylation events when a steroidogenic hormone, such as OEH I or an insulin-like peptide, binds to the extracellular α subunit. When postvitellogenesis begins 30 to 48 h after a bloodmeal, the follicle cells switch from ecdysteroid production to chorion production and the MIR is not found on the developing oocyte. Finally, after oviposition, the MIR is once again expressed in the follicle cells as

Figure 3.8: Expression of MIR in immature mosquitoes. Protein extracts from three larvae, three tissue equivalents of pupae tissue, and approximately 100 eggs were loaded onto the gel. White pupae had pupated less then 2 h prior to protein isolation. A 5-day-old ovary extract was included as a positive control. **A.** MIR was not detected on immunoblots of immatures treated with a polyclonal antibody recognizing the carboxy terminal of the β subunit at various stages under non-reducing conditions. **B.** The MIR transcript could not be detected with northern blot analysis in immature mosquitoes. Adult ovaries were used as a positive control and actin was used as a loading control. 10μg of total RNA was loaded into each lane.


A
~400 kDa
Eggs (2 days post-oviposition)
2nd 4th
Laval instar
White Pupae
Pharate Adult
Ovary Control
B
9.5 kb
8 kb
2 12
Eggs (days post-oviposition)
2nd 4th
Laval instar
White Pupae
Pharate Adult
Ovary Control

the secondary follicle matures to the previtellogenic arrest stage, repeating the oogenic cycle.

Qualitative analysis of the immunoblots and northern blots demonstrate that during the first 3 days after adult emergence, expression of the MIR protein and mRNA transcripts in the ovary increase, followed by steady levels of expression from 3 to 21 days post-eclosion. In *Ae. aegypti*, the primary follicles increase in size from 40 μM to 110 μM during the first 60 h post-eclosion, after which the follicles enter previtellogenic arrest until a blood meal is ingested (Hagedorn et al. 1977). During this period, the number of follicle cells surrounding the oocyte increases from approximately 20 cells at eclosion to over 200 at arrest (Laurence and Simpson 1974). Results from immunocytochemistry in this and other studies have shown that the MIR is localized in the membranes of follicle cells (Helbling and Graf 1998). Thus, the increased MIR protein levels in the first three days post eclosion may be due to division and differentiation of follicle cells as the ovary develops. MIR transcript levels appear to increase in ovaries during the first 3 days post-eclosion when compared to actin transcript levels. If the increase in MIR levels is only due to the increase in the number of follicle cells during this time, actin levels should increase proportionally, resulting in a constant ratio of MIR to actin, which is not observed. The increased expression indicates that the amount of MIR transcript and most likely protein per follicle cell is increasing as the ovary matures. In the arrest stage (3 to 21 days post-eclosion), steady levels of MIR mRNA are present in ovaries, but the level of MIR protein does not increase. This suggests that there is either a high turnover rate of the MIR protein in the follicle cells during the arrested state, or little translation of the MIR transcript occurs.

Changes in the expression of MIR after a blood meal are thought to reflect a shift from steroidogenesis to chorion production by follicle cells. When a bloodmeal is consumed, the previtellogenic arrest stage ends, and the initiation phase of vitellogenesis begins (Sappington and Raikhel 1999). During this phase, the number of follicle cells increase from approximately 200 during the arrest stage to 470 by 10 h pbm (Clements 1992). No significant increase in the level of MIR protein is seen on immunoblots in the first 12 h pbm despite this two to threefold increase in follicle cell numbers. Although this suggests a decrease in the number of receptors per follicle cell, constant staining in the follicle cells was observed with immunocytochemistry during the initiation phase. Most likely, the sensitivity of the immunoblots was not sufficient to detect a significant increase in MIR levels during this time. The bloodmeal also stimulates the release of the neuropeptide, ovary ecdysteroidogenic hormone I (OEH I), resulting in an increasing titer of ecdysteroids in the hemolymph (Brown et al. 1998) that initiates the trophic phase and stimulates the fat body to begin vitellogenin synthesis (Hagedorn et al. 1975). As early as 32 h pbm, chorionic plaques begin to fuse and the follicle cells separate from the oocyte (Sappington and Raikhel 1999). Between 24 and 48 h pbm, the follicles cells are shifting from steroid to chorion production, no MIR was detected in reducing and non-reducing immunoblots. Immunocytochemical results between 24 and 48 h pbm show reduced levels of MIR in the follicle cell membranes at 24 h pbm, diffuse immunostaining across the cell surface at 36 h pbm, and finally, a complete lack of MIR immunostaining by 48 h pbm in the primary follicle. It is likely that by 24 h pbm, after steroid production is completed, the MIR is no longer essential and is degraded by the follicle cells in preparation for chorion production. Between 48 and 72 h pbm, the

secondary follicles develop to the previtellogenic arrest stage (Sappington and Raikhel 1999). Once again, the presence of MIR protein on immunoblots after oviposition is due to expression on the arrested secondary follicle, as confirmed by immunocytochemistry.

Two peaks of MIR transcription occurred in mosquito ovaries after a blood meal, one immediately following a bloodmeal and the other after oviposition. A high level of MIR mRNA transcript relative to actin transcript is observed 2 h pbm. By 6 h pbm the amount of transcript has fallen and a low level of transcript is present during the next 48 h. This low level of transcript is present even after the protein is no longer detectable. During this time the transcript likely is being produced in the secondary follicles that begin to form in response to a rising ecdysteroid titer, 12 to 24 h pbm. MIR transcript levels increased after oviposition, presumably to produce mature MIR for the next reproductive cycle.

This study further characterizes the involvement of the MIR in the signal transduction pathway that activates steroidogenesis after a bloodmeal. As demonstrated, MIR is phosphorylated on tyrosine residues when ovaries are stimulated with the heterologous bovine insulin and the tyrosine phosphatase inhibitor, sodium orthovanadate. Both are known stimulators of steroidogenesis by *Aedes* ovaries in vitro (Riehle and Brown 1999). This tyrosine phosphorylation most likely activates the PI3K/PKB signaling cascade that regulates ecdysteroid production (Riehle and Brown 1999). The endogenous ligand for MIR is most likely a member of the insulin superfamily. Such an insulin-like peptide has been localized in the lateral neurosecretory cells of the mosquito brain using an antibody generated against the *Drosophila* insulin-

like peptide (Cao and Brown 2001). A second potential ligand for the MIR is OEH I since it is the only known steroidogenic hormone identified to date.

A low level of tyrosine phosphorylation is observed on the MIR during the previtellogenic arrest stage however, it is probably not sufficient to activate the PI3K/PKB signaling cascade. MIR phosphorylation begins to increase within 1 min in the presence of bovine insulin and reaches a plateau within 5 min, both in the cell membrane and cytosol/organelle extracts. The speed of this phosphorylation is consistent with studies on the human insulin receptor that begins to be internalized within 30 seconds and reaches an equilibrium by 6 min (Marshall 1985). The presence of the MIR within the cytosol/organelle samples suggests that the receptor may be internalized and recycled in a manner similar to many vertebrate RTKs. Nevertheless, most of the MIR remains in the cell membrane.

Insulin receptor orthologues and the PI3K/PKB signaling cascade regulate a number of physiological events including growth, body size, cell proliferation, life span and reproduction in *Drosophila* and *C. elegans* (Ailion et al. 1999; Chen et al. 1996; Kimura et al. 1997; Marin-Hincapie and Garofalo 1999; Tatar et al. 2001; Tissenbaum and Ruvkun 1998). As demonstrated for female *Ae. aegypti*, the structure of the mature MIR in ovaries is similar to these and other members of the insulin receptor family. Immunoblot analysis with the β subunit antibody did not identify a higher molecular weight band that would suggest the presence of a β subunit with the C terminal extension seen in *Drosophila*, *C. elegans*, and *Lymnaea* (Kimura et al. 1997; Roovers et al. 1995; Ruan et al. 1995). Two MIR transcripts (8 kb and 9.5 kb) that were detected on northern blots of ovary total RNA are comparable to those identified in *Drosophila* embryos and

adults (8.6 kb and 11 kb) (Garofalo and Rosen 1988). The two DIR transcripts are thought to be alternative RNA processing events, and the same may be true in *Ae. aegypti*. Insulin receptors with a C-terminal extension in *Drosophila* and *C. elegans* regulate development and are best characterized in the immature stages by overexpression and mutational analysis (Garofalo and Rosen 1988). Interestingly, the MIR protein and mRNA transcript are not detected in earlier lifestages of mosquitoes (Fig. 7), suggesting that the MIR is specific to adult females, although expression of MIR in males has not been studied. In addition, the MIR may be expressed, not only in the ovary, but in other adult tissues. Although only a single insulin receptor orthologue is present in the genomes of *Drosophila* and *C. elegans*, it is possible that a second RTK with a C terminal extension exists in the immature stages of mosquitoes.

In *C. elegans* and *Drosophila*, the insulin receptor and its signaling cascade have been shown to affect dauer formation and reproductive diapause, a state similar to the arrest stage of *Ae. aegypti*, respectively and shown to regulate life span in both species. Dauer formation in *C. elegans* is affected by mutations in the insulin receptor, *daf*-2, and the PI 3-kinase, *age*-1, resulting in developmental arrest at the dauer larval stage (Riddle et al. 1997). In addition, other mutations in these genes result in a two fold increase in adult life span (Kimura et al. 1997). In *Drosophila*, mutations to the insulin receptor gene resulted in an 85% increase in adult life span (Tatar et al. 2001). Mutations in the *Drosophila* insulin receptor and CHICO, the *Drosophila* insulin receptor substrate, resulted in smaller ovaries characteristic of flies in reproductive diapause (Tatar and Yin 2001). The insulin signaling cascade may be the critical switch between arrest and initiation of reproduction not only in mosquitoes, but in other organisms as well. Since

reproduction is a metabolically demanding event and may reduce longevity, it is not surprising that mutations reducing fecundity in *C. elegans* and *Drosophila* often result in long-lived adults.

Acknowledgements

This work was supported by Public Health Service grant AI33108 from the National Institute of Allergy and Infectious Diseases. Special thanks to Anna Majzlik and Stacie Thompson for maintaining the mosquito colonies, and to Chun Cao, Christina Reeves, and Carl Lowenburger for their technical assistance and insight.

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CHAPTER 4

CHARACTERIZATION OF PROTEIN KINASE B IN THE FEMALE MOSQUITO *Aedes aegypti*[1]

[1]Riehle, M.A. and Brown, M.R. 2002. To be submitted to Insect Molecular Biology.

ABSTRACT

The cDNA for a key protein in the insulin signaling pathway has been characterized from the yellow fever mosquito *Aedes aegypti*. The mosquito protein kinase B (MPKB) transcript is 2239 nucleotides long and encodes a 529 amino acid protein with a putative molecular weight of 60 kDa. Like other members of this family, MPKB possesses a pleckstrin homology domain for binding to the membrane through PtdIns(3,4,5)P_3 and a kinase domain. MPKB possesses a conserved Thr at amino acid position 345 on the regulatory T-loop that is believed to cause a conformational change and expose the catalytic domain when phosphorylated. A conserved Ser, phosphorylated on many PKBs, is found at amino acid 504 near the carboxy terminus. The MPKB became phosphorylated on a Thr when ovaries were stimulated by bovine insulin in vitro, as determined by immunoblot analysis. However, antiphosphoserine antibodies did not recognize MPKB in the same ovary extracts. The MPKB transcript was expressed in the ovary throughout a reproductive cycle, with an apparent increase in expression late in oogenesis (24 to 48 h post bloodmeal), and is also expressed early in embryogenesis (0 to 6 h post oviposition). The transcript could not be detected later in embryogenesis, in other life stages, or in other adult tissues. Likewise the MPKB protein, immunolocalized to the cytosol of epithelial cells surrounding the primary follicles on the ovary, was most abundant late in the reproductive cycle. This expression pattern, consistent with that of the PKB orthologue in *Drosophila*, suggests that MPKB may have an important role in late oogenesis and early embryogenesis.

INTRODUCTION

Egg development in the female mosquito *Aedes aegypti* is initiated by the ingestion of a blood meal that stimulates the release of steroidogenic gonadotropins from neurosecretory cells in the brain (4). These neuropeptides stimulate the ovaries to secrete ecdysteroids that in turn initiate production of vitellogenin in the fat body for uptake by the developing oocyte. Two hormones known to exist in the medial neurosecretory cells of the brain, ovary ecdysteroid hormone I (OEH I) and a putative insulin-like peptide, are both members of the insulin family (4, 6). OEH I regulates steroid production in vitro and egg development in vivo (4), while the putative insulin-like peptide, recognized by antibody staining, has not been isolated and its physiological role has not been determined. In vitro, the heterologous hormone bovine insulin stimulates ecdysteroid production by ovaries through a receptor tyrosine kinase (RTK)/ phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (PKB) signaling pathway (21). The mosquito insulin receptor is thought to be the key receptor in this cascade and has been well characterized (21), but other components of this signaling cascade have yet to be defined. An activator of PKB, the phosphatase inhibitor okidaic acid, stimulated steroid production in the mosquito ovary in the absence of a steroidogenic hormone such as OEH I (21). This finding suggests that PKB is a likely intermediate of the steroidogenic signaling cascade in the mosquito ovary.

Originally discovered 10 years ago, PKB, also known as Akt or Rac, mediates insulin's metabolic effects, cell proliferation, and apoptosis in mammals (23). PKB is a member of the AGC kinase family named for three of its members, protein kinases A, G, and C (20). Other members of this family include $p70^{S6K}$, phosphoinositide-dependent

kinase 1 (PDK1) and PKC-related kinases 1 and 2 (PRK1 and 2). Although this family of kinases has a wide range of physiological functions, all phosphorylate their substrates on serine/threonine residues and share several structural characteristics, including an activation loop and a conserved phosphorylation site at the carboxy-terminus.

In mammals, the metabolic effects of insulin signaling are regulated through at least two distinct phosphorylation events. The phosphorylation of phosphatidylinositol (PtdIns)(4,5)P_2 to PtdIns(3,4,5) P_3 by PI3K provides a binding site for the pleckstrin homology (PH) domain of PKBα in humans (11, 13). Phosphatidylinositol dependent kinase 1 (PDK1), associated with the cell membrane through binding of its PH domain to PtdIns(3,4) P_2, PtdIns(3,4,5) P_3, or PtdIns(4,5) P_2, is known to phosphorylate the Thr^{308} residue of human PKBα (23). Thr^{308} lies on a flexible peptide loop termed the activation loop or T-loop. In its unphosphorylated state, the T-loop inhibits the kinase activity of PKB, but when phosphorylated a conformational change is thought to allow ATP and substrate access to the kinase domain (1, 2). In addition, phosphorylation of a second amino acid residue, Ser^{473}, is required for full activation of human PKBα (24). Ser^{473} was thought to be phosphorylated by a second kinase tentatively called PDK2, although recent evidence suggests that Ser^{473} may be phosphorylated by PKB itself (23).

PKB is reported in the literature from only two invertebrate species, *Drosophila melanogaster* and *Caenorhabditis elegans*. In the fruit fly *D. melanogaster* a single copy of the PKB gene, named *Dakt1*, is translated into two proteins of 66 kDa and 86 kDa (3, 12). Early in embryogenesis, PKB and other proteins in the insulin signaling pathway have been shown through mutational analysis to induce apoptosis (22, 25). Later in

embryonic development, this signaling cascade regulates cell size without affecting apoptosis (22, 26).

As in *Drosophila*, the PKB signaling cascade in *C. elegans* regulates development, metabolism, and lifespan (17). Two PKB genes, termed Akt-1 and Akt-2, have been identified in the genome of *C. elegans* (19). Both Akt-1 and 2 suppress *Daf*-16, the forkhead transcription factor orthologue in *C. elegans*, since expression of both must be reduced to cause dauer arrest (19). However Akt-1 is more biologically active due to the presence of two phosphorylation sites (Thr-308 and Ser-473) compared to a single site (Thr-308) on Akt-2 (19). An activating mutation or overexpression of Akt-1 in *age*-1 and *daf-2* mutants results in a shift from dauer formation to L4 and adult development. However, this shift is only partial in *daf-2* mutants suggesting a second signaling cascade for dauer formation (19).

PKB has been shown in mammals, *Drosophila*, and *C. elegans* to be a nexus in the metabolic branch of the insulin signaling pathway. Evidence also suggests that a PKB orthologue in the mosquito ovary is a key regulator of steroidogenesis. This paper identifies and characterizes protein kinase B, a second member of the steroidogenic signaling cascade in the ovary of mosquito *Aedes aegypti.*

MATERIALS AND METHODS

Insects

Mosquitoes were maintained at 27° C in a 16h light/8h 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 two days, and subsequently were given only distilled water. Adults used in the age time course were given access to 10% sucrose solution

continuously. Adults used for post-bloodmeal experiments were fed on an anesthetized rat until engorged, separated from unfed and partially fed individuals, and maintained on water until needed.

PKB gene identification

Messenger RNA from the ovaries of 3 day old female mosquitoes was isolated using oligo-dT paramagnetic beads (Dynal). First strand cDNA was synthesized from the purified ovary mRNA using the First-Strand cDNA synthesis kit (Pharmacia-Biotech). Degenerate primers to conserved regions of the PKB family were generated (Forward primer: 5'-CARTGGACNACNGTNATYGA-3' (amino acids 79 to 85 of human PKBα) and Reverse primer: 5'-CCRCACATCATYTCRTACAT-3' (amino acids 339 to 345 of human PKBα)) and used to amplify products from ovary cDNA using "touchdown" PCR (95° C for 5 sec, 60° C to 50° C (-2° C every 3 cycles) for 7 sec, 72° C for 10 sec, 17 cycles followed by 95° C for 5 sec, 48° C for 7 sec, 72° C for 10 sec, 40 cycles) in glass capillary tubes with a Rapidcycler PCR machine (Idaho Technologies). After the products were separated on a gel, a 900 bp fragment was gel purified, cloned (TOPO TA cloning kit, invitrogen), sequenced, and found have sequence similarity to the PKB family. The First Choice RLM-Race kit (Ambion) was used to complete the 5' and 3' ends. Nested, specific primers (5' Outer: CGATACGACCCAATAAACC; 5' Inner: CCCAATAAACCTCAGACATACC; 3' Outer: TCCTTCTGCCTTGCTATTC; 3' Inner: CCTTGCTATTCAAAACCTC) were designed to amplify by PCR the entire mosquito PKB (MPKB) sequence from ovary cDNA. Full-length MPKB cDNAs from the ovaries of three separate mosquitoes were sequenced in both the forward and reverse direction to confirm the MPKB nucleotide sequence.

Sequence alignment and phylogenetic tree

The MPKB protein sequence was aligned with those for Dakt (XP_081482), human AKTα (AAL55732), AKT1 from *C. elegans* (T43234) and a PKB orthologue from *Anopheles gambiae* (compiled from the genomic trace data of the *Anopheles gambiae* genome project: sequence submitted to http://www.ncbi.nlm.nih.gov/) using the ClustalW program in the GCG Wisconsin package. The pairwise distances between these sequences were determined with the Jukes-Cantor distance correction method using the Distance program in the GCG Wisconsin package, and a phylogenetic tree was generated using the UPGMA tree construction method in the Growtree program.

Northern Blotting

RNA probes labeled with digoxigenin (Dig) were synthesized from the 900 bp PKB fragment cloned above. The forward primer for PKB (see above) and a M13 (-20) forward primer were used to amplify product by PCR for PKB from the pCR II vector (Invitrogen) that possessed a T7 promoter. This product was used as a template in the MAXIscript i*n vitro* transcription kit (Ambion) to generate an antisense RNA probe containing dUTP-Dig (Roche). The probe was aliquoted and stored at -80° C. Additional probes were generated in the same way to the complete MPKB cDNA and the 5' region of MPKB.

Twenty pairs of ovaries were dissected from female mosquitoes in *Aedes* saline solution (128 mM NaCl, 4.7 mM KCl, and 1.9 mM $CaCl_2$), immediately transferred to RNAlater (50 µl, Ambion) and stored at -20° C until processed. Total RNA was isolated from the ovaries using the RNAeasy mini kit (Qiagen). Loading buffer (5X: 32%

formamide, 2.4% formaldehyde, 4X 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 ten minutes and loaded onto a precast 1.25% Reliant MOPS gel (BMA), and separated at 3 volts/cm for 3 hours in 1X MOPS running buffer. The gel was denatured in 0.05M NaOH for 30 minutes followed by neutralization in 1 M Tris for 30 minutes. The RNA was then vacuum transferred onto nylon membrane (MSI) for one hour and autocrosslinked (120,000 j/cm Stratalinker® UV crosslinker, Stratagene). The blot was prehybridized in ULTRAhyb (7 ml, Ambion) at 65° C for 1 hour in small roller bottles. The labeled probes were denatured for 10 min at 80° C, added to the prehybridization solution (1:20,000 final concentration), and hybridized overnight at 65° C. After hybridization, the blot was washed 3 times (20 min/wash) in 0.5X SSC/0.1% SDS at 65° C followed by a 1 hour block in 1X blocking reagent (Roche). The blot was incubated with an antiDig-alkaline phosphotase antibody (1:10,000) and 1X blocking reagent for 30 minutes followed by 3 washes (20 min/wash) in maleic acid buffer (0.1M maleic acid, 0.15 M NaCl, pH 7.5) and 0.3% Tween 20. The MPKB transcript was detected with CDP-Star (Roche) and visualized with Biomax light X-ray film (Kodak) or an Alpha Inotech chemiluminescent imager. Densitometry was performed with the Alpha Inotech chemiluminescent imager. Blots were replicated at least three times for each experiment.

Immunocytochemisty and confocal microscopy

Ovaries were dissected from mosquitoes in fixative solution (4% paraformaldehyde in PBS (2.5 mM NaH_2PO_4, 8.5 mM Na_2PO_4, and 175 mM NaCl, pH

7.4)), and transferred into fresh fixative solution for 1 to 2 hours at room temperature followed by a 30 min wash in PBST (PBS and 0.5% Triton 100) at room temperature. Ovaries were subjected to three freeze/thaw cycles at -80° C to permeate the membrane, washed in PBST (30 min, RT), blocked in 5% BSA (2 hour, 4° C), and incubated with the diluted human PKB antibody (1:200 dilution; Affinity Bioreagents Inc (ABR)) in PBST overnight at 4° C. Tissues were washed in PBST 3X for 60 min each (4° C), and incubated with a goat anti-rabbit secondary antibody (Alexa^{Tm} 488 goat anti-rabbit IgG (H+L) conjugate, 1:1000; Molecular Probes, Inc) for 12 hours at 4° C. The ovaries were washed twice (15 min each) in PBST and incubated with a donkey anti-goat secondary antibody (Alexa^{Tm} 488 donkey anti-goat IgG (H+L) conjugate, 1:1000; Molecular Probes, Inc) for 12 hours. Three additional 20 minute washes were performed (4° C). Tissues were mounted on a slide in media (PBS:Glycerol 1:1) and examined with a Leica TCS/SP2 confocal microscope.

Immunoblotting

Ovaries were dissected from mosquitoes in medium (139 mM NaCl, 4.05 mM KCl, 1.85 mM $CaCl_2$, 12.5 mM HEPES, 2.5 mM trehalose, 0.3 mM $MgCl_2$, and 0.9 mM $NaHCO_3$; pH 6.5, adjusted with NaOH) and immediately transferred to a solution of 2X protease inhibitor cocktail (Roche) and water. Equal volumes of reducing (2X: 0.125 M Tris-Cl, 4% SDS, 20% glycerol, 0.2 M DTT, 0.02% bromophenol blue, pH 6.8) or non-reducing sample buffer (2X: 0.125 M Tris-Cl, 4% SDS, 50% glycerol, 0.02% bromophenol blue, pH 6.8) were added to the samples. The samples were homogenized, frozen for 15 minutes, vortexed to shear genomic DNA and reduce viscosity, boiled for 5 minutes, and plunged into an ice bath. The treated samples and molecular weight

markers (10-250 kDa, Rainbow Markers, Amersham) were loaded onto a 7.5% Tris-glycine gel (BioRad), separated by SDS polyacrylamide gel electrophoresis (10x Tris/Glycine/SDS running buffer, Biorad), and tank transferred (10 mM Tris, 100 mM Glycine, 10% methanol) to a PVDF membrane (Biorad). After the transfer, blots were blocked for 1 hour in TBST containing 5% BSA. Antibodies to the *D. melanogaster* (generously provided by M. Birnbaum and characterized in (14)) and human PKB (ABR) were added to the blocking buffer at a 1/5,000 and 1/500 dilution respectively and incubated overnight at 4° C with gentle rocking. Blots were washed in TBST (3X, 20 min), incubated with an anti-rabbit antibody conjugated to horseradish peroxidase (1/20,000 dilution, Sigma) in TBST and 2% GS (1 hour, RT), and washed an additional 3 times in TBST (20 min/wash). The immunoreactive proteins were detected using the Renaissance chemiluminescent detection kit (NEN) and Kodak Biomax light film, and quantified with an Alpha Inotech chemiluminescent imager. Experimental immunoblots were replicated three or more times.

Phosphorylated Serine and Threonine studies

Ovaries (20 pairs) were incubated in *Aedes* saline by themselves or with bovine insulin (17 μM), bovine insulin and phosphatase inhibitor cocktail (Sigma), or phosphatase inhibitor alone, for 30 min at 30° C with gentle shaking. After 30 minutes the ovaries were homogenized in loading buffer with 5X protease inhibitor cocktail and 1X phosphatase inhibitor, and prepared for immunoblot analysis as described above. Blots were incubated with polyclonal antibodies against phosphorylated Ser or Thr residues (1:20,000; Zymed) overnight at 4° C. Goat anti-rabbit HRP antibody at a 1:20,000 dilution was incubated with the blots for 1 h at RT.

Tertiary structure modeling

Due to the high percentage of protein sequence identity MPKB and *Dakt*, a model of MPKB's tertiary structure was generated. The amino acid sequence of MPKB was submitted to Swiss-model (http://www.expasy.ch/swissmod/) and used to construct the 3D model based on the structure of Dakt and the human protein kinase A. The amino and carboxy termini of the sequence, including the conserved Ser involved in kinase activation, was not modeled due to low sequence identity.

RESULTS

Identification of a PKB in the mosquito

The complete nucleotide sequence of the MPKB cDNA from the ovaries of female mosquitoes was determined (Figure 4.1). The MPKB cDNA is a 2239 bp transcript that encodes a 529 amino acid open reading frame. Sequence comparisons with other members of the PKB family demonstrate that the PKB gene in the mosquito *Aedes aegypti* has 83% sequence similarity (78% identity) to the *An. gambiae* PKB, 81% sequence similarity (76% identity) to *Dakt1*, 65% and 66% sequence similarity (50% and 52% identity) to Akt 1 and 2 in *C. elegans* respectively, and 73% sequence similarity (61% identity) to the human PKB beta (Figure 4.2). Phylogenetic analysis of vertebrate and invertebrate PKBs shows a close relationship between dipteran PKBs (Figure 4.3). The dipteran PKBs are more closely related to the vertebrate PKBs than the three PKBs identified in another invertebrate, the nematode *C. elegans*. As with other members of the PKB family, the MPKB possesses a pleckstrin homology (PH) domain (AA 28-105), shown to be important in binding of the protein to PI-3,4,5. A Ser/Thr-type protein kinase domain (AA 184-441) is responsible for the phosphorylation of downstream

```
   1 AAAAGTTCTCATCGCGCTCGCTAATAGGGCAGCGTGTGTTTCGAAGTTCGGTGAAAGTGC  60

  61 ACGGGTGCACACGCGAGAGCTTTGGAATTAGCAAGTTGTGGAAGTCGTCATCGTCGGAAG 120

 121 CGATACGACCCAATAAACCTCAGACATACCTCAGAAATTGTGACTTTACCAGTAACAAGG 180

 181 GCTGACTAGAGAAGGAGAGATAGTGACTTGCACCAAAGTGTTACTCGACAAGACATCCAA 240

 241 TCATAATGTCTTCATCGGACACCACTCAACCACCGGCTGTGCCGGTTACACAGCCTGCCC 300
   1     M  S  S  S  D  T  T  Q  P  P  A  V  P  V  T  Q  P  A  R  19

 301 GTGTCATCCAGCCATCTGCAGCGTTGATCGTGAAGGAAGGCTGGCTGTACAAACGTGGCG 360
  20   V  I  Q  P  S  A  A  L  I  V  K  E  G  W  L  Y  K  R  G  E  39

 361 AACACATCAAGAACTGGCGCTCGCGTTATTTCATTCTGCGCGACGATGGGACACTCGTCG 420
  40   H  I  K  N  W  R  S  R  Y  F  I  L  R  D  D  G  T  L  V  G  59

 421 GGTACAAAAATCGTCCAGATGCATCGTTCCAGGCTGAACCATCGAACAACTTCACCGTAC 480
  60   Y  K  N  R  P  D  A  S  F  Q  A  E  P  S  N  N  F  T  V  R  79

 481 GAGGCTGTCAGATAATGTCCGTGGACAGACCGCGGCCATTCACCTTCATCATCCGAGGCC 540
  80   G  C  Q  I  M  S  V  D  R  P  R  P  F  T  F  I  I  R  G  L  99

 541 TACAATGGACCACTGTCATCGAACGCATGTTCCATGTGGAGGAAGAACGGGAGCGCCAGG 600
 100   Q  W  T  T  V  I  E  R  M  F  H  V  E  E  E  R  E  R  Q  E 119

 601 AGTGGGTAGAGGCAATCCGCAGCGTTGCCAATCGCCTCACGGAAGCCGAAGCCTATCAGG 660
 120   W  V  E  A  I  R  S  V  A  N  R  L  T  E  A  E  A  Y  Q  G 139

 661 GATCCCAGTCGAATGGGGACGGCGATGTGGAGATGGCCTCGATTGCCGAGGACGAACTGT 720
 140   S  Q  S  N  G  D  G  D  V  E  M  A  S  I  A  E  D  E  L  L 159

 721 TGACGGAAAAGTTCTCGGTACAGGGCACGTCAACGGGCAAAATCAGTGGCAGAAAGAAAG 780
 160   T  E  K  F  S  V  Q  G  T  S  T  G  K  I  S  G  R  K  K  V 179

 781 TGACGCTGGAGAATTTCGAGTTCTTGAAAGTGCTCGGAAAGGGAACCTTCGGCAAAGTTA 840
 180   T  L  E  N  F  E  F  L  K  V  L  G  K  G  T  F  G  K  V  I 199

 841 TCCTGTGTCGTGAGAAAACCACCGCCAAGCTGTACGCTATCAAGATCCTGAAGAAGGAGG 900
 200   L  C  R  E  K  T  T  A  K  L  Y  A  I  K  I  L  K  K  E  V 219

 901 TCATCGTACAAAAGGATGAAGTCGCTCACACAATGGCCGAGAATCGCGTACTTAAAAAGA 960
 220   I  V  Q  K  D  E  V  A  H  T  M  A  E  N  R  V  L  K  K  T 239

 961 CCAATCATCCTTTCCTGATATCATTGAAGTATTCTTTTCAAACGGTGGACCGCCTGTGCT 1020
 240   N  H  P  F  L  I  S  L  K  Y  S  F  Q  T  V  D  R  L  C  F 259

1021 TCGTTATGCAATACGTCAACGGTGGGGAATTGTTTTTCCATCTCAGTCGTGAGCGCGTTT 1080
 260   V  M  Q  Y  V  N  G  G  E  L  F  F  H  L  S  R  E  R  V  F 279

1081 TCTCGGAGGATAGAACCAGATTTTATGGCGCAGAAATTATATCGGCACTCGGGTATCTAC 1140
 280   S  E  D  R  T  R  F  Y  G  A  E  I  I  S  A  L  G  Y  L  H 299

1141 ATTCACACGAAATCGTCTATCGAGACTTGAAACTGGAGAATCTCTTATTGGATAAGGACG 1200
 300   S  H  E  I  V  Y  R  D  L  K  L  E  N  L  L  L  D  K  D  G 319
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1201 GTCACATCAAAATAGCCGACTTCGGCCTCTGCAAGGAACAGATCACCTACGGACGCACAA 1260
 320   H  I  K  I  A  D  F  G  L  C  K  E  Q  I  T  Y  G  R  T  T 339

1261 CGAAGACCTTCTGCGGCACGCCGGAATATCTGGCGCCGGAGGTACTAGAAGACAACGATT 1320
 340   K  T  F  C  G [T] P  E  Y  L  A  P  E  V  L  E  D  N  D  Y 359

1321 ACGGTCTAGCGGTCGACTGGTGGGGAACGGGTGTGGTAATGTACGAAATGATGTGCGGCC 1380
 360   G  L  A  V  D  W  W  G  T  G  V  V  M  Y  E  M  M  C  G  R 379

1381 GGTTGCCATTCTACAATCGCGATCACGATATTCTGTTCACGCTGATCCTGATGGAAGAGG 1440
 380   L  P  F  Y  N  R  D  H  D  I  L  F  T  L  I  L  M  E  E  V 399

1441 TTAAGTTTCCGCGAAGCATCAGTGCCAACGCACGTGATTTGCTCGCCGGTTTACTGATGA 1500
 400   K  F  P  R  S  I  S  A  N  A  R  D  L  L  A  G  L  L  M  K 419

1501 AGCAACCTAGAGATCGTCTCGGCGGTGGACCAAACGACGTTAAGGAAATTATGGTGCATC 1560
 420   Q  P  R  D  R  L  G  G  G  P  N  D  V  K  E  I  M  V  H  P 439

1561 CATTTTTCTCCAGCATCAACTGGACAGATCTGGTGCAGAAGCGCATAGCACCTCCCTTCA 1620
 440   F  F  S  S  I  N  W  T  D  L  V  Q  K  R  I  A  P  P  F  K 459

1621 AACCACAGGTCACGTCCGACACCGATACCCGTTACTTCGATTCCGAGTTCACAGGTGAAA 1680
 460   P  Q  V  T  S  D  T  D  T  R  Y  F  D  S  E  F  T  G  E  S 479

1681 GCGTCGAGCTAACGCCTCCCGACAACAATGGACCGCTGGGGGCGGTCCAGGAGGAACCGC 1740
 480   V  E  L  T  P  P  D  N  N  G  P  L  G  A  V  Q  E  E  P  H 499

1741 ATTTTTCACAGTTCAGTTACCAGGATATGGCATCTACATTGAATACGCCTTCGTTTATCA 1800
 500   F  S  Q  F [S] Y  Q  D  M  A  S  T  L  N  T  P  S  F  I  N 519

1801 ACAATCCGAACAGCTATGTGTCGATGCAGTGAAACTATCGAGCATAATTTGAGATGCTGT 1860
 520   N  P  N  S  Y  V  S  M  Q  *                              529

1861 GTACTGCAATTGATGGGGCAGTAAACGTGACTTATGCCTTTCGGGGTACTCAATATCGCT 1920

1921 AACACTATCCCATGCTCGATTCAGAATGAGAGTGAGCGTTGCTGCCCTCACAAAGCATTC 1980

1981 GGAACCGTGTGGATTTGCTACGTAAACCAGGTGGGCCTTTTCGTGTTGATTATTGTTTGC 2040

2041 AGTTCGGGAGTGATAAAGAGGACACACAGTTCAGAATATTTAGTTACGGTAACAG**AAATA** 2100

2101 **A**CAAGCAGAGGTTTTGAATAGCAAGGCAGAAGGAGCCAGTAGTTGCTTCAATTACAGTCA 2160

2161 AACTAGACGGACTAGTGTTTAACACAGGACAAGACAATGGTCCTGAAATGGACGCTCAGA 2220

2221 CCCCGCAACAAAAAAAAAA 2239
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Figure 4.1: Nucleotide and protein sequences of MPKB. The MPKB cDNA is 2239 bps long and encodes a 529 amino acid protein. Phosphorylated Thr^{345} and Ser^{504} (boxed), and a putative polyadenylation site are indicated (in bold).

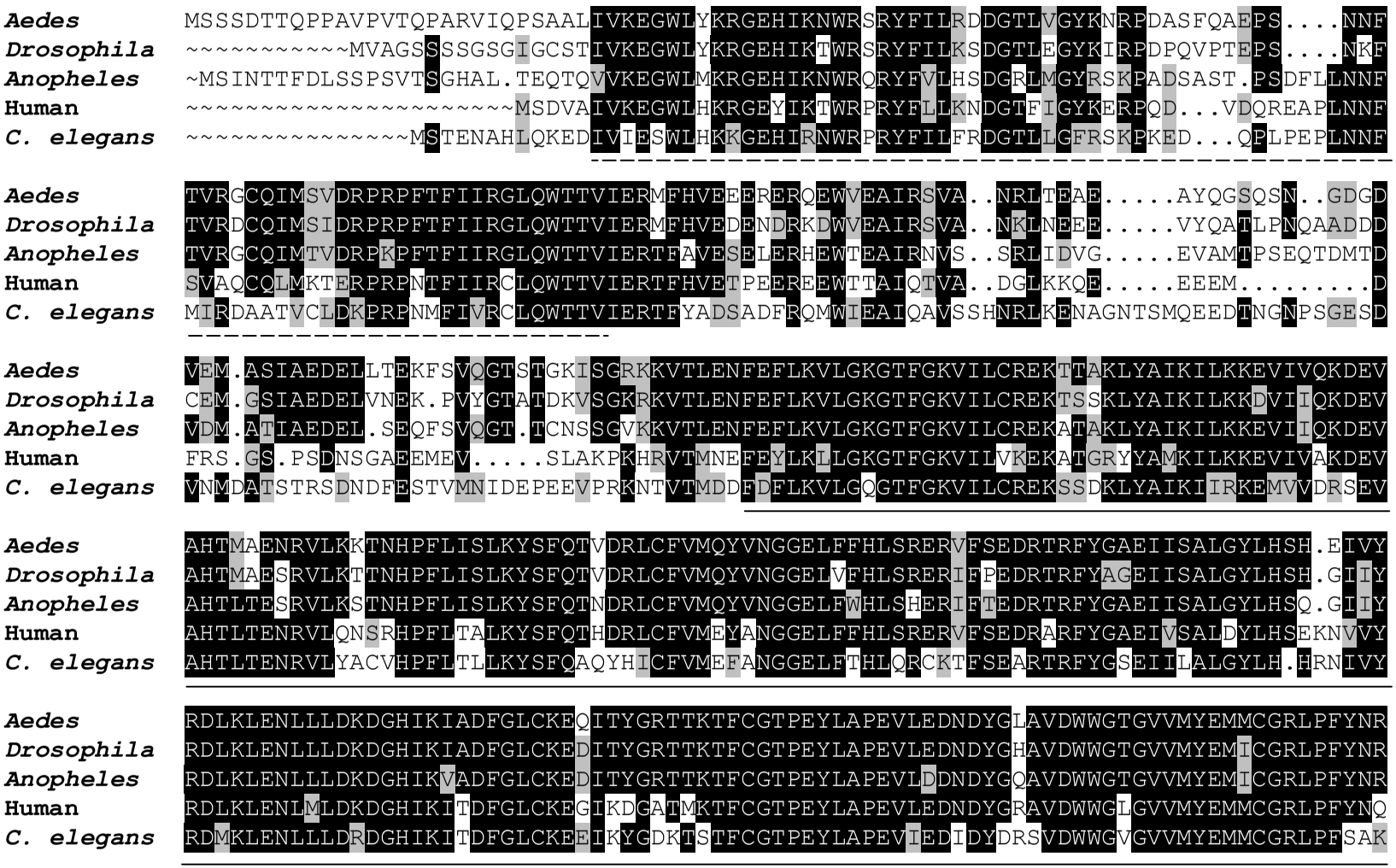
Aedes
Drosophila
Anopheles
Human
C. elegans

Figure 4.2: Pileup analysis of MPKB and related members of the PKB family. Identical residues in at least 50% of the sequences are highlighted in black. Conserved residues in at least 50% of the sequences are shaded grey. The PH domain is underlined with a dashed line and the kinsae domain with a solid line. The *D. melanogaster, C. elegans*, and human PKB sequences were obtained from Genbank. The *Anopheles gambiae* sequence was compiled from the genomic trace data of the *Anopheles* genome project.

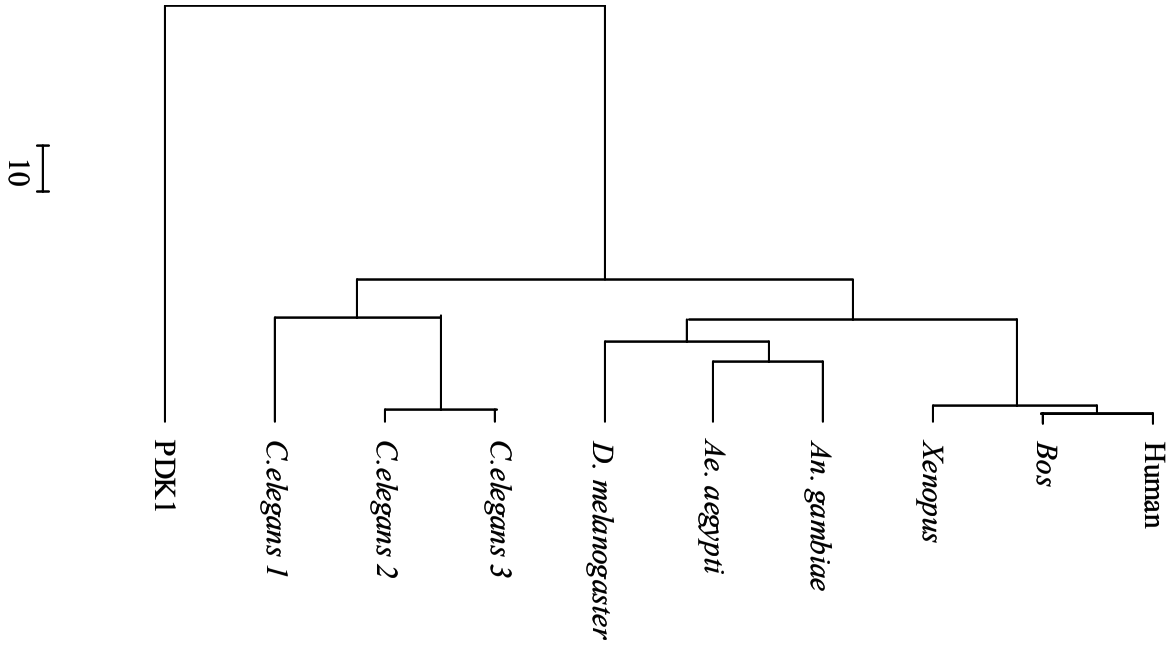

Figure 4.3: Phylogenetic tree of the PKB family. MPKB is most closely related to the two other Dipteran species *An. Gambiae* and *D. melanogaster*. Surprisingly, MPKB is more closely related to the vertebrate PKBs then to three other invertebrate PKB in *C. elegans*. The tree was generated using the Distances program in the GCG package and phosphatidylinositol dependant kinase 1 (PDK1) from *Drosophila* was used as an unrelated outlier.

substrates. It possesses a Ser residue at position 524 in a carboxy-terminal hydrophobic region and a Thr residue at position 365 in the T-loop, which are potential phosphorylation sites that may be needed to activate the catalytic domain. The amino acid sequence from 191 to 196 and the Lys residue at position 215 conform to a consensus ATP binding motif (G-X-G-X-X-G). The cDNA has a 245 bp 5' UTR and a 396 bp 3' UTR not including the polyA tail. The 3' UTR has an AAATAA region thought to regulate polyadenylation and an mRNA destabilization signal (ATTTA) immediately upstream of the polyadenylation site.

With the Swissmodel program, the tertiary structure of the MPKB kinase domain was modeled from the closely related Dakt1, which was used as a backbone (Figure 4.4). Considering the sequence identity between these proteins (76%), one would expect a model with an accuracy of approximately 1Å. The 3D model of MPKB clearly shows the flexible T-loop critical for activation of the kinase domain. On the T-loop resides Thr^{365} that is phosphorylated by PDK1 and likely causes the conformational change in the T-loop that allows substrate access to the catalytic core.

MPKB expression in immature mosquitoes and adult tissues

Expression of the MPKB transcript was found to be specific to the adult ovary and early embroys. Using northern analysis, we detected a 2.5 and 2.3 KB mRNA transcript in both bloodfed and non-bloodfed ovaries and in 0 to 3 hour embryos. The MPKB mRNA was not detected in 10 μg of total RNA from other stages (6+ h embryos, larvae, pupae, and pharate adults; Figure 4.5A) or in head, midgut, thoracic muscle, and body wall of females (Figure 4.5B). A high molecular weight band (~5 KB) was seen in

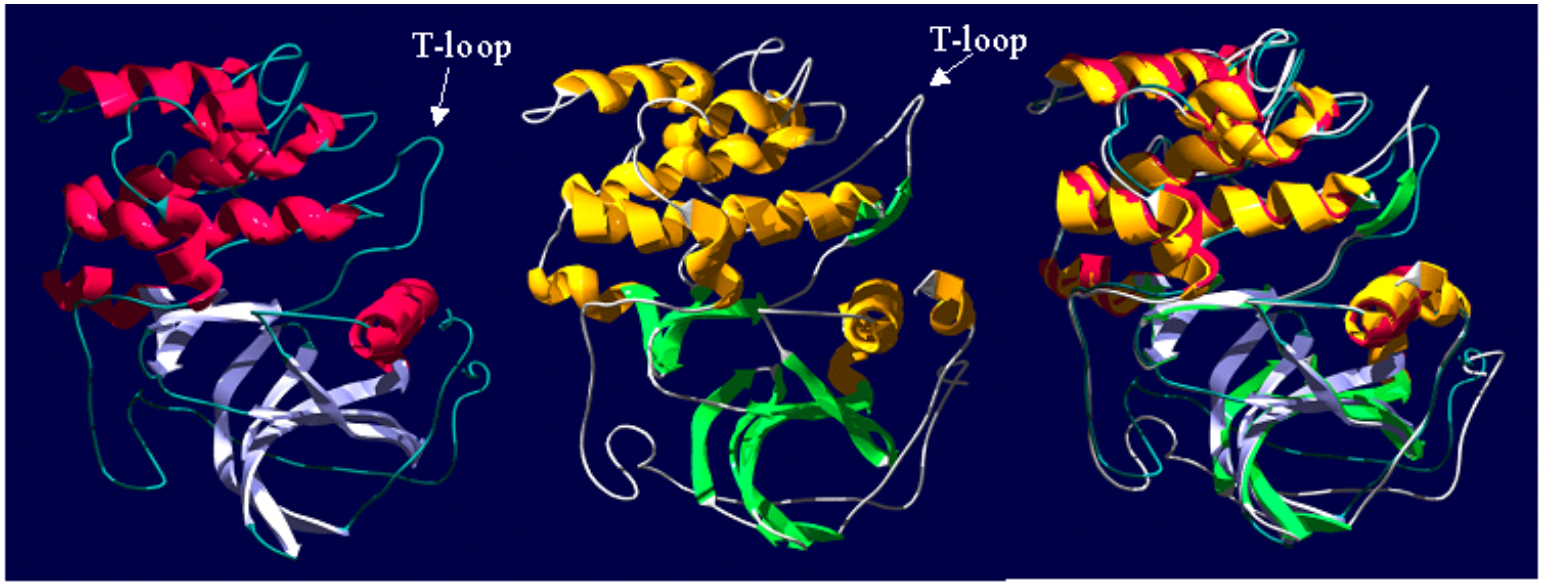

Figure 4.4: Putative tertiary structure of the MPKB kinase domain compared with *Dakt*. The high conservation of the kinase domain of the AKT family results in a 3D model, generated with Swissmodel, with an accuracy of approximately 1Å. Note the conserved T loop that when phosporylated on a Thr residue moves to expose the catlytic core.

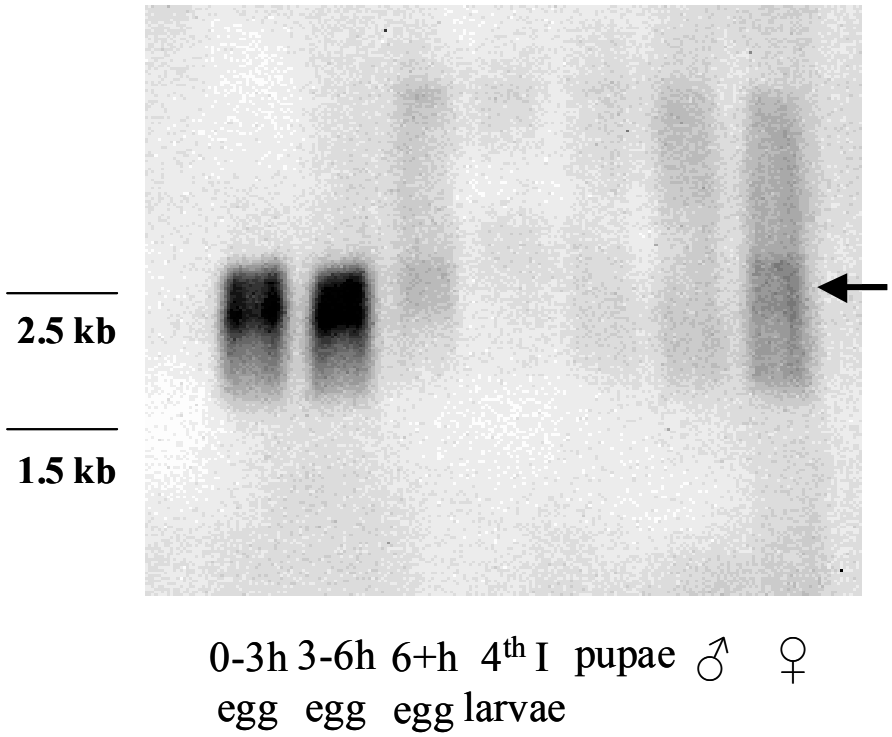

Figure 4.5A: MPKB transcript in eggs, larvae, pupae, and adults

Northern blot analysis showing MPKB transcript only in 0 to 6 h embryos and adult females (♀). Expression in adult females is low (arrow) since Total RNA was obtained from whole bodies and MPKB is only expressed in the ovary. MPKB transcript could not be detected in 4th instar larvae, pupae, or males (♂). Total RNA (10 µg) was loaded into each lane.

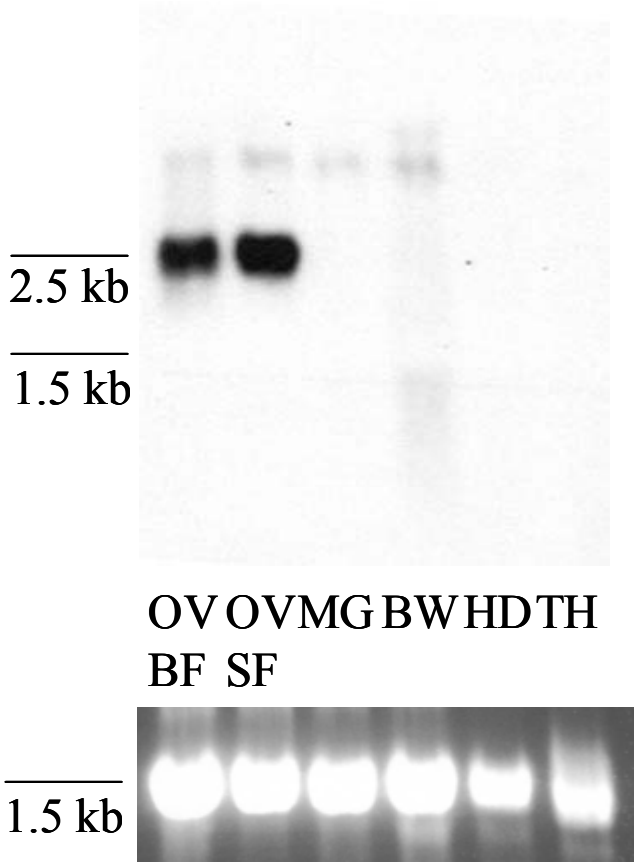

Figure 4.5B: Northern blot analysis of MPKB in female tissues

Northern blot analysis showing MPKB transcript only in 24 h bloodfed (OV BF) and sugarfed (OV SF) ovary. MPKB transcript could not be detected in midgut (MG), body wall (BW), head (HD), or thorax (TH). Total RNA (10 μg) was loaded into each lane. Lower gel is stained with ethidium bromide to demonstrate RNA integrity and as a loading control.

the various adult tissues and most likely represents the closely related and considerable larger protein kinase C.

In females, expression of the 2.5 and 2.3 kb MPKB transcript varied during the first week after adult emergence. For the first 24 hours after eclosion, little MPKB transcript was detected from 10 ovary equivalents of total RNA (Figure 4.5C). From 1 to 7 days after eclosion, levels of MPKB transcripts remained fairly constant with a slight increase at the end of previtellogenesis, 3 days post-eclosion.

MPKB expression during a reproductive cycle

The expression pattern of MPKB protein and transcript was examined in ovaries during a reproductive cycle. Total RNA from 10 pairs of ovaries was used to standardize the loading of RNA since rRNA levels rise and fall drastically between a blood meal and oviposition. MPKB transcript decreased immediately after a blood meal compared to prebloodmeal levels (Figure 4.6a). From 12 to 48 hours post blood meal (PBM) a two to three fold increase in MPKB levels was observed. By 72 hours PBM, after oviposition, the level of MPKB transcript dropped to a quarter of the 48-hour level.

Immunoblot analysis using a human PKB polyclonal antibody detected a 56-kDa protein between 24 and 48 hours after a bloodmeal. No PKB protein was detected in sugar fed mosquitoes, during the first 24 hours after a bloodmeal, or after oviposition. Immunoblot analysis using a *Drosophila* PKB polyclonal antibody detected MPKB (60 kDa) between 36 and 72 hours after a bloodmeal, with no PKB detected in sugarfed females or during the first 36 hours after a bloodmeal (Figure 4.6b and c). These heterologous antibodies had a low affinity for MPKB and most likely could not detect low levels of MPKB protein at the earlier time points.

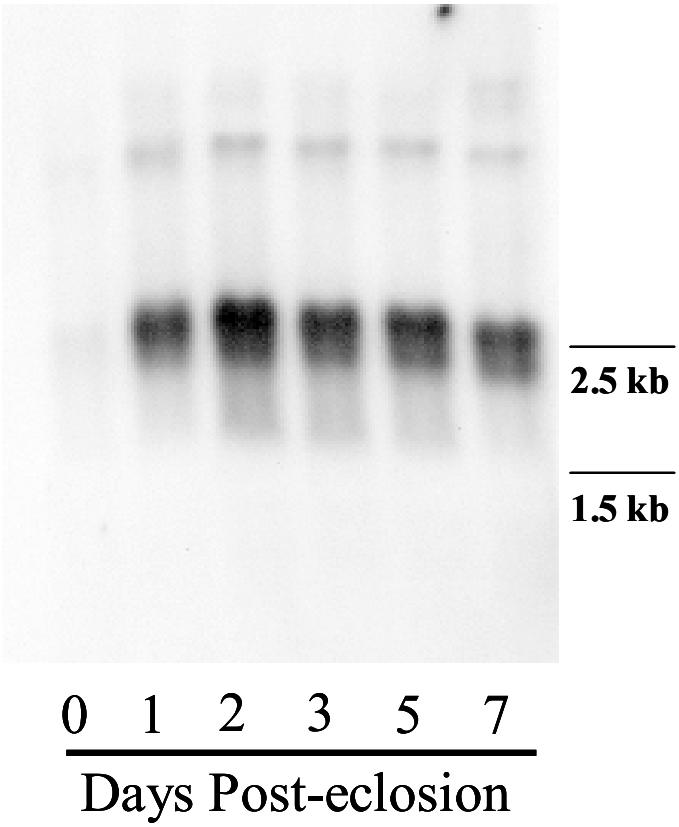

Figure 4.5C: Northern blot analysis of MPKB in ovaries of females after eclosion

MPKB transcript is barely detectable immediately after eclosion, but increases dramatically during the first 2 days after eclosion. During previtellogenic arrest (3-7 days) MPKB transcript levels remained steady. Total RNA from 10 pairs of ovaries was loaded into each lane.

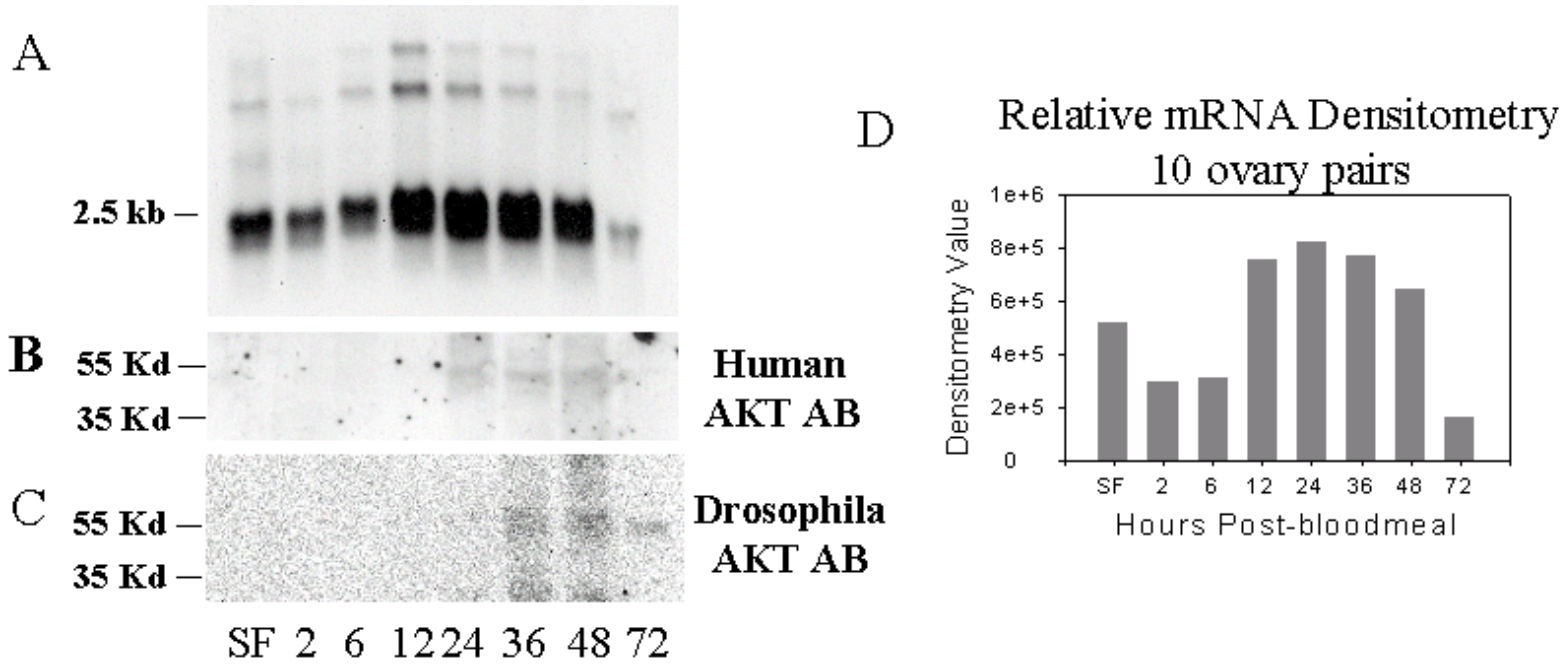

Figure 4.6: Expression of the MPKB transcript and protein before and during a reproductive cycle. A. Representative northern blot of ovary RNA incubated with an MPKB probe. Total RNA from 10 pairs of ovaries was used at each time point. B. Immunoblot incubated with an antibody against the human AKT. A ~55 kDa band is detected from 24 to 48 h PBM. C. Immunoblot incubated with an antibody against the *Drosophila* AKT. A ~55 kDa band is detected from 24 to 72 h PBM. D. Relative densitometry of MPKB transcript after a blood meal. An increase in transcript is observed from 12 to 48 h PBM corresponding to the increase in protein expression observed on the immunoblots.

Immunolocalization of MPKB in the ovary

Immunocytochemistry using antibodies against both the *Drosophila* and human PKB detected the MPKB in the follicle cells surrounding the oocyte in the primary follicle (Figure 4.7). The immunostaining was localized in the cytoplasm and not on the membrane surface or nucleus, even after ovaries were stimulated for 30 min with bovine insulin, a heterologous ligand known to stimulate steroidogenesis and the insulin signaling cascade in the ovary.

A putative MPKB protein is phosphorylated on threonine after insulin stimulation

On immunoblots incubated with an anti-phosphothreonine antibody, a single 60 kDa protein was strongly phosphorylated on threonine in whole ovary extracts when stimulated with bovine insulin, a known activator of steroidogenesis and the insulin signaling cascade in the ovary (Figure 4.8). This protein has the expected size of MPKB and shows a minor but insignificant increase in Thr phosphorylation when stimulated with bovine insulin, a 2.3 fold increase with bovine insulin and phosphatase inhibitor added to prevent constitutive dephosphorylation, and a 1.7 fold increase when incubated with phosphatase inhibitor alone as a control. Two additional proteins, at 105 and 120 kDa respectively, in the ovary extract were also strongly phosphorylated on threonine residues but phosphorylation of these proteins did not significantly increase with bovine insulin stimulation (not shown). We did not observe any Ser phosphorylation on proteins extracted from ovaries stimulated with bovine insulin.

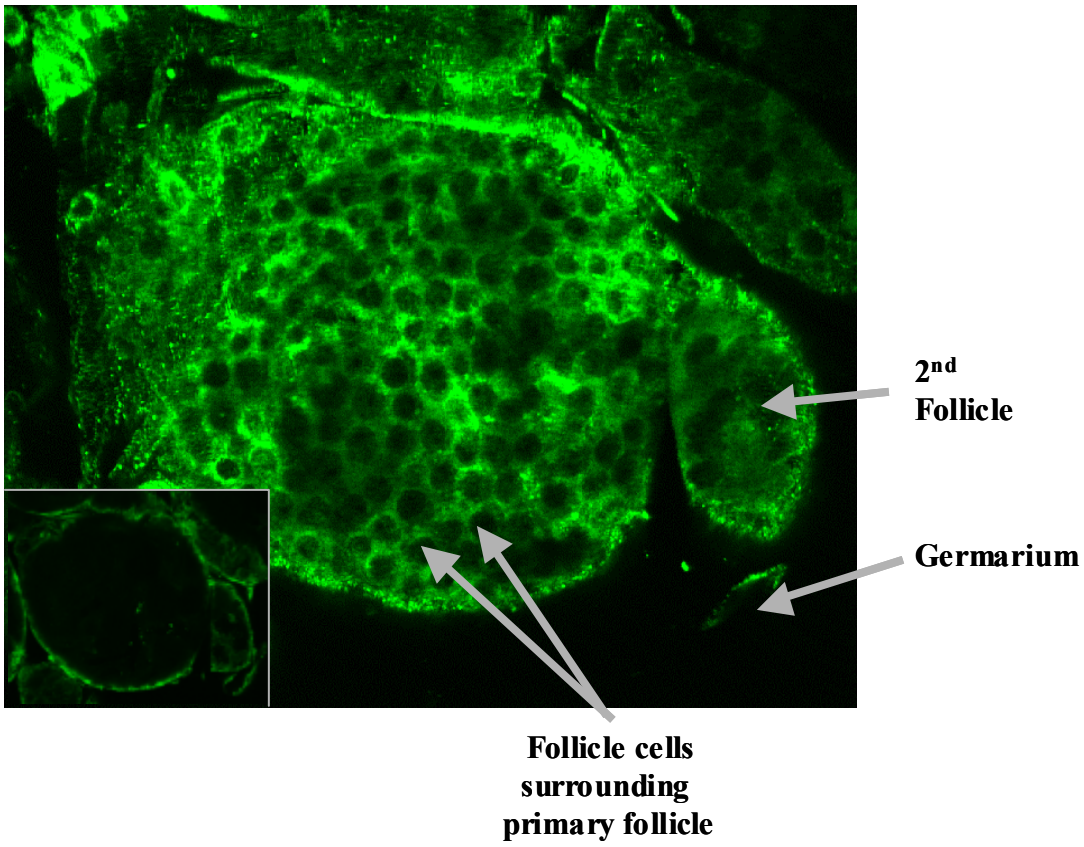

Figure 4.7: Immunolocalization of the MPKB in the follicle cells surrounding the primary follicle. The surface of a ovariole from a 3 day old female mosquito prior to a bloodmeal was immunostained with the human AKT antibody. Immunostaining is observed only in the cytoplasm of the follicle cells, not at the cell membrane or nucleus. Inset: Cross section of the same ovariole immunostained with the human AKT antibody.

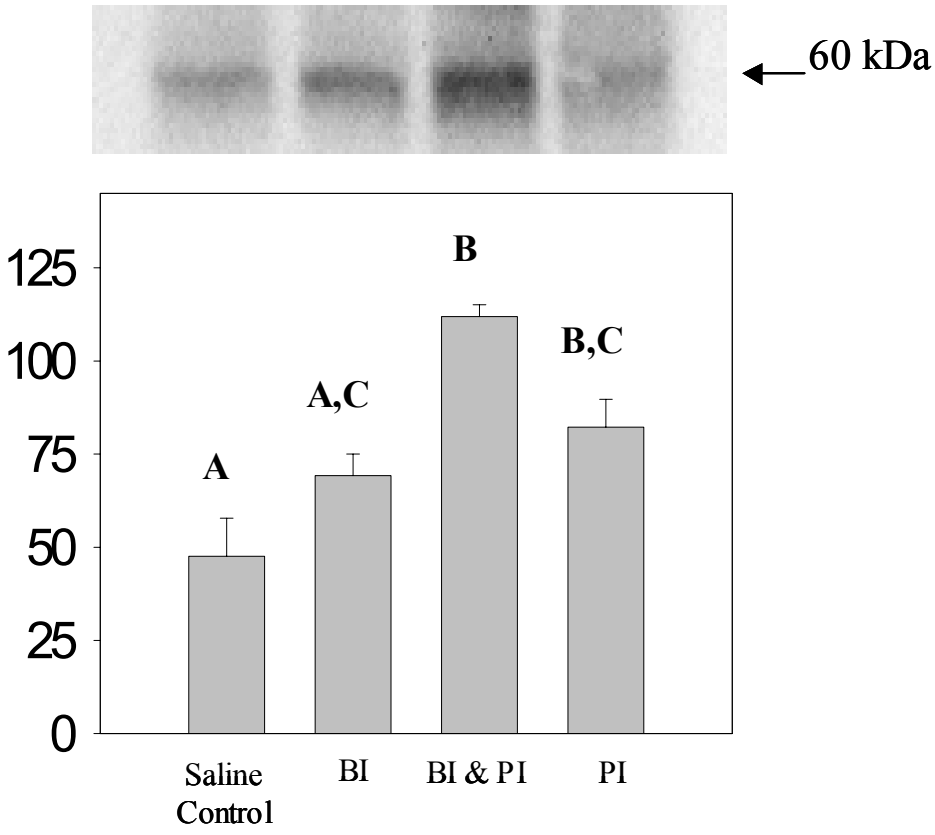

Figure 4.8: Immunoblot analysis of threonine phosphorylated proteins in the ovary. A 60 kDa ovarian protein, potentially MPKB, was threonine phosphorylated in the presence of 17 μM bovine insulin (BI) and phosphatase inhibitor (PI) or phosphatese inhibitor alone. An anti-phosphothreonine antibody was used to detect the protein. Experiments were replicated three times and statistically analyzed with ANOVA and Tukey's test. Significantly different samples are denoted with a unique letter.

DISCUSSION

In the mosquito *Aedes aegypti*, the insulin signaling cascade is a critical regulator of steroidogenesis during egg development in the ovaries as demonstrated in vitro using a variety of activators and inhibitors of this cascade (21). A critical component of this signaling cascade is protein kinase B (also termed *akt* and RAC). This paper has characterized a protein kinase B cDNA in the mosquito *Aedes aegypti* and examined its expression patterns.

The MPKB shares considerable sequence identity with other members of the PKB family, in particular PKBs from other dipteran species, with 79% sequence identity to the mosquito *An. gambiae* and 78% sequence identity to the fruit fly *D. melanogaster*. Furthermore, the dipteran PKBs are more closely related to the vertebrate PKBs than to the other known invertebrate PKBs from *C. elegans*.

The MPKB, a globular, cytosolic protein of approximately 50 to 60 kDa in weight, has the same features as other members of the PKB family. The kinase domain of MPKB from amino acids 184 to 441 is highly conserved and contains a conserved Thr at position 365. Phosphorylation of this residue most likely results in a conformational change to the highly flexible T-loop that is thought to allow substrate and ATP access to the kinase domain. The Thr phosphorylation of a 60 kDa protein in extracts from ovaries stimulated with bovine insulin most likely reflects phosphorylation of Thr^{365} on MPKB. Within the kinase domain is also an ATP binding motif at positions 191 to 196 to bind ATP and provide phosphates for the substrates. The pleckstrin homology domain, located at amino acids 28 to 105 of MPKB, is another defining characteristic of the PKB family. The PH domain of PKB binds to PtdIns(3,4,5)P_3 at the cell membrane allowing it

to be phosphorylated and activated by PDK1. Finally, a conserved Ser is found at position 524 near the carboxy terminus of MPKB. Unlike other PKBs, MPKB did not appear to be Ser phosphorylated in extracts of ovaries stimulated with bovine insulin.

PKB is a key component of the insulin signaling pathway in mammals and regulates insulin's metabolic effects, apoptosis, and cell proliferation. PKB regulates insulin's metabolic effects through the phosphorylation and inactivation of glycogen synthase kinase 3 (GSK3) resulting in increased protein and glycogen synthesis (8), and the translocation of GLUT4 glucose transporters from intracellular vesicles to the cell surface (16). Mice deficient in PKBβ have a phenotype similar to Type II diabetes mellitus in humans, including insulin resistance and elevated glucose levels (7). Evidence also suggests that PKB can phosphorylate insulin receptor substrate (IRS) resulting in a negative feedback loop after prolonged insulin stimulation (18). Cell survival is promoted by PKB through the direct or indirect phosphorylation and inactivation of several apoptotic genes including BAD, several caspases, and possible GSK3 (5, 9, 10, 16). Furthermore forkhead transcription factors appear to regulate expression of some apoptotic genes, such as the Fas ligand, and inactivation of the transcription factors by PKB phosphorylation may promote cell survival (5). Originally identified as an oncogene, PKB also promotes cell proliferation. Members of the p21 family that reversibly inhibit the cell cycle can be phosphorylated and inactivated by PKB resulting in cell proliferation (27). PKB also increases cell proliferation through the increased translation of Cyclin D proteins, key regulators of cell cycle progression (15).

In invertebrates, PKB is a key regulator of growth, apoptosis, and reproduction, and in female *Ae. aegypti*, the insulin signaling cascade, including PKB, regulates steroid

production in the ovary. However, its role in growth and development in mosquitoes is not known. We examined the expression pattern of the MPKB transcript at a variety of developmental stages and adult tissues to determine when and where MPKB mRNA is expressed. Expression of the 2.3 and 2.5 kb MPKB mRNA only occurs in the adult ovary, most likely playing a role in steroid production, and in early embryos where it may be involved in embryonic development. In *Drosophila*, two Dakt transcripts, 4 kb and 2.7 kb respectively are generated as a result of multiple polyadenylation sites on the gene. Interestingly, the smaller 2.7 kb transcript has a size and expression pattern (0-3 h embryos and adult females only) identical to that of MPKB. In contrast, the 4 kb Dakt transcript is strongly expressed during the first 12 hours in the embryo, moderately expressed in older embryos and larvae, and weakly expressed in adult females. The two Dakt proteins, 66 kDa and 85 kDa respectively are translated from a single gene in *Drosophila* with the larger protein resulting from a weak translational start site 81 amino acids upstream of the primary start site. The 85 kDa protein is moderately expressed in early embryos, weakly expressed in late embryos, pupae and adult, and not expressed in larvae. The 66 kDa Dakt 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 Dakt in adults, although less abundant than in embryos, has an intrinsic kinase activity ~8 fold higher than in embryos (3).

In the mosquito, MPKB is thought to be a critical component of the steroidogenic-signaling cascade. Steroid production occurs in the follicle cells of the developing ovaries during the first 36 hours after a bloodmeal. Between 36 and 48 hours postbloodmeal, the role of the follicle cells switch from steroid production to chorion

production. Throughout the reproductive cycle, the MPKB transcript could be detected in the ovaries, but surprisingly the greatest expression occurred 24 to 48 hours pbm, a time when the follicle cells are producing chorion not ecdysteroids. The MPKB protein, like the transcript, was highly expressed during late egg development. But in contrast to transcript expression, the protein was not detected with immunoblot analysis prior to a bloodmeal or during early egg development. However, the heterologous antibodies we used tended to bind poorly on immunoblots, and when sufficient ovaries were used (>100 pairs) the MPKB could be detected in sugarfed ovaries using immunoblotting (data not shown). In contrast, using immunocytochemistry with the same antibodies, MPKB was readily detected in the follicle cells of sugarfed mosquitoes. MPKB was localized in the cytosol of the follicle cells, not on the cell membrane or nucleus. This is not surprising since PKB proteins only associate with the membrane after PI3K phosphorylates PtdIns(4,5)P_2 on the third position, providing a binding site for PKB at the membrane. However, immuncytochemistry studies did not demonstrate any association of MPKB with the follicle cell membrane after 5 min and 20 min stimulation with bovine insulin (data not shown). The antibody's inability to recognize PKB on immunoblots may be due to the loss of antigenicity when bound to a membrane substrate, a modification in the antigenic site under reducing and denaturing conditions, or simply due to the lower concentration of antibody used. In summary, it appears that the MPKB transcript and protein is present at low levels during steroid production in the follicle cells and becomes more abundant during the later stages of egg development.

The MPKB is the second protein characterized in the insulin-like steroidogenic signaling cascade and provides another target for disruption of egg development. Future

studies will examine the effects of MPKB on reproduction and steroidogenesis through the use of dsRNA interference assays, and identify downstream targets of MPKB, in particular transcription factors such as forkhead and the genes they regulate.

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CHAPTER 5

PARTIAL CHARACTERIZATION OF A CLASS 1 AND CLASS 3 PHOSPHATIDYLINOSITOL 3-KINASE FROM THE OVARIES OF THE MOSQUITO *Aedes aegypti*

Introduction

Steroidogenesis in the ovaries of the female mosquito *Aedes aegypti* is initiated by the ingestion of a blood meal that stimulates the release of steroidogenic gonadotropins from the brain (1). These neuropeptides stimulate the ovaries to secrete ecdysteroids that in turn initiate production of vitellogenin in the fat body for uptake by the developing oocyte. Two of these steroidogenic peptides, ovary ecdysteroid hormone I (OEHI) and an insulin-like peptide, both members of the insulin family, are known to exist in the medial neurosecretory cells of the brain. In vitro, the heterologous hormone bovine insulin stimulates ecdysteroid production by ovaries through a receptor tyrosine kinase (RTK) and a phosphoinositide 3-kinase (PI3K)/ protein kinase B (PKB) signaling pathway (9). The mosquito insulin receptor (MIR), the key receptor in this cascade, has had its expression pattern and steroidogenic role well characterized (9, Riehle and Brown, in press), and mosquito PKB (MPKB) has recently been characterized in *Aedes aegypti* (Chapter 4). However the signaling molecules between the MIR and MPKB have not been isolated. An important protein transducing the signal between the receptor and the metabolic branch of the insulin signaling cascade is PI3K.

Three classes of PI3K, varying in their physiological role and substrate specificity, have been described in mammals. The role of PI3K in both vertebrates and invertebrates is to phosphorylate inositol lipids in the cell membrane at the D3 position of the inositol ring (3, 8, 13). Class I PI3Ks are activated by cell-surface receptors and have a broad substrate specificity, being able to phosphorylate phosphatidylinositol (PtdIns), PtdIns(4)*P*, and PtdIns(4,5)P_2. In mammals, a Class 1 PI3K is involved in insulin receptor signaling and consists of 2 subunits, an 85 kDa regulatory subunit and a 110 kDa catalytic subunit (13). Phosphorylated tyrosine residues on IRS with the motif YXXM act as docking sites for the SH2 domain on the regulatory subunit, and binding of the p85 regulatory subunit releases the p110 catalytic subunit, activating its kinase activity and allowing it to translocate to the cell membrane. At the cell membrane it phosphorylates one of the above PtdIns at the third position creating PtdIns(3)P, PtdIns(3,4)P_2, or PtdIns(3,4,5)P_3.

Class 1 PI3Ks have been described for a number of non-vertebrate species including *C. elegans*, *D. melanogaster*, *Dictystelium*, yeast, and soybean. As with other components of this signaling cascade they have been best characterized in the two model invertebrates, *D. melanogaster* and *C. elegans*. In *Drosophila*, the catalytic subunit of the Class 1 PI3K has been termed Dp110 (4). The regulatory subunit binding to Dp110 is a 60 kDa protein that immunoprecipitates with Dp110 antibodies (15). The p60/Dp110 complex is found in *Drosophila* larvae, pupae, and adults (15). As with DIR and *Chico*, Dp110 is involved in the regulation of growth and body size. Overexpression of Dp110 in eye and wing imaginal discs result in enlargement of the respective tissues (14). In contrast, expression of a nonfunctional Dp110 results in reduced wings and eyes. It is

interesting to note that the differences in wing size resulted from changes in both cell size and number, indicating a role for Dp110 in both growth and mitosis, whereas differences in eye size resulted from changes in cell size only. Although it is believed the insulin signaling cascade, and thus PI3K, is involved in regulating adult lifespan in *Drosophila*, no evidence yet exists for the involvement of Dp110 in this role.

The *C. elegans* p110 subunit, *age*-1, was the first gene found to regulate longevity. One mutation in *age*-1 results in a two-fold increase in *C. elegans* lifespan, dauer arrest, and fat accumulation (7). *Age*-1 is a 1155 amino acid protein and contains the expected domains of the PI3K catalytic subunit, including a kinase domain and a p85 binding domain. Ruvkun et al have submitted the sequence of a putative regulatory subunit named aaap-1, a protein similar to p55, a regulatory subunit already described (Unpublished Genbank submission, AAF28335).

Members of the second class of PI3Ks, including PI3K_68D in humans, have not been well characterized. They have an N-terminal extension with several novel motifs but interactions with other proteins are unknown. They are capable of phosphorylating both PtdIns and PtdIns(4)*P* in vitro. Members of the final class of PI3Ks, class 3, are orthologues of the *S. cerevisiae* protein Vps34. Vps34 was originally identified through a phenotype of defective sorting and delivery of hydrolases between the Golgi and the vacuole. Thus the primary role of the class appears to be the regulation of protein trafficking events. The Class 3 PI3Ks have been shown to specifically phosphorylate PtdIns only, creating PtdIns(3)*P*.

The insulin signaling cascade regulates a wide range of metabolic and mitogenic processes in both mammals and invertebrates. In the mosquito *Aedes aegypti*,

components of the insulin signaling cascade both upstream (MIR) and downstream (MPKB) of PI3K have been characterized but PI3K in the mosquito has not. This study presents the partial sequencing and characterization of the catalytic subunits from Class 1 and Class 3 PI3Ks.

Materials and Methods

Insects

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 two days, and subsequently were given only distilled water, whereas those used in the age time course were given continual access to 10% sucrose solution. To initiate a reproductive cycle, adults were fed on an anesthetized rat until engorged, separated from unfed and partially fed individuals, and maintained on water until needed.

Class 1 PI3K Gene Identification

Total RNA from the ovaries of 3 day old female mosquitoes was isolated using the RNAeasy mini kit (Qiagen). First strand cDNA was synthesized from the total RNA using the First-Strand cDNA synthesis kit (Pharmacia-Biotech). Degenerate primers to conserved regions of the Class 1 PI3K family were generated (Forward primer: 5'-CTGTTCCAGTACCTGCTGCARYTNGTNCA-3' and Reverse primer: 5'-CGCAGATCATCGCCGTTYTTRAADAT-3') and used to amplify products from ovary cDNA by touchdown PCR (95° C for 15 sec, 65° C to 50° C (-1° C every cycle) for 15 sec, 72° C for 30 sec, 15 cycles followed by 95° C for 15 sec, 50° C for 15 sec, 72° C for 30 sec, 30 cycles) in a gradient PCR machine (Eppendorf). A fragment of the expected

size (500 bp) was gel purified, cloned (TOPO TA cloning kit, invitrogen), and sequenced. When the nucleotide sequence was translated it was found to have sequence similarity to the Class 1 PI3K family. The First Choice RLM-Race kit (Ambion) was used to obtain the complete 3' end, however 5' RACE was unsuccessful.

Class 3 PI3K Gene Identification

Messenger RNA from the ovaries of 3 day old female mosquitoes was isolated using oligo-dT paramagnetic beads (Dynal). First strand cDNA was synthesized from the purified ovary mRNA using the First-Strand cDNA synthesis kit (Pharmacia-Biotech). Degenerate primers to conserved regions of the class 2 PI3K family were generated (Foward primer: 5'-GGNGAYGAYYTNMGNCARGA-3': amino acids 807 to 813 of human PI3Kβ and Reverse primer: 5'-ATNACRCARTANCCNGCRCA-3': amino acids 905 to 911 of human PI3Kβ) and used to amplify products from ovary cDNA using touchdown PCR (95° C for 5 sec, 60° C to 50° C (-3° C every 3 cycles) for 7 sec, 72° C for 10 sec, 17 cycles followed by 95° C for 5 sec, 45° C for 7 sec, 72° C for 10 sec, 30 cycles) in glass capillary tubes using a Rapidcycler PCR machine (Idaho Technologies). A 200 bp fragment was gel purified, cloned (TOPO TA cloning kit, Invitrogen), sequenced, and found have sequence similarity to the Class 3 PI3K family. The 200 bp product was used as a probe to screen a whole body cDNA library, and two positive clones were isolated. The larger clone was a 1797 bp fragment that encoded the 3' ORF and 176 bp of 3' UTR of a Class 3 PI3K.

Sequence alignment and phylogenetic tree

The partial Class 1 and 3 PI3K amino acid sequences in *Ae. aegypti* were aligned with the amino acid sequences of Dp110, *age* 1 and 2 from *C. elegans*, and several

vertebrate p110s using the ClustalW program in the GCG Wisconsin package. The pairwise distances between these sequences was determined with the Jukes-Cantor distance correction method using the Distance program in the GCG Wisconsin package and a phylogenetic tree was generated using the UPGMA tree construction method in the Growtree program.

Northern Analysis

Northern blots were used to determine the expression pattern of the mosquito Class 1 PI3K in adult tissues and in the ovary during a reproductive cycle. Digoxigenin (Dig) labeled RNA probes for northern blot analysis were synthesized from the 500 bp Class 1 p110 fragment cloned above. The forward primer for p110 (see above) and a M13 (-20) forward primer were used to generate PCR products for PI3K from the plasmid that possessed a T7 promoter. This product was used as a template in the MAXIscript *in vitro* transcription kit (Ambion) to generate an antisense RNA probe containing dUTP-Dig (Roche). The probe was aliquoted and stored at -80° C. Twenty pairs of ovaries, head, and body wall were dissected from female mosquitoes in *Aedes* saline solution (128 mM NaCl, 4.7 mM KCl, and 1.9 mM $CaCl_2$), immediately transferred to RNAlater (50 µl, Ambion) and stored at -20° C until processed. Total RNA was isolated from the tissues using the RNAeasy mini kit per kit instructions (Qiagen). Loading buffer (5X: 32% formamide, 2.4% formaldehyde, 4X MOPS, 20% glycerol, 4 mM EDTA, 0.1 mg/ml ethidium bromide, and bromophenol blue) was added to total RNA from 10 tissue equivalents, and RNAse free water was added to bring the final volume to 20 µl. The RNA was denatured at 80° C for 10 min and loaded onto a precast 1.25% Reliant MOPS gel (BMA). The gel was electrophoresed at 3 volts/cm for

3 hours in 1X MOPS running buffer. The gel was denatured in 0.05M NaOH for 30 minutes followed by neutralization in 1 M Tris for 30 minutes. The RNA was then vacuum transferred onto nylon membrane (MSI) for one hour and autocrosslinked (120,000 j/cm Stratagene). The blot was prehybridized in ULTRAhyb (7 ml, Ambion) at 65° C for 1 hour in small roller bottles. Dig labeled probes were denatured for 10 min at 80° C, added to the prehybridization solution (1:20,000 final concentration), and hybridized overnight at 65° C. After hybridization, the blot was washed 3 times (20 min/wash) in 0.5X SSC/0.1% SDS at 65° C followed by a 1 hour block in 1X blocking reagent (Roche). The blot was incubated with an antiDig-alkaline phosphotase antibody (1:10,000) and 1X blocking reagent for 30 minutes followed by 3 washes (20 min/wash) in maleic acid buffer (0.1M maleic acid, 0.15 M NaCl, pH 7.5) and 0.3% Tween 20. The MIR transcript was detected with CDP-Star (Roche) and visualized an Alpha Inotech chemiluminescent imager.

Results and Discusion

Partial cDNA sequences to both Class 1 and 3 PI3Ks have been obtained for the mosquito *Ae. aegypti*. However, both sequences lack the 5' end of the open reading frame (ORF) and the 5' UTR sequence. A 2495 bp fragment for the Class 1 PI3K, named *Ae*p110, encodes 775 amino acids of the ORF and the entire 3' UTR (Figure 5.1). Approximately 300 amino acids of the ORF and the 5' UTR have not been sequenced. The Class 1 PI3K family has a standard domain structure consisting of a catalytic domain at the C-terminus, a p85 subunit binding domain at the N-terminus, and in between a C2 domain, responsible for binding to phospholipids and bringing the catalytic domain to the cell membrane, and an accessory domain whose function is unknown. At the C-terminus

of the *Ae*p110, from amino acids 505 to 769, lies the catalytic domain that phosphorylates PtdIns on the third position. Farther upstream, from amino acids 240 to 428, lies the accessory domain and at the 5' end of the fragment, from amino acids 54 to 197, is the C2 domain. Presumably, a regulatory subunit binding domain exists upstream of the C2 domain, but the sequence has yet to be determined for *Ae*p110.

The partial cDNA sequence of a Class 3 PI3K in *Aedes* is 1797 bps long and encodes 540 amino acids of the ORF and 176 bp of 3' UTR. Approximately 360 amino acids of the ORF and the 5' UTR have not yet been determined (Figure 5.2). Like *Ae*p110, the Class 3 PI3K sequence contains both a catalytic (AA 295-544) and an accessory domain (AA 133-196). However, the nucleotide sequence for the C2 domain and regulatory subunit binding domain have yet to be obtained.

Sequence alignment of *Ae*p110 and the Class 3 PI3Ks in *Aedes* reveals considerable sequence homology with other PI3Ks in mammals and invertebrates, particularly at the carboxy terminus where the catalytic domain is found (Figure 5.3). Both of the PI3K genes partially sequenced in *Aedes* are closely related to PI3Ks found in *Drosophila*. *Ae*p110 shares 78% sequence identity and 86% sequence similarity to Dp110, whereas the Class 3 PI3K shares 73% sequence identity and 83% sequence similarity to the *Drosophila* gene P13K_59F, a class 3 PI3K.

A growtree phylogram comparing numerous vertebrate species, *Aedes*, *Drosophila*, and *C. elegans* was consistant with the sequence alignment (Figure 5.4). In both classes the *Aedes* and *Drosophila* PI3Ks are grouped together and are distinct from the related vertebrate PI3Ks. Interestingly, the *C. elegans* PI3K, Age-1, is in a distinct branch only slightly more related to the Class 1 PI3Ks than the Class 2.

```
   1 TCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCGGCACGAGCCGGCAGTAGGCAA
   1 S  R  S  T  V  S  I  S  L  I  S  N  S  A  R  A  G  S  R  Q

  61 CCTGCGCACACTTTGCGGAAGGTGAAATACGTTCCTAGCTGGGATACGGAAGGCACATTC
  21 P  A  H  T  L  R  K  V  K  Y  V  P  S  W  D  T  E  G  T  F

 121 ATGTGCATGGTGAAAGAAATCGACGCCCTGAACTGTGACTCGAATCGCAATGTGGAGGTT
  41 M  C  M  V  K  E  I  D  A  L  N  C  D  S  N  R  N  V  E  V

 181 GGCATCACGCTCGGGTTGTTTCACGGAGGAAAATCTCTTTGTAAACCGATGAAAACGGAA
  61 G  I  T  L  G  L  F  H  G  G  K  S  L  C  K  P  M  K  T  E

 241 TTGGTGGTGCTAAATGGAGGTCGTGCGGTGTGGAATGAAACGATAGTTTTCGATATTGAT
  81 L  V  V  L  N  G  G  R  A  V  W  N  E  T  I  V  F  D  I  D

 301 GTAAAAAATGTTCCTAGGATGGCGCGGTTATGTTTAGTAATTTATGAACTGGTCAGGACG
 101 V  K  N  V  P  R  M  A  R  L  C  L  V  I  Y  E  L  V  R  T

 361 GCGAAGGGTTCCGGCGTGAGGGCCCGCCGCACCAAGGATGGGTTTATGAATCCCATTGCT
 121 A  K  G  S  G  V  R  A  R  R  T  K  D  G  F  M  N  P  I  A

 421 TGGGTGAACACCATGCTGTACGATTACAAAAGTCAGCTCAAATCCGGCCCGGTAACGTTG
 141 W  V  N  T  M  L  Y  D  Y  K  S  Q  L  K  S  G  P  V  T  L

 481 TACACGTGGACCTATGCGGAAGATTTGCAATCGGAGGACGTGCTGCATCCACTCGGTACA
 161 Y  T  W  T  Y  A  E  D  L  Q  S  E  D  V  L  H  P  L  G  T

 541 GTGGAACCGAACCCGCGACGGGACGAATGTTCCTCGATAATTTTAAGTTTTCACAGCTAC
 181 V  E  P  N  P  R  R  D  E  C  S  S  I  I  L  S  F  H  S  Y

 601 TACAATGAACACAAGATCGTGGTGTATCCTTCGGAGGAGCAGCTGCTGGACCATGCCAGC
 201 Y  N  E  H  K  I  V  V  Y  P  S  E  E  Q  L  L  D  H  A  S

 661 AAGATGCGAAAGATCAATAGGCATTTAAACCGTGACAGTGCGGAGGAGGTGCGACCTATC
 221 K  M  R  K  I  N  R  H  L  N  R  D  S  A  E  E  V  R  P  I

 721 AAGGAGATTCTGCTGCCGTACATGCACAACGATAAGCTGAACGATATGCACGAACAGGAT
 241 K  E  I  L  L  P  Y  M  H  N  D  K  L  N  D  M  H  E  Q  D

 781 CGTAATGCGATTTGGGCGAAGCGGAGAGAGTGCATGACGCTGGATCCGGACGGGTTGCCG
 261 R  N  A  I  W  A  K  R  R  E  C  M  T  L  D  P  D  G  L  P

 841 TGTTTGCTCTACTGCGTGGAGTGGAACGACAGGAATGAGGTAGCGGAAATCGTCTCCCTG
 281 C  L  L  Y  C  V  E  W  N  D  R  N  E  V  A  E  I  V  S  L

 901 CTGCAGGAGTGGCCCATCCGTCCGGTGGAGCGTGCCCTCGAGCTGCTCGACTACGCCTAC
 301 L  Q  E  W  P  I  R  P  V  E  R  A  L  E  L  L  D  Y  A  Y

 961 GCCGACCAGTACGTCCGGCGCTATGCCGTCAAGTGTTTGCGCACCATCCAAGACGACGAA
 321 A  D  Q  Y  V  R  R  Y  A  V  K  C  L  R  T  I  Q  D  D  E

1021 CTACTCCTCTACCTGCTGCAGCTGGTGCAAGCCATGAAGCACGAATCCTACATCTACAGC
 341 L  L  L  Y  L  L  Q  L  V  Q  A  M  K  H  E  S  Y  I  Y  S

1081 GATCTGGTAGATTTCCTCCTGCAGCGATCCCTCAACAACCAGCACATTGGGCACTTCTTC
 361 D  L  V  D  F  L  L  Q  R  S  L  N  N  Q  H  I  G  H  F  F
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1141 TTCTGGCATTTACGATCGGAAATCCTGGTTCCCTCGGTCCAAGTGCGGTTCAGCCTTATC
 381 F  W  H  L  R  S  E  I  L  V  P  S  V  Q  V  R  F  S  L  I

1201 CTCGAAGCATACTTGAAGGGCAGTCAGGAACATATATCGATACTGCTGAAGCAGATGCAG
 401 L  E  A  Y  L  K  G  S  Q  E  H  I  S  I  L  L  K  Q  M  Q

1261 TGTTTGCGGAAGCTTCAACATGGGTCCGAGTTGGTCAAGAAAGGCAACAAGGAAAAGGGG
 421 C  L  R  K  L  Q  H  G  S  E  L  V  K  K  G  N  K  E  K  G

1321 AAATCTCTGCTGATGGACTACCTTCAGGACAAACCCGTCGGCGATGCATTGTCCGACGTA
 441 K  S  L  L  M  D  Y  L  Q  D  K  P  V  G  D  A  L  S  D  V

1381 ATAAGTCCGCTTAATCCTAGCTTTCGCTGTAAGACCGTTCGCAAAGAAAAGTGCAAAGTG
 461 I  S  P  L  N  P  S  F  R  C  K  T  V  R  K  E  K  C  K  V

1441 ATGGATTCCAAGATGCGACCCCTATGGATTGTGTACCAAAATTCCGATAGCAATGGCGAC
 481 M  D  S  K  M  R  P  L  W  I  V  Y  Q  N  S  D  S  N  G  D

1501 GATATTAACATGATTTTCAAAAACGGCGATGATCTCCGGCAGGACATGCTGACGCTACAA
 501 D  I  N  M  I  F  K  N  G  D  D  L  R  Q  D  M  L  T  L  Q

1561 ATGCTTCGAATTATGGATCGAATATGGAAAAGTCACGGGTATGATTTCCGAATGAATCCC
 521 M  L  R  I  M  D  R  I  W  K  S  H  G  Y  D  F  R  M  N  P

1621 TACAGTTGCATAAGCACCGACCGGCGGTTAGGCATCATCGAGGTGGTGCTCAATGCGGAA
 541 Y  S  C  I  S  T  D  R  R  L  G  I  I  E  V  V  L  N  A  E

1681 ACCATTGCCAACATCCAGAAGGAACGAGGGATGTTCTCGGCCACTTCGCCGTTCAAGAAG
 561 T  I  A  N  I  Q  K  E  R  G  M  F  S  A  T  S  P  F  K  K

1741 GGATCGCTTCTGACGTGGCTCAAGGAACACAACACCACGGAGGAAATGCTAGCGAAAGCA
 581 G  S  L  L  T  W  L  K  E  H  N  T  T  E  E  M  L  A  K  A

1801 ATACAGGAATTCACGCTCAGCTGTGCCGGGTACTGTGTGGCAACTTACGTGCTGGGGGTG
 601 I  Q  E  F  T  L  S  C  A  G  Y  C  V  A  T  Y  V  L  G  V

1861 GCCGATCGGCATTCCGATAATATCATGGTGAAAAAGACTGGTCAGCTGTTCCACATCGAC
 621 A  D  R  H  S  D  N  I  M  V  K  K  T  G  Q  L  F  H  I  D

1921 TTCGGTCGCATATTGGGCCATTTCAAGGAGAAGTTCGGTTTTAGGCGCGAGCGAGTTCCG
 641 F  G  R  I  L  G  H  F  K  E  K  F  G  F  R  R  E  R  V  P

1981 TTCGTGTTAACGCACGACTTTGTGTATGTGATCAACAATGGACGGACCGATCGGGAAGCT
 661 F  V  L  T  H  D  F  V  Y  V  I  N  N  G  R  T  D  R  E  A

2041 AAGGAGTTTCGACAGTTCCAGCATCTTTGCGAAGAGGCATTCCTAATCCTACGAAAGCAC
 681 K  E  F  R  Q  F  Q  H  L  C  E  E  A  F  L  I  L  R  K  H

2101 GGATGTCTAATCCTGTCACTGTTCGCCATGATGATCTCGACCGGTTTGCCGGAGCTGTCA
 701 G  C  L  I  L  S  L  F  A  M  M  I  S  T  G  L  P  E  L  S

2161 TCGGAGAAGGATCTCAACTATCTGCGGGAAACTTTGGTACTGGATCTGCCAGAGGAGGAC
 721 S  E  K  D  L  N  Y  L  R  E  T  L  V  L  D  L  P  E  E  D

2221 GCCCGCACCCACTTCAAGTCCAAATTCAGCGAAGCCCTGGCCAACTCGTGGAAAACGTCC
 741 A  R  T  H  F  K  S  K  F  S  E  A  L  A  N  S  W  K  T  S
```

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2281 CTCAACTGGGCCTCGCACAATTTCTCCAAGAACAACAAACAGTGACAGTTGCGGTACAAT
 761 L  N  W  A  S  H  N  F  S  K  N  N  K  Q  *

2341 CTCACCATGTAAAATCCAAGCAGTACGGTGTAGGTTCTAGGAGCGGATCGACCGTTTCCG
2491 GGGGAACCATCAACCGTTGCGCTTTTCCGGAAAGGATTGCGCTTGTCATAGTACCTATGT
2461 TATGTGGAGTGCAGATGCTAATAAAACCTCGTGCC
```

Figure 5.1: Sequence of *Ae*p110 in the mosquito *Aedes aegypti*. The C2 domain is highlighted in GREEN, the accessory domain is highlighted in YELLOW, and the catalytic domain is highlighted in BLUE. A putative polyadenylation site is shown in bold.

```
   1 TGGAAGCCGATGGATGTGGAGGACGCACTAGAACTACTGAATTCATCGTTCAACCATCCT
   1 W  K  P  M  D  V  E  D  A  L  E  L  L  N  S  S  F  N  H  P

  61 ACTGTGAGACGTTATGCTATATCCCGATTGGATCAGGCACTGGACGATGACTTGTTACTC
  21 T  V  R  R  Y  A  I  S  R  L  D  Q  A  L  D  D  D  L  L  L

 121 TATTTGCTGCAACTCGTACAAGCGCTCAAATATGAACATTTTGATGAAATTCTTAAATCT
  61 Y  L  L  Q  L  V  Q  A  L  K  Y  E  H  F  D  E  I  L  K  S

 181 TGCAATAATCTTGCATTGGAAAAAGACATGCTGAAATCAGTAGAAGAAAATCCAGTGGAA
  81 C  N  N  L  A  L  E  K  D  M  L  K  S  V  E  E  N  P  V  E

 241 ACAAATTTTGAAGTGTTTACTGAATCAGATGAGGGTAATTCTATGTATCAGGACGCAGCT
 101 T  N  F  E  V  F  T  E  S  D  E  G  N  S  M  Y  Q  D  A  A

 301 CACGATATAATAGGATCCAAATCAATGAGTCAAACCGATAATAACTCGAATGCAAATGAG
 121 H  D  I  I  G  S  K  S  M  S  Q  T  D  N  N  S  N  A  N  E

 361 TCGAACGTAAATAGCAGTGAGGATGCACAAGTACCTTCAAATTTGGCCATGTTTCTCATT
 141 S  N  V  N  S  S  E  D  A  Q  V  P  S  N  L  A  M  F  L  I

 421 CAAAGGGCATGTAAAAATTCTACATTGGCAAATTATTTGTATTGGTACCTGTCGATAGAA
 161 Q  R  A  C  K  N  S  T  L  A  N  Y  L  Y  W  Y  L  S  I  E

 481 TGCGAGGAAGAAACAGTAAGGAAACAAGATGAACGCGTTAGGAAAATGTACCGCACAGTT
 181 C  E  E  E  T  V  R  K  Q  D  E  R  V  R  K  M  Y  R  T  V

 541 CTACATATATTTCTACGGCAGCTTTCTACAGGAAACCCCGAACTGAGGACTATACATCGC
 201 L  H  I  F  L  R  Q  L  S  T  G  N  P  E  L  R  T  I  H  R

 601 AGCTTGAAGGAGCAACAGAAATTCATCGATAATCTGGTGAAACTCATAAAAATTGTCGCA
 221 S  L  K  E  Q  Q  K  F  I  D  N  L  V  K  L  I  K  I  V  A

 661 AAAGAACCGGGTAACCGAAAGAAAAAGACGGAAAAATTCCAAATGTTGCTCTCCGACACC
 241 K  E  P  G  N  R  K  K  K  T  E  K  F  Q  M  L  L  S  D  T

 721 GATGCGTTGAAAATCAATTTCACCAAATTTGAGCCCCTATCCTTTCCGCTGGATCCTAAT
 261 D  A  L  K  I  N  F  T  K  F  E  P  L  S  F  P  L  D  P  N

 781 GTCCGAATTCGTGGCATAATTGCCGAAAAAGTCACTCTTTTCAAGAGCGCCTTGATGCCC
 281 V  R  I  R  G  I  I  A  E  K  V  T  L  F  K  S  A  L  M  P

 841 TCAAAGTTAACATTCCTAACTACGGGACCGTCTGAATATGTAGCGATCTTCAAGCACGGT
 301 S  K  L  T  F  L  T  T  G  P  S  E  Y  V  A  I  F  K  H  G

 901 GACGATCTACGGCAGGATCAGTTGATACTGCAAATGATTACTCTGATGGATAAATTGCTA
 321 D  D  L  R  Q  D  Q  L  I  L  Q  M  I  T  L  M  D  K  L  L

 961 CAGAAAGAAAACCTAGATCTGAAACTAACGCCTTATCGCGTCCTGGCCACCAGTTCCAAG
 341 Q  K  E  N  L  D  L  K  L  T  P  Y  R  V  L  A  T  S  S  K

1021 CATGGATTCATGCAGTACATAGATTCCATCACGGTCGCCGAAGTTCTCAATGCTGAAGGA
 361 H  G  F  M  Q  Y  I  D  S  I  T  V  A  E  V  L  N  A  E  G

1081 AGCATTTTGAACTTTTTCCGCAAGCATCACCCGTGCGAAACAGGACCCTATGGAATTGTG
```

```
 381 S  I  L  N  F  F  R  K  H  H  P  C  E  T  G  P  Y  G  I  V

1141 GCTGACGTTATGGAGACGTACATTAAAAGCTGTGCCGGCTATTGTGTGATAACTTATTTA
 401 A  D  V  M  E  T  Y  I  K  S  C  A  G  Y  C  V  I  T  Y  L

1201 TTAGGTGTGGGTGATCGTCATTTGGACAATTTGCTGCTGACGAATTCCGGAAAGTTGTTC
 421 L  G  V  G  D  R  H  L  D  N  L  L  L  T  N  S  G  K  L  F

1261 CACATTGATTTCGGGTATATACTGGGAAGAGACCCGAAACCGATGCCGCCGCCGATGAAG
 441 H  I  D  F  G  Y  I  L  G  R  D  P  K  P  M  P  P  P  M  K

1321 CTTAGCAAAGAAATGGTAGAAGCGATGGGTGGATTGAACTCCGAGTACTATCAAGAATTT
 461 L  S  K  E  M  V  E  A  M  G  G  L  N  S  E  Y  Y  Q  E  F

1381 AGGAAGCTGTGCTACACCGCTTTCCTCCACCTGCGCAGACACGCCAACGTGATGTTGAAT
 481 R  K  L  C  Y  T  A  F  L  H  L  R  R  H  A  N  V  M  L  N

1441 CTTTTCGGGCTGATGGTAGATGCGTCCATTCCGGATATCGCTTTGGAACCCGACAAGGCG
 501 L  F  G  L  M  V  D  A  S  I  P  D  I  A  L  E  P  D  K  A

1501 GTTAAGAAGGTAGAAGACAATTTACGTTTAGATTTATCTGACGAGGAAGCTGTGCAGCAT
 521 V  K  K  V  E  D  N  L  R  L  D  L  S  D  E  E  A  V  Q  H

1561 TTACAAAATCTACTTGACCTCTCGATAACAGCTGTGATGCCAGCTTTAGTTGAACAGATT
 541 L  Q  N  L  L  D  L  S  I  T  A  V  M  P  A  L  V  E  Q  I

1621 CACAAATTAGCACAGTATTGGAGGAAATAATTTTATTGCAAAAGTTATATGCAATTACAC
 561 H  K  L  A  Q  Y  W  R  K  *

1681 ACGACACACTTATCTGCATGTTTATTGATCTATGAAAATATATAGACAGCTCGTGCCGAA
1741 TTCCTGCAGCCCGGGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAG
1801 CTTTTGTTCCCTTTAGTGAGGGTTAATA
```

Figure 5.2: Sequence of the Class 3 PI3K in the mosquito *Aedes aegypti*. The accessory domain is highlighted in YELLOW and the catalytic domain is highlighted in BLUE.

```
C3 Rat     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C3 Human   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C3 Dros    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C3 Aedes   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C1 HumanA  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MPPRPS
C1 Mouse   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MPPRPS
C1 Aedes   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C1 Dros    ~~~~~~~~~~~~~~~~~~~~~~~~~~~MNMMDNRALAYVAHQPKYETPPEEAEPPCMRF
C1 C.egn   KPWSSRSDCWTRTELRRISQMHVNILHPQLQTMVEQWQMRERPSLETENGKGSL.LLEN

C3 Rat     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C3 Human   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C3 Dros    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C3 Aedes   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C1 HumanA  SGELWGIHLMPPRILVECLLPNGMIVTLECLREATLVTIKHELFREARKYPLHQLLQDET
C1 Mouse   SGELWGIHLMPPRILVECLLPNGMIVTLECLREATLVTIKHELFKEARKYPLHQLLQDES
C1 Aedes   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C1 Dros    SVNLWKNEMLNWVDLI.CLLPNGFLLELRVNPANTIQVIKVEMVNQAKQMPLGYVIKEAC
C1 C.egn   EGVADIITMCPFGEVISVVFP.WFLANVRTSLEIKLSDFKHQLFELIAPMKWGTYSVKPQ

C3 Rat     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C3 Human   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C3 Dros    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C3 Aedes   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C1 HumanA  SYIFVSVTQEAEREEFFDETRRLCDLRLF..QPFLKVIEPVGN...REEKILNREIGFVI
C1 Mouse   SYIFVSVTQEAEREEFFDETRRLCDLRLF..QPFLKVIEPVGN...REEKILNREIGFAI
C1 Aedes   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C1 Dros    EYQVYGIS.TFNIEPYTDETKRLSEVQPY..FGILSLGERTDTTSFSSDYELTKMVNGMI
C1 C.egn   DYVFRQLNNFGEIEVIFNDDQPLSKLELHGTFPMLFLYQPDG...INRDKELMSDISHCL

C3 Rat     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MGEAEKFHYIYSCDLDINVQLKIGSLE
C3 Human   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MGEAEKFHYIYSCDLDINVQLKIGSLE
C3 Dros    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MDQPDDHFRYIHSSSLHERVQIKVGTLE
C3 Aedes   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C1 HumanA  GMPVCEFDMVKDPEVQDFRRNILNVCKEAVDLRDLNSPHSRAMYVYPPNVESSPELPKHI
C1 Mouse   GMPVCEFDMVKDPEVQDFRRNILNVCKEAVDLRDLNSPHSRAMYVYPPHVESSPELPKHI
C1 Aedes   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C1 Dros    GTTFDHNRTHGSPEIDDFRLYMTQTCDNIELERSAYTWQQRLLYEHPLRLANSTKMPELI
C1 C.egn   GYSLDKLEESLDEELRQFRASLWARTKKTCLTRGLEGT.SHYAFPEEQYLCVGESCPKDL

C3 Rat     GKREQKSYKAVLEDPMLKFSGLYQETCSDLYVTCQVFAEGKPLALPVRTSYKPFSTRWNW
C3 Human   GKREQKSYNAVLEDPMLKFSGLYQETCSDLYVTCQVFAEGKPSALPVRTSYKAFSTRWNW
C3 Dros    GKKRQPDYEKLLEDPILRFSGLYSEEHPSFQVRLQVFNQGRPYCLPVTSSYKAFGKRWSW
C3 Aedes   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C1 HumanA  YNKLDKGQIIV.VIW....VIVSPNNDK.QKYTLKINHDCVPEQVIAEAIRKKTRSMLLS
C1 Mouse   YNKLDRGQIIV.VIW....VIVSPNNDK.QKYTLKINHDCVPEQVIAEAIRKKTRSMLLS
C1 Aedes   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C1 Dros    RERHPTRTFLI.V........VKNENDQ.STFTLSVNEQDTPFSLTESTLQKMNRSQMKM
C1 C.egn   ESKVKAAKLSYQMFWRKRKAEINGVCEKMMKIQIEFNPNETPKSLLHTFLYEMRKLDVYD

C3 Rat     NEWLKLPVKYPDLPRNAQVALTIWDVYGPGRAVPVGGTTVSLFGKYGMFRQGMHDLKVWP
C3 Human   NEWLKLPVKYPDLPRNAQVALTIWDVYGPGKAVPVGGTTVSLFGKYGMSRQGMHDLKVWP
C3 Dros    NEWVTLPLQFSDLPRSAMLVLTILDCSGAGQTTVIGGTSISMFGKDGMFRQGMYDLRVWL
C3 Aedes   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C1 HumanA  SEQL..KLCVLEY.QGK..YI....LKVCGCD..EYFLEKYPLSQYKYIRSCIMLGRM..
C1 Mouse   SEQL..KLCVLEY.QGK..YI....LKVCGCD..EYFLEKYPLSQYKYIRSCIMLGRM..
C1 Aedes   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C1 Dros    NDR...........TSD..YI....LKVSGRD..EYLLGDYPLIQFLYIQEMLSDSAV..
C1 C.egn   TDDPADEGWFLQL.AGRTTFVTNPDVKLTSYDGVRSELESYRCPGFVVRRQSLVLKDY..
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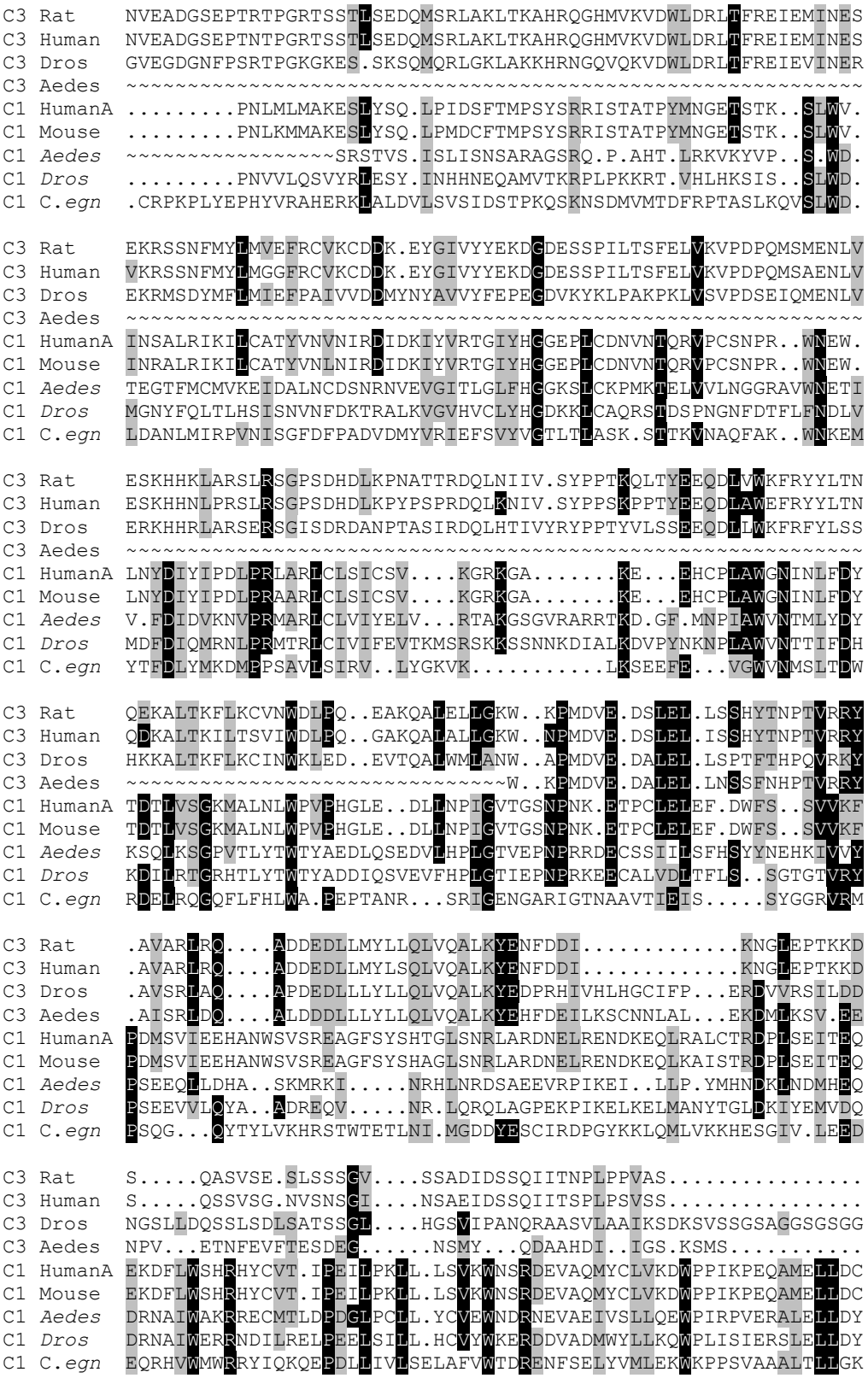
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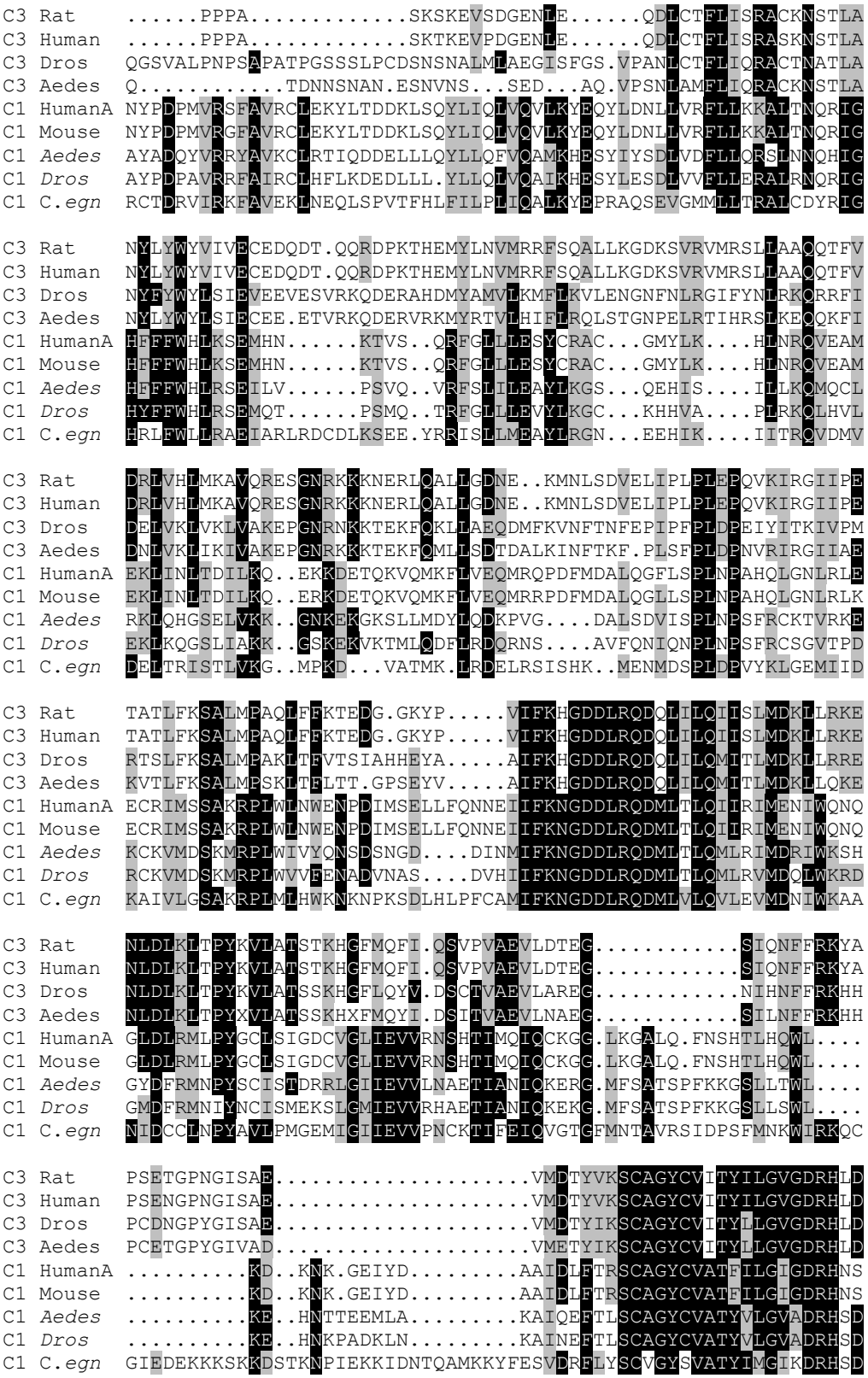
C3 Human NVEADGSEPTNTPGRTSSTLSEDQMSRLAKLTKAHRQGHMVKVDWLDRLTFREIEMINES
C3 Dros GVEGDGNFPSRTPGKGKES.SKSQMQRLGKLAKKHRNGQVQKVDWLDRLTFREIEVINER
C3 Aedes ~~~~~~
C1 HumanA .........PNLMLMAKESLYSQ.LPIDSFTMPSYSRRISTATPYMNGETSTK..SLWV.
C1 Mouse .........PNLKMMAKESLYSQ.LPMDCFTMPSYSRRISTATPYMNGETSTK..SLWV.
C1 Aedes ~~~~~~~~~~~~~~~~~SRSTVS.ISLISNSARAGSRQ.P.AHT.LRKVKYVP..SLWD.
C1 Dros .........PNVVLQSVYRLESY.INHHNEQAMVTKRPLPKKRT.VHLHKSIS..SLWD.
C1 C.egn .CRPKPLYEPHYVRAHERKLALDVLSVSIDSTPKQSKNSDMVMTDFRPTASLKQVSLWD.
C3 Rat EKRSSNFMYLMVEFRCVKCDDK.EYGIVYYEKDGDESSPILTSFELVKVPDPQMSMENLV
C3 Human VKRSSNFMYLMGGFRCVKCDDK.EYGIVYYEKDGDESSPILTSFELVKVPDPQMSAENLV
C3 Dros EKRMSDYMFLMIEFPAIVVDDMYNYAVVYFEPEGDVKYKLPAKPKLVSVPDSEIQMENLV
C3 Aedes ~~~~~~
C1 HumanA INSALRIKILCATYVNVNIRDIDKIYVRTGIYHGGEPLCDNVNTQRVPCSNPR..WNEW.
C1 Mouse INRALRIKILCATYVNLNIRDIDKIYVRTGIYHGGEPLCDNVNTQRVPCSNPR..WNEW.
C1 Aedes TEGTFMCMVKEIDALNCDSNRNVEVGITLGLFHGGKSLCKPMKTELVVLNGGRAVWNETI
C1 Dros MGNYFQLTLHSISNVNFDKTRALKVGVHVCLYHGDKKLCAQRSTDSPNGNFDTFLFNDLV
C1 C.egn LDANLMIRPVNISGFDFPADVDMYVRIEFSVYVGTLTLASK.STTKVNAQFAK..WNKEM
C3 Rat ESKHHKLARSLRSGPSDHDLKPNATTRDQLNIIV.SYPPTKQLTYEEQDLVWKFRYYLTN
C3 Human ESKHHNLPRSLRSGPSDHDLKPYPSPRDQLKNIV.SYPPSKPPTYEEQDLAWEFRYYLTN
C3 Dros ERKHHRLARSERSGISDRDANPTASIRDQLHTIVYRYPPTYVLSSEEQDLLWKFRFYLSS
C3 Aedes ~~~~~~
C1 HumanA LNYDIYIPDLPRLARLCLSICSV....KGRKGA.......KE...EHCPLAWGNINLFDY
C1 Mouse LNYDIYIPDLPRAARLCLSICSV....KGRKGA.......KE...EHCPLAWGNINLFDY
C1 Aedes V.FDIDVKNVPRMARLCLVIYELV...RTAKGSGVRARRTKD.GF.MNPIAWVNTMLYDY
C1 Dros MDFDIQMRNLPRMTRLCIVIFEVTKMSRSKKSSNNKDIALKDVPYNKNPLAWVNTTIFDH
C1 C.egn YTFDLYMKDMPPSAVLSIRV..LYGKVK.....LKSEEFE...VGWVNMSLTDW
C3 Rat QEKALTKFLKCVNWDLFQ..EAKQALELLGKW..KPMDVE.DSLEL.LSSHYTNPTVRRY
C3 Human QDKALTKILTSVIWDLFQ..GAKQALALLGKW..NPMDVE.DSLEL.ISSHYTNPTVRRY
C3 Dros HKKALTKFLKCINWKLED..EVTQALWMLANW..APMDVE.DALEL.LSPTFTHPQVRKY
C3 Aedes ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~W..KPMDVE.DALEL.LNSSFNHPTVRRY
C1 HumanA TDTLVSGKMALNLWPVPHGLE..DLLNPIGVTGSNPNK.ETPCLELEF.DWFS..SVVKF
C1 Mouse TDTLVSGKMALNLWPVPHGLE..DLLNPIGVTGSNPNK.ETPCLELEF.DWFS..SVVKF
C1 Aedes KSQLKSGPVTLYTWTYAEDLQSEDVLHPLGTVEPNPRRDECSSIILSFHSYYNEHKIVVY
C1 Dros KDILRTGRHTLYTWTYADDIQSVEVFHPLGTIEPNPRKEECALVDLTFLS..SGTGTVRY
C1 C.egn RDELRQGQFLFHLWA.PEPTANR...SRIGENGARIGTNAAVTIEIS.....SYGGRVRM
C3 Rat .AVARLRQ....ADDEDLLMYLLQLVQALKYENFDDI.............KNGLEPTKKD
C3 Human .AVARLRQ....ADDEDLLMYLSQLVQALKYENFDDI.............KNGLEPTKKD
C3 Dros .AVSRLAQ....APDEDLLLYLLQLVQALKYEDPRHIVHLHGCIFP...ERDVVRSILDD
C3 Aedes .AISRLDQ....ALDDDLLLYLLQLVQALKYEHFDEILKSCNNLAL...EKDMLKSV.EE
C1 HumanA PDMSVIEEHANWSVSREAGFSYSHTGLSNRLARDNELRENDKEQLRALCTRDPLSEITEQ
C1 Mouse PDMSVIEEHANWSVSREAGFSYSHAGLSNRLARDNELRENDKEQLKAISTRDPLSEITEQ
C1 Aedes PSEEQLLDHA..SKMRKI.....NRHLNRDSAEEVRPIKEI..LLP.YMHNDKLNDMHEQ
C1 Dros PSEEVVLQYA..ADREQV.....NR.LQRQLAGPEKPIKELKELMANYTGLDKIYEMVDQ
C1 C.egn PSQG...QYTYLVKHRSTWTETLNI.MGDDYESCIRDPGYKKLQMLVKKHESGIV.LEED
C3 Rat S.....QASVSE.SLSSSGV....SSADIDSSQIITNPLPPVAS.................
C3 Human S.....QSSVSG.NVSNSGI....NSAEIDSSQIITSPLPSVSS.................
C3 Dros NGSLLDQSSLSDLSATSSGL....HGSVIPANQRAASVLAAIKSDKSVSSGSAGGSGSGG
C3 Aedes NPV...ETNFEVFTESDFG.....NSMY...QDAAHDI..IGS.KSMS............
C1 HumanA EKDFLWSHRHYCVT.IPEILPKLL.LSVKWNSRDEVAQMYCLVKDWPPIKPEQAMELLDC
C1 Mouse EKDFLWSHRHYCVT.IPEILPKLL.LSVKWNSRDEVAQMYCLVKDWPPIKPEQAMELLDC
C1 Aedes DRNAIWAKRRECMTLDPDGLPCLL.YCVEWNDRNEVAEIVSLLQEWPIRPVERALELLDY
C1 Dros DRNAIWERRNDILRELPEELSILL.HCVYWKERDDVADMWYLLKQWPLISIERSLELLDY
C1 C.egn EQRHVWMWRRYIQKQEPDLLIVLSELAFVWTDRENFSELYVMLEKWKPPSVAAALTLLGK


C3 Rat .....PPPA.....SKSKEVSDGENLE.....QDLCTFLISRACKNSTLA
C3 Human .....PPPA.....SKTKEVPDGENLE.....QDLCTFLISRASKNSTLA
C3 Dros QGSVALPNPSAPATPGSSSLPCDSNSNALMLAEGISFGS.VPANLCTFLIQRACTNATLA
C3 Aedes Q.....TDNNSNAN.ESNVNS...SED...AQ.VPSNLAMFLIQRACKNSTLA
C1 HumanA NYPDPMVRSFAVRCLEKYLTDDKLSQYLIQLVQVLKYEQYLDNLLVRFLLKKAITNQRIG
C1 Mouse NYPDPMVRGFAVRCLEKYLTDDKLSQYLIQLVQVLKYEQYLDNLLVRFLLKKAITNQRIG
C1 Aedes AYADQYVRRYAVKCLRTIQDDELLLQYLLQFVQAMKHESYIYSDLVDFLLQRSLNNQHIG
C1 Dros AYPDPAVRRFAIRCLHFLKDEDLLL.YLLQLVQAIKHESYLESDLVVFLLERALRNQRIG
C1 C.egn RCTDRVIRKFAVEKLNEQLSPVTFHLFILPLIQALKYEPRAQSEVGMMLLTRALCDYRIG
C3 Rat NYLYWYVIVECEDQDT.QQRDPKTHEMYLNVMRRFSQALLKGDKSVRVMRSLLAAQQTFV
C3 Human NYLYWYVIVECEDQDT.QQRDPKTHEMYLNVMRRFSQALLKGDKSVRVMRSLLAAQQTFV
C3 Dros NYFYWYLSIEVEEVESVRKQDERAHDMYAMVLKMFLKVLENGNFNLRGIFYNLRKQRRFI
C3 Aedes NYLYWYLSIECEE.ETVRKQDERVRKMYRTVLHIFLRQLSTGNPELRTIHRSLKEQQKFI
C1 HumanA HFFFWHLKSEMHN......KTVS..QRFGLLLESYCRAC...GMYLK....HLNRQVEAM
C1 Mouse HFFFWHLKSEMHN......KTVS..QRFGLLLESYCRAC...GMYLK....HLNRQVEAM
C1 Aedes HFFFWHLRSEILV......PSVQ..VRFSLILEAYLKGS...QEHIS....ILLKQMQCL
C1 Dros HYFFWHLRSEMQT......PSMQ..TRFGLLLEVYLKGC...KHHVA....PLRKQLHVL
C1 C.egn HRLFWLLRAEIARLRDCDLKSEE.YRRISLLMEAYLRGN...EEHIK....IITRQVDMV
C3 Rat DRLVHLMKAVQRESGNRKKKNERLQALLGDNE..KMNLSDVELIPLPLEPQVKIRGIIPE
C3 Human DRLVHLMKAVQRESGNRKKKNERLQALLGDNE..KMNLSDVELIPLPLEPQVKIRGIIPE
C3 Dros DELVKLVKLVAKEPGNRNKKTEKFQKLLAEQDMFKVNFTNFEPIPFPLDPEIYITKIVPM
C3 Aedes DNLVKLIKIVAKEPGNRKKKTEKFQMLLSDTDALKINFTKF.PLSFPLDPNVRIRGIIAE
C1 HumanA EKLINLTDILKQ..EKKDETQKVQMKFLVEQMRQPDFMDALQGFLSPLNPAHQLGNLRLE
C1 Mouse EKLINLTDILKQ..ERKDETQKVQMKFLVEQMRRPDFMDALQGLLSPLNPAHQLGNLRLK
C1 Aedes RKLQHGSELVKK..GNKEKGKSLLMDYLQDKPVG....DALSDVISPLNPSFRCKTVRKE
C1 Dros EKLKQGSLIAKK..GSKEKVKTMLQDFLRDQRNS....AVFQNIQNPLNPSFRCSGVTPD
C1 C.egn DELTRISTLVKG..MPKD...VATMK.LRDELRSISHK..MENMDSPLDPVYKLGEMIID
C3 Rat TATLFKSALMPAQLFFKTEDG.GKYP.....VIFKHGDDLRQDQLILQIISLMDKLLRKE
C3 Human TATLFKSALMPAQLFFKTEDG.GKYP.....VIFKHGDDLRQDQLILQIISLMDKLLRKE
C3 Dros RTSLFKSALMPAKLTFVTSIAHHEYA.....AIFKHGDDLRQDQLILQMITLMDKLLRRE
C3 Aedes KVTLFKSALMPSKLTFLTT.GPSEYV.....AIFKHGDDLRQDQLILQMITLMDKLLQKE
C1 HumanA ECRIMSSAKRPLWLNWENPDIMSELLFQNNEIIFKNGDDLRQDMLTLQIIRIMENIWQNQ
C1 Mouse ECRIMSSAKRPLWLNWENPDIMSELLFQNNEIIFKNGDDLRQDMLTLQIIRIMENIWQNQ
C1 Aedes KCKVMDSKMRPLWIVYQNSDSNGD....DINMIFKNGDDLRQDMLTLQMLRIMDRIWKSH
C1 Dros RCKVMDSKMRPLWVVFENADVNAS....DVHIIFKNGDDLRQDMLTLQMLRVMDQLWKRD
C1 C.egn KAIVLGSAKRPLMLHWKNKNPKSDLHLPFCAMIFKNGDDLRQDMLVLQVLEVMDNIWKAA
C3 Rat NLDLKLTPYKVLATSTKHGFMQFI.QSVPVAEVLDTEG.....SIQNFFRKYA
C3 Human NLDLKLTPYKVLATSTKHGFMQFI.QSVPVAEVLDTEG.....SIQNFFRKYA
C3 Dros NLDLKLTPYKVLATSSKHGFLQYV.DSCTVAEVLAREG.....NIHNFFRKHH
C3 Aedes NLDLKLTPYXVLATSSKHXFMQYI.DSITVAEVLNAEG.....SILNFFRKHH
C1 HumanA GLDLRMLPYGCLSIGDCVGLIEVVRNSHTIMQIQCKGG.LKGALQ.FNSHTLHQWL....
C1 Mouse GLDLRMLPYGCLSIGDCVGLIEVVRNSHTIMQIQCKGG.LKGALQ.FNSHTLHQWL....
C1 Aedes GYDFRMNPYSCISTDRRLGIIEVVLNAETIANIQKERG.MFSATSPFKKGSLLTWL....
C1 Dros GMDFRMNIYNCISMEKSLGMIEVVRHAETIANIQKEKG.MFSATSPFKKGSLLSWL....
C1 C.egn NIDCCLNPYAVLPMGEMIGIIEVVPNCKTIFEIQVGTGFMNTAVRSIDPSFMNKWIRKQC
C3 Rat PSETGPNGISAE.....VMDTYVKSCAGYCVITYILGVGDRHLD
C3 Human PSENGPNGISAE.....VMDTYVKSCAGYCVITYILGVGDRHLD
C3 Dros PCDNGPYGISAE.....VMDTYIKSCAGYCVITYLLGVGDRHLD
C3 Aedes PCETGPYGIVAD.....VMETYIKSCAGYCVITYLLGVGDRHLD
C1 HumanA .....KD..KNK.GEIYD.....AAIDLFTRSCAGYCVATFILGIGDRHNS
C1 Mouse .....KD..KNK.GEIYD.....AAIDLFTRSCAGYCVATFILGIGDRHNS
C1 Aedes .....KE..HNTTEEMLA.....KAIQEFTLSCAGYCVATYVLGVADRHSD
C1 Dros .....KE..HNKPADKLN.....KAINEFTLSCAGYCVATYVLGVADRHSD
C1 C.egn GIEDEKKKSKKDSTKNPIEKKIDNTQAMKKYFESVDRFLYSCVGYSVATYIMGIKDRHSD

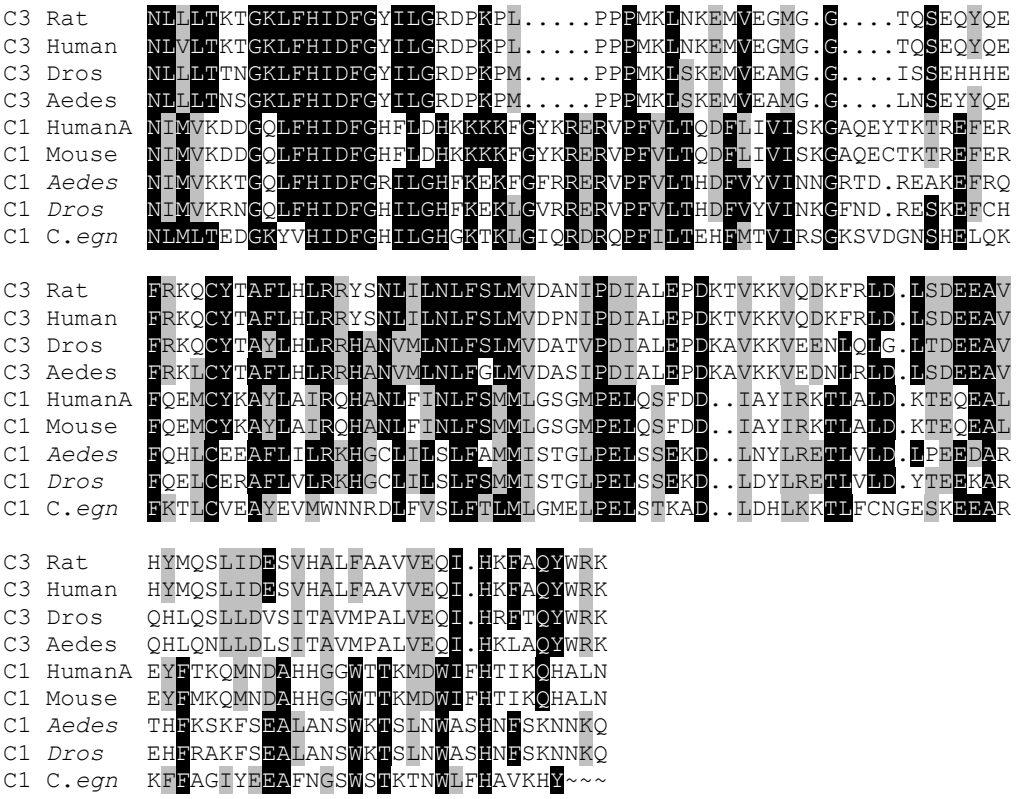


Figure 5.3: Pileup comparison of Class 1 (p110) and Class 3 (Cls3) PI3Ks in *D. melanogaster* (*Dros)*, *A. aegypti* (*Aedes*), *C. elegans* (*C.egn*), and vertebrates. Class 1 PI3Ks have a 120 to 150 amino acid extension on the amino terminus compared with class 3 PI3Ks. Both families have strong sequence similarities in the catalytic domain at the C-terminus but share little sequence homology at the N-terminus.

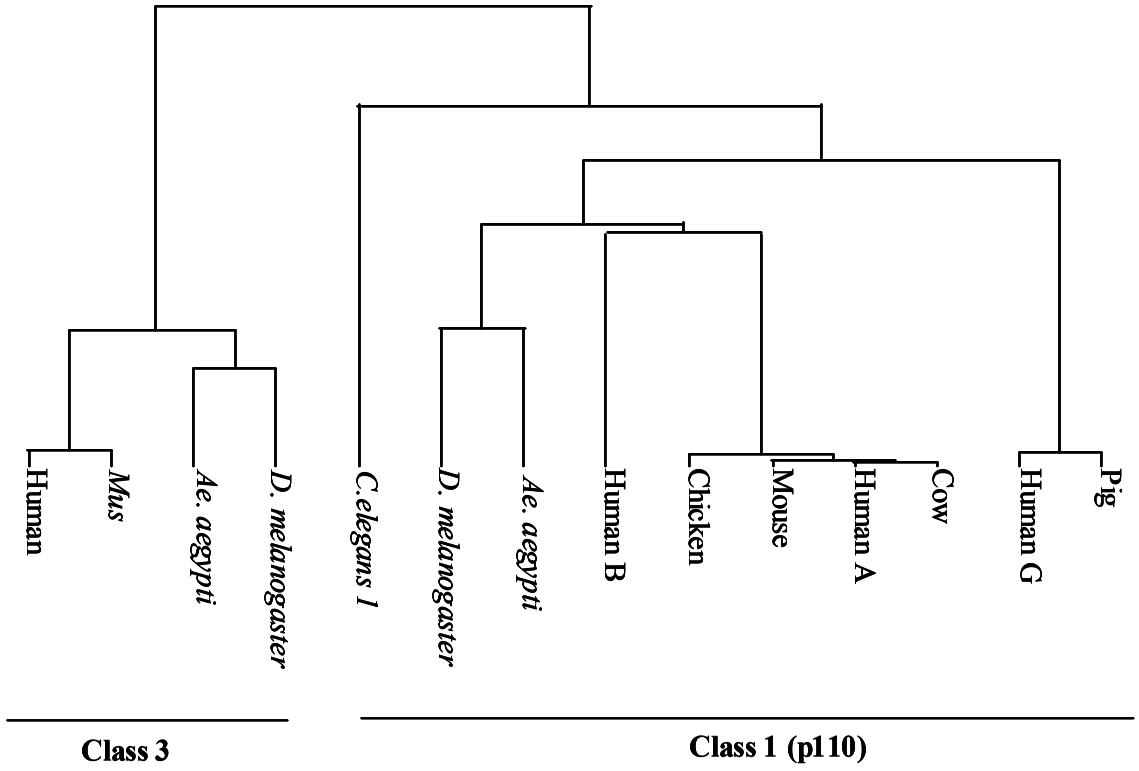


Figure 5.4: Phylogenetic tree of class 1 and 3 PI3Ks in a variety of vertebrates and invertebrates. The catalytic subunits of mosquito PI3Ks are most closely related to the *Drosophila* PKBs. Like PKB, the class 1 PI3K in *Aedes* is moe closely related to the vertebrate PI3Ks then to the invertebrate *C. elegans*.

The *Ae*p110 transcript is expressed in a variety of tissues in female mosquitoes. Northern blot analysis reveals two transcripts, of approximately 5 and 6 kb in size, in head, ovary, and body wall (Figure 5.5A). This is in contrast to MPKB that is only found in the ovary and early embryo. Blots were performed under high stringency conditions, and it is unlikely that the transcripts represent both the Class 1 and 3 transcripts. More likely the two transcripts are due to multiple transcriptional start sites as is seen in other components of this signaling cascade, or a closely related Class 1 PI3K. However this analysis will have to await the complete sequence of the 5' UTR is known. Comparing the ratio of rRNA to transcript, it appears as though the head possesses considerably more transcript then either the body wall or ovary. The presence of *Ae*p110 in multiple adult tissues suggests that, in addition to its putative steroidogenic role, it most likely regulates other metabolic processes in the mosquito. These roles may include the regulation of lipid stores as is seen in both *Drosophila* and *C. elegans* and the regulation of lifespan observed in *C. elegans* and regulated by an upstream signaling component in *Drosophila* (2, 5, 11, 12, 16). Complete characterization of these proteins in *Ae. aegypti* will elucidate their role in various tissues, life stages, and metabolic states.

A representative northern blot of *Ae*p110 transcript levels in the ovary during a reproductive cycle was performed (Figure 5.5B). Densitometry analysis found a decrease in *Ae*p110 transcript immediately after a bloodmeal (2 h) and a significant increase from 12 to 24 hours after a bloodmeal (Figure 5.5C). Examining the ratio of *Ae*p110 transcript to ribosomal RNA levels revealed a pattern similar to straight densitometry indicating a real increase in *Ae*p110 levels (Figure 5.5D). The exception is at 72 hours, shortly after

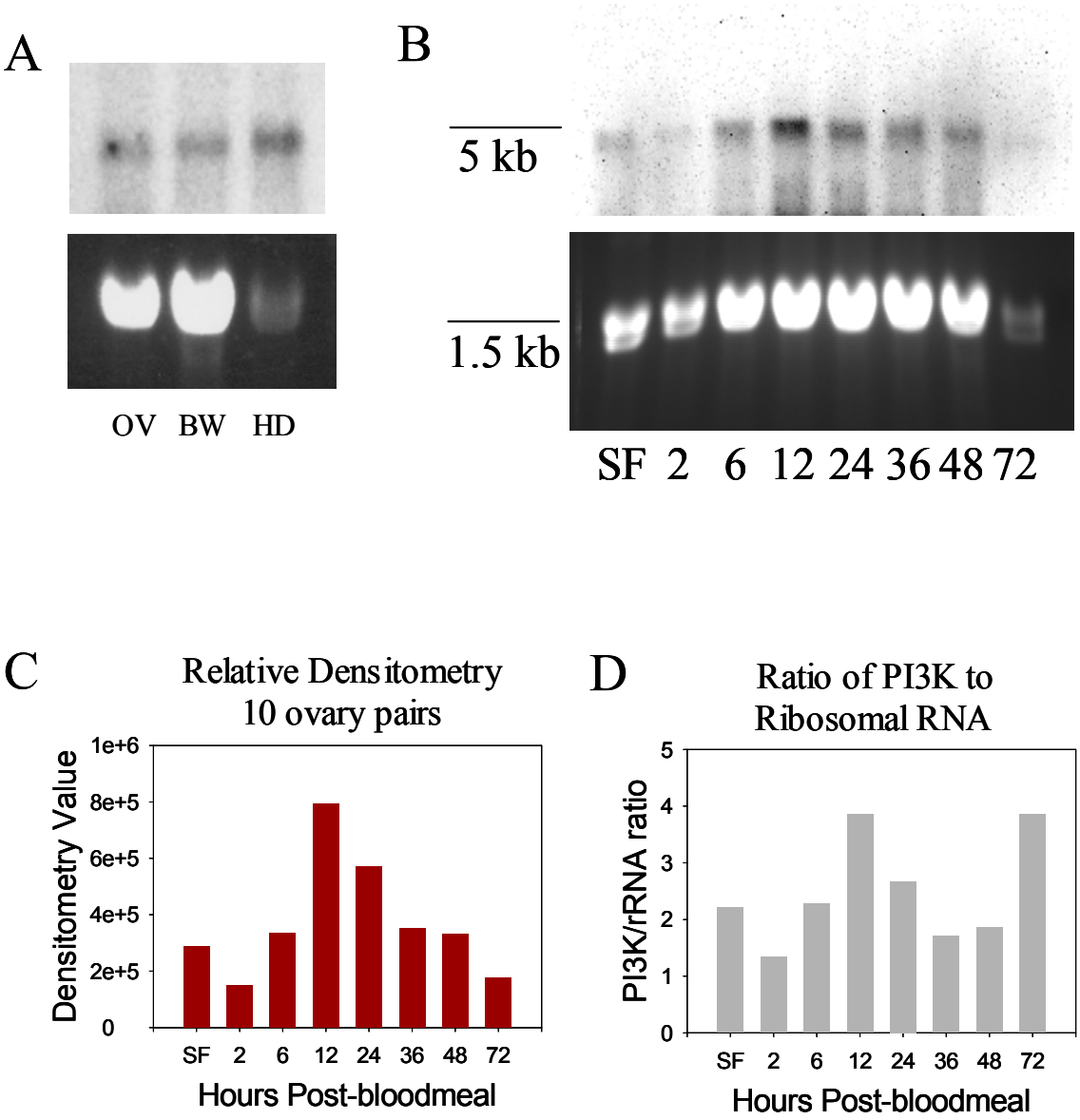


Figure 5.5: Expression of *Ae*p110 in different tissues during a reproductive cycle. A. Representative northern blot showing expression of *Ae*p110 in ovary (OV), body wall (BW), and head (HD). **B.** Representative northern blot examining *Ae*p110 expression in 10 pairs of ovaries after a bloodmeal. **C.** Densitometry analysis of the northern blot in B. **D.** Ratio of *Ae*p110 to ribosomal RNA levels.

egg deposition, when the ratio indicates a large increase in transcript due to the low levels of rRNA.

Only partial sequences from *Ae*p110 and a Class 3 PI3K have been isolated from *Ae. aegypti*, yet this information has still revealed a wealth of knowledge. Both classes of kinases were found to have key domains required for their catalytic activity, and *Ae*p110 has a C2 binding domain required for association with PtdIns. However, to completely understand the functional domains found in these proteins, the complete sequence will need to be determined, their expression patterns in immature and adult stages examined, and their roles elucidated through knockout analysis.

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CHAPTER 6

CONCLUSIONS

In this dissertation I have demonstrated that steroid production in the ovary, a key process in egg maturation, is regulated through an insulin-like signaling cascade in the mosquito *Aedes aegypti*. Stimulation of steroidogenesis by bovine insulin in vitro suggests that an endogenous insulin-like hormone, at least in part, may activate steroid production in the mosquito ovary. Inhibitors and activators to various components of the RTK/PI3K/PKB signaling pathway were able to inactivate or activate steroidogenesis respectively in vitro. An inhibitor of tyrosine kinase receptors, HNMPA-$(AM)_3$, prevented ovaries stimulated with bovine insulin from producing steroids in a dose dependant manner in vitro (ID_{50} of 14.2 μM). In contrast, pervanadate, known to stimulate insulin signaling in vertebrates by preventing the constitutive dephosphorylation of the insulin receptor, significantly activated steroid production at concentration ranging from 250 μM to 1 mM but was not as potent as bovine insulin. In mammals, the insulin signaling cascade branches at insulin receptor substrate (IRS) into a mitogenic MAP kinase cascade and a metabolic PI3K/PKB cascade. The MAPK inhibitor, PD098059, did not inhibit steroid production in vitro even at high concentrations, ruling out the mitogenic MAPK branch as a regulator of steroidogenesis. However, two inhibitors of PI3K, wortmannin and LY294003, inhibited steroid production at concentrations similar to those used in mammalian studies (4, 6) indicating that signaling for steroidogenesis occurred throughout the metabolic PI3K/PKB signaling

branch and not the mitogenic MAPK branch. This was further shown by the stimulation of PKB by okidaic acid, a phosphatase inhibitor known to activate PKB. Since steroidogenesis appeared to occur through the RTK/PI3K/PKB signaling cascade, several main components of this cascade, including the RTK, PI3K, and PKB were characterized further. The first component examined in detail was the RTK, termed mosquito insulin receptor (MIR).

The MIR was first described in the mosquito *Ae. aegypti* by Graf et al. (3). Members of the insulin receptor family are translated as a proreceptor consisting of an α and β subunit and separated by a tetrabasic processing site. During processing in the Golgi complexes or secretory granule, these subunits are proteolytically separated yet remain bound together through a disulfide bridge. Once the processed receptor is inserted into the cell membrane, it dimerizes with a second α/β subunit to form the mature receptor. Although the MIR possessed a tetrabasic processing site that may separate the α and β subunits, the protein, when inserted into a baculovirus and expressed in an insect cell line, was not cleaved, leaving doubt as to whether the receptor was cleaved natively (3). Antibodies generated against both the α and β subunit were used to examine the native receptor in the ovary under reducing and non-reducing conditions and the molecular weights were consistant with the typical tertrameric structure, two α and two β subunits, of an insulin receptor. Under reducing conditions, the α and β subunits migrated at 110 and 90 kDa respectively as determined by SDS-PAGE analysis. When linked by disulfide bridges, the complete tetrameric receptor migrated at ~400 kDa. Using antiphosphotyrosine antibodies, it was demonstrated that the MIR became tyrosine phosphorylated when incubated with bovine insulin, providing strong evidence that the

MIR was in fact the RTK stimulating steroidogenesis in vitro. In addition to the protein characterization of the MIR, it was important to determine its expression patterns during development and a reproductive cycle.

Detectable expression of the MIR in the mosquito occurs only in the adult. Neither the MIR protein nor the transcript could be detected in embryos, larvae, early pupae, or pharate adults. This is in contrast to insulin receptor orthologues in other invertebrates that have a crucial role in development during the immature stages. In females, expression of the MIR in the ovary increased rapidly during the first three days after adult eclosion, when the ovaries undergo rapid development in preparation for the reproductive cycle. By three days after eclosion ovary development ends and the mosquito enter a state of previtellogenic arrest. Throughout the arrest stage the insulin receptor was expressed at high but constant levels in anticipation of a bloodmeal.

One of the most intriguing results was found while studying the receptor's expression pattern during a reproductive cycle. The MIR was expressed on the plasma membrane of the follicle cells only during previtellogenesis and the first 12 hours post-bloodmeal, as confirmed both by immunoblots and immunocytochemistry. This expression pattern is not surprising, since the role of the follicle cells switch from steroid to chorion production between 24 and 36 hours post blood meal. After the switch to chorion production there is no need for the steroidogenic receptor on the follicle cells, and the receptor was not expressed again until after oviposition when the secondary follicles develop into the primary follicles and a new reproductive cycle began.

Following the further characterization of the MIR gene, an orthologue of PKB, a nexus for many of insulin's metabolic effects in mammals, was characterized in the ovary

and early embryo of *Ae. aegypti*. The primary sequence of mosquito PKB (MPKB) shares 81% and 73% sequence similarity with the *Drosophila* and human PKBs respectively, and this high degree of sequence conservation allowed us to generate a 3D structural model of the tyrosine kinase domain. Immunocytochemistry was used to localize MPKB in the cytosol of follicle cells surrounding the primary follicle, and its subcellular localization did not appear to be affected by insulin stimulation. The protein could not be detected with immunoblots prior to a bloodmeal due to the use of heterologous antibodies that did not bind to MPKB well. However, 36 to 48 hours after a bloodmeal, MPKB protein levels were sufficient to be detected on immunoblots. This high level of expression corresponds with the increase in MPKB transcript observed using northern blots. Although we are not certain why MPKB expression increased late in the reproductive cycle, it is likely that, as in *Drosophila*, the MPKB plays a role in early embryonic development and may be a maternally transmitted mRNA (1). Additional evidence for this theory is the high level of MPKB mRNA expression during the first 6 hours after oviposition, an expression pattern identical to *Dakt* in *Drosophila* (1, 2). Stimulation of ovaries with bovine insulin resulted in the threonine phosphorylation of a 60 kD protein, the putative MPKB. Surprisingly, there was no serine phosphorylation detected in ovaries after insulin stimulation as would be expected if Ser^{524} was required for full activation of MPKB. It is possible that the Ser residue may be quickly dephosphorylated or may not be required for MPKB activation. Activation of PKB through the phosphorylation of Thr only has been observed in the Akt-2 gene of *C. elegans* that does not possess a phosphorylated Ser at the C-terminus (5).

Finally, the partial sequences of the catalytic subunit from two different classes of PI3K have been identified, a Class 1 PI3K believed to be involved in the steroidogenic signaling cascade and a Class 3 PI3K with an unknown function. The Class 1 PI3K, *Aedes* p110 (*Ae*p110), shares 86% sequence similarity to the *Drosophila* Dp110 protein while the Class 3 PI3K in *Aedes* shares 83% sequence similarity with the *Drosophila* PI3K_59F protein. As with other members of the PI3K family, the class one PI3K in *Aedes* possesses a catalytic, accessory, and C2 domains and the class 3 possesses a catalytic and accessory domain. A p85 binding domain will most likely be found upstream in the unsequenced region of *Ae*p110. In contrast to the MPKB, the *Ae*p110 is expressed in a wide range of tissues including head, abdomen, and ovary where it likely has additional metabolic and mitogenic signaling roles.

This dissertation has elucidated critical steps in the steroidogenic signaling cascade in the ovary of the mosquito *Aedes aegypti* (Fig 6.1). In summary, an insulin-like peptide or OEH I binds to the MIR causing a conformational change that results in the autophosphorylation and activation of the β subunit. The kinase activity of the β subunit may phosphorylate an IRS orthologue or the phosphorylated tryosines may bind downstream signaling components directly. The SH2 domain on the regulatory subunit of PI3K binds to phosphotyrosines on either the MIR or an IRS orthologue resulting in a conformational change in the regulatory subunit and the release of the catalytic subunit, *Ae*p110. The C2 domain of the catalytic subunit then binds to PtdIns and phosphorylates PtdIns(4,5)P_2 on the third position creating PtdIns(3,4,5)P_3. This provides a binding site for the PH domains of both MPKB and an unidentified Ser/Thr kinase, most likely PDK1. The binding of these proteins to PtdIns(3,4,5)P_3 brings them into close

association resulting in the phosphorylation and activation of MPKB on a Thr residue. Phosphorylation of the Thr residue causes a conformational shift in the T-loop allowing substrates and ATP access to PKB's kinase domain. PKB may activate transcription factors such as members of the forkhead family to translate members of the ecdysteroid synthesis cascade, and it may be important in recruiting cholesterol into the cell for conversion into ecdysteroids. In addition, this cascade may have a role in nutrient metabolism, the regulation of lifespan, and follicle cell division in the mosquito.

Although this dissertation has laid the groundwork for understanding steps in the steroidogenic signaling cascade, additional studies need to be performed. Other signaling components, including IRS, p85, PDK1, PTEN, mTOR and forkhead transcription factors need to be isolated from *Ae. aegypti* and their potential role in steroidogenesis examined. Furthermore, and perhaps more importantly, the MIR ligand, whether it is OEH I, an insulin-like peptide, or a novel peptide, needs to be determined. Finally, we need to determine the precise role these signaling proteins have in steroidogenesis. To that end overexpression and knockout (RNAi) experiments need to be performed on key signaling molecules in this complex steroidogenic signaling cascade.

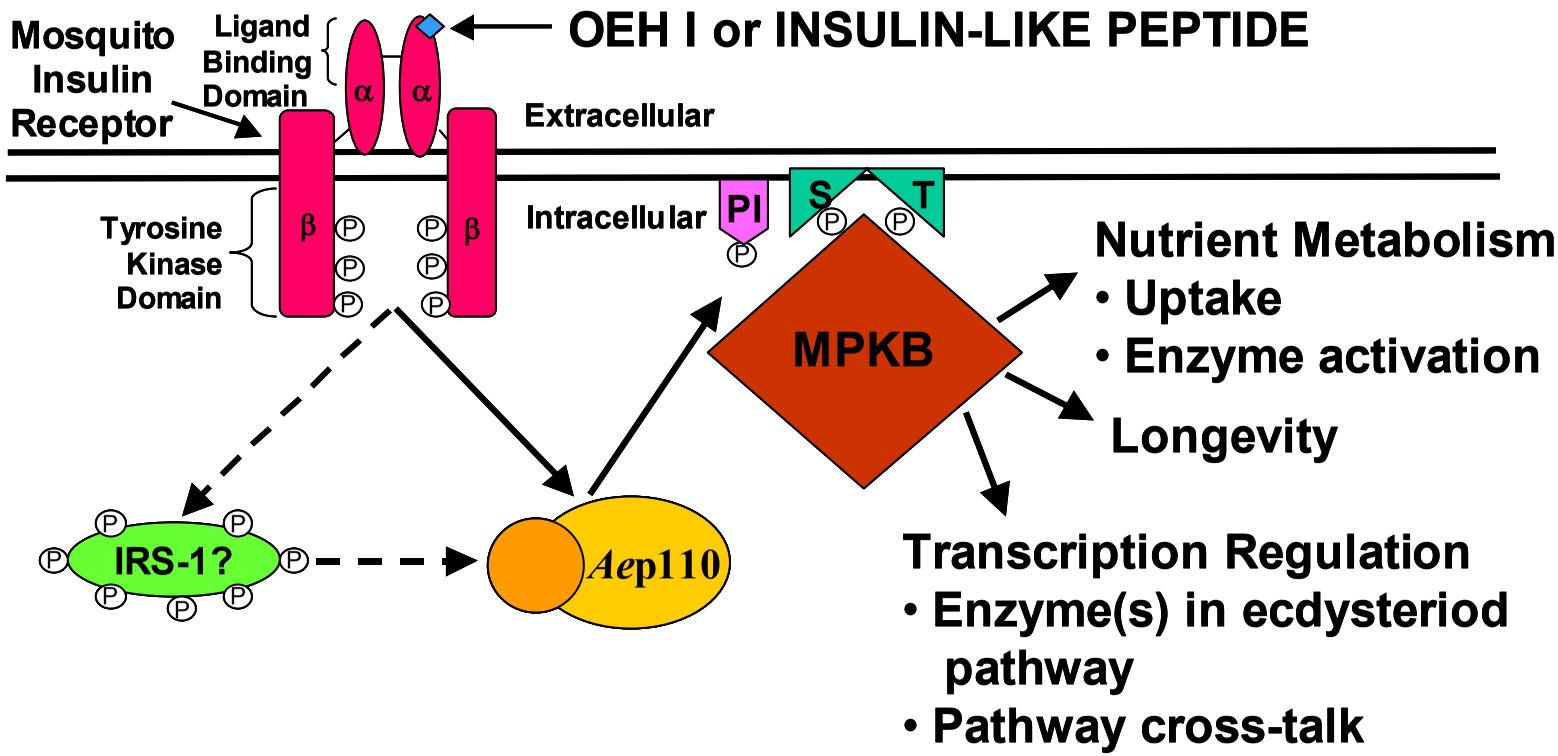

Figure 6.1: Putative steroidogenic signaling cascade in the ovaries of the mosquito *Ae. aegypti*. The MIR ligand binds to the extracellular subunit activating the receptors kinase domain. MIR may phosphorylate an IRS molecule or activate PI3K directly. The *Ae*p110 subunit phosphorylates PI on the third position providing a binding site for MPKB. MPKB may in turn activate a wide range of physiological responses including steroidogenesis.

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