Subcellular location and function of a putative juvenile hormone esterase binding protein in *Drosophila melanogaster*

by

Zhiyan Liu

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Program of Study Committee:
Bryony C. Bonning, Major Professor
Russell Jurenka
Kristen Johansen
Clark Coffman
Jack Girton

Iowa State University

Ames, Iowa

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ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

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ABSTRACT

Insect development, metamorphosis and reproduction are regulated in part by the action of juvenile hormone (JH). The titer of JH is regulated in turn by the action of the enzymes juvenile hormone epoxide hydrolase and juvenile hormone esterase (JHE). Because of the potential for disruption of regulation of insect development through perturbation of the action of JH, the biology of JHE has been well studied. A putative juvenile hormone esterase binding protein, P29 was identified in the tobacco hormworm, *Manduca sexta*. Following sequencing of the *Drosophila melanogaster* genome, we identified a homolog of P29 in *D. melanogaster*, and used this insect for analysis of the biology and function of P29 in relation to JHE.

The gene encoding *D. melanogaster* P29 (DmP29), CG3776 was cloned, recombinant DmP29 expressed in *E. coli* and two anti-DmP29 antisera raised. *In vitro* binding of the P29 homolog to *Drosophila* JHE was confirmed. P29 mRNA and an immunoreactive protein of 25 kDa were detected in *Drosophila* larvae, pupae and adults. The predicted size of the protein is 30kD. *Drosophila* P29 is predicted to localize to mitochondria (MitoProt; 93% probability) and has a 6kD N-terminal targeting sequence. Subcellular organelle fractionation and confocal microscopy of *Drosophila* S2 cells confirmed that the immunoreactive 25kD protein is present in mitochondria but not in the cytosol. Expression of P29 without the predicted N-terminal targeting sequence in High Five™ cells showed that the N-terminal targeting sequence is shorter than predicted, and that a second, internal mitochondrial targeting signal is also present. An immunoreactive

protein of 50 kDa in the hemolymph does not result from alternative splicing of CG3776 but may result from dimerization of P29.

We investigated the potential ligands of DmP29 by testing three hypotheses: (i) DmP29 binds to *D. melanogaster* JHE: We produced a stably transformed insect cell line that expresses DmJHE and confirmed that DmP29 binds to *D. melanogaster* P29. DmJHE binds to both the 25 kD and 50 kD immunoreactive proteins. (ii) DmP29 binds other, non-specific esterases including two esterases predicted to be targeted to the mitochondria: We did not detect any interaction between DmP29 and non-specific esterases. (iii) DmP29 binds to other proteins in *D. melanogaster*: Ligand blot analysis, immunoprecipitation experiments and affinity binding experiments showed that larval serum protein 1 binds the 25 kD P29. The possible biological relevance of the *in vitro* DmP29-JHE interaction is provided by detection of JHE activity in *D. melanogaster* mitochondrial fractions; 0.48 nmol JH hydrolysed/min/mg mitochondrial protein, 97% of which was inhibited by the JHE-specific inhibitor OTFP. However, the DmP29-LSP interactions may not be biologically relevant, given the high abundance, and "sticky" nature of these proteins. Interaction of DmP29 with LSP may result from non-specific associations.

We used P29 hypo- and hyper-expression mutants to elucidate the function of P29 and the potential interaction of P29 with JHE. The hypomorphic mutant EP835 of P29 had reduced JHE activity when compared to wild type flies. Hyperexpression of P29 in EP/Gal4 during the early larval stages was lethal, while hyperexpression during the third

instar resulted in reduced size of adult flies. This phenotype showed that overexpression of P29 interfered with insect development. Hyperexpression in newly eclosed but not in older females resulted in reduced fecundity, indicating that overexpression of P29 affected ovarian development. Fecundity was not affected by P29 hyperexpression in the male. Hypermorphic adults exhibited male-male courtship behavior. Hyperexpressed females showed reduced receptivity to males. Hyperexpressed females had decreased production of courtship pheromone, cis, cis-7, 11-hepta cosadiene, which resulted in male flies being unable to locate female flies. Hyperexpression of P29 in males resulted in decreased production of the aggregation pheromone, cis-vaccenyl acetate. For EP835/Gal4, the hypermorphic mutant, all hyperexpression phenotypes were consistent with a reduced JH titer in *Drosophila*. Flies that hypo- or hyper- expressed P29 had a significantly shorter lifespan: Reduced lifespan correlated with increased egg production (hypomorphic flies) and hyperactivity (hypermorphic flies), respectively. Hence, the titer of P29 appeared to be positively correlated with the titer of JHE and negatively correlated with the titer of JH. Based on the collective phenotypes and detection of JHE activity in mitochondria, we hypothesize that JHE is stored in mitochondria and that P29 functions in transport of JHE to the cytosol.

CHAPTER 1

General introduction

Juvenile Hormone

Juvenile hormone (JH) is a sesquiterpenoid insect hormone which is produced by the corpora allata in the brain. It has a methyl ester on one end and an epoxide on the other end. There are six natural forms of JH that have been detected in different orders and at different specific developmental stages: JH0, JHI, JHII, JHIII, JH bis-epoxide (JHB_3) and methyl farnesoate. JH I was the first form of JH to be identified (Roller and Dahm, 1968). JH has now been identified in about 100 insect species in at least 10 insect orders (Baker, 1990). JH III is the predominant homolog (Schooley *et al.*, 1984) while other forms of JH have a more restricted distribution. All forms of JH are produced by the corpora allata. In the Diptera the corpora allata and corpora cardiaca are fused to form a ring gland.

JH, in conjunction with ecdysteroids, regulates insect development, metamorphosis and reproduction. Regulation of the larval - pupal transition has been particularly well characterized. The presence of JH at a larval molt directs maintainence of the larval stage, and prevents the development of the pupal form (Kumaran, 1990). JH functions in the metamorphosis of holometabolous insects as follows: a high level of JH signals a larval/larval molt, a low level of JH signals a larval/pupal molt and no JH signals a pupal/adult molt (Schneiderman and Gilbert, 1964; Gilbert and King, 1973). In most insects, JH is involved in vitellogenesis which is required for oocyte development (Shapiro *et al.*, 1986; Bownes and Rembold, 1987; Sappington *et al.*, 1998). JH

stimulates vitellogenin production and uptake by the ovaries. JH also regulates pheromone production and calling in longer lived lepidopteran adults (McNeil, 1987; Cusson *et al.*, 1990). However, pheromone production appears to be regulated by ecdysone in some Diptera (Barth and Lester, 1973; Jean-Marc, 1984). JH affects the behavioral response to aggregation pheromone in desert locusts (Ignell *et al.*, 2001). JH also functions in various other processes including the development of both male and female gonadotrophic organs (Yamamoto *et al.*, 1988), caste determination (Rachinsky *et al.*, 1990) and diapause (Denlinger and Tanaka, 1989; Zera and Tiebel, 1989).

JH Synthesis

The titer of JH is controlled by the relative rates of JH biosynthesis and degradation. JH is synthesized by the corpora allata, but is not stored there. JH biosynthesis is regulated by two kinds of peptide hormones, stimulated by allatotropins and inhibited by allatostatins, which are produced by neurosecretory cells of the brain. In the tobacco hornworm *Manduca sexta*, allatotropinis are produced in adults by the gene Mas-AT which encodes three prehormones which are generated by alternative splicing of the gene (Kataoka *et al.*, 1989; Taylor *et al.*, 1996). Allatotropic activity was found in the subesophageal ganglion of crickets (Lorenz and Hoffmann, 1995), but no gene was isolated. A 20kD peptide identified as allatotropin from *Galleria mellonella* larval brain, may share common epitopes with Mas-AT (Bogus and Scheller, 1996). In the vinegar fly, *Drosophila melanogaster,* it has been suggested that a sex-peptide synthesized by the male sex gland and transferred to the female is a source of allatotropin (Moshitsky *et al.*, 1996). Allatostatin (Mas-AS) was purified and sequenced from *M. sexta* (Kramer *et al.*,

1991). In *Drosophila*, the Mas-AS like protein does not have an allatostatin function (Jansons *et al.*, 1996). There is evidence for a cerebral allatostatin in *D. melanogaster* (Richard *et al.*, 1990; Altaratz *et al.*, 1991).

Proteins Involved in Juvenile Hormone Binding and Regulation

Several proteins are involved in binding and metabolizing JH. Juvenile hormone binding proteins (JHBPs) identified in 1972 have been found in many species (Whitmore and Gilbert, 1972; Gilbert *et al.*, 2000). JHBPs have high affinity for JH, but low affinity for JH degradation products. The function of JHBP is to keep JH in solution in the hemolymph, to prevent non-tissue-specific uptake of JH, to prevent non-specific degradation of JH and to assist in the interaction between JH and JH specific degradation enzymes (Goodman *et al.*, 1990; Trowell, 1992). There are three types of hemolymph JH binding proteins. One is a 30kD protein found in Lepidoptera which has a single peptide and a single JH binding site (Kramer *et al.*, 1974; Goodman *et al.*, 1978). The second is lipophorin, which is a predominant hemolymph protein and main lipid carrier in the hemolymph (DeKort and Granger, 1996). The third JHBP is a 566kD protein found in *Locusta migratoria* that has six JH binding sites (Koopmanschap and deKort, 1988).

JH degradation is attributed to juvenile hormone esterase (JHE), which is present in the hemolymph and tissues, and to juvenile hormone epoxide hydrolase (JHEH), which is tissue/membrane bound (DeKort and Granger, 1996). JHE hydrolyzes the ester of JH to produce JH acid. JHEH hydrolyzes the epoxide of JH to produce JH diol (JHD), but JHEH only functions in cells (Halarnkar *et al.*, 1993) (Fig 1). The cumulative activities of the two enzymes convert JH to juvenile hormone acid diol (JHAD) for which no activity

has been discovered (Fig 2). Most JH is bound to JHBP and hence JH is protected from degradation by non-specific esterases with low binding affinities (Touhara *et al.*, 1993; Touhara and Prestwich, 1993; Touhara *et al.*, 1995). JHE is the only enzyme in the hemolymph that has a high affinity for JH, and hence is the only hemolymph esterase important in JH degradation (Gilbert *et al.*, 2000) (Fig 2). A polar JH acid ester was also discovered to be synthesized and released from the corpora allata of *M. sexta,* but its function is unknown. (Granger *et al.*)

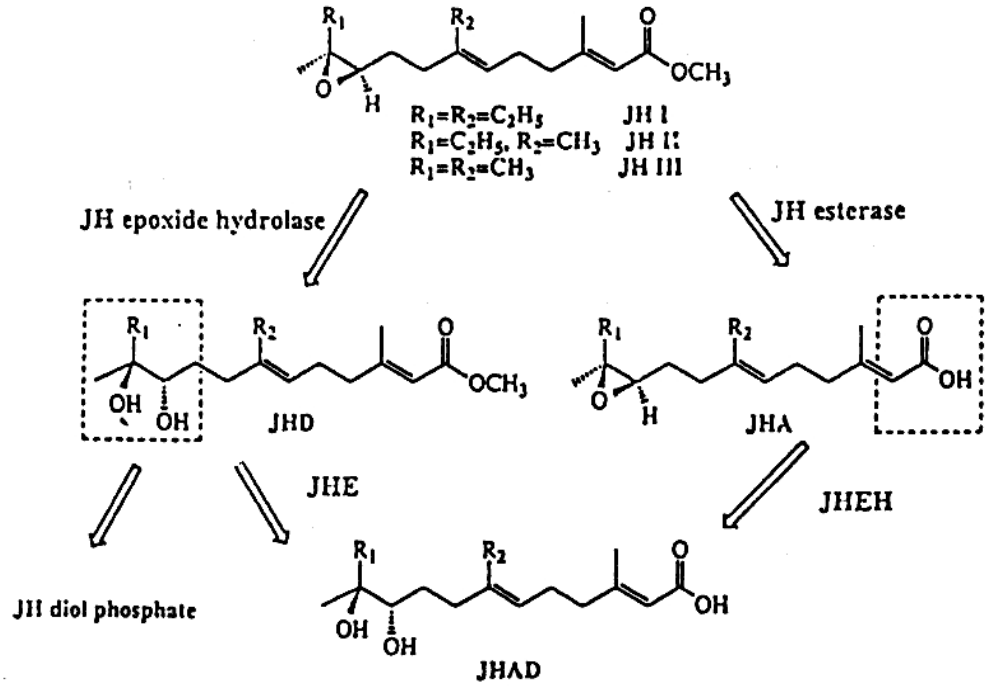

Fig 1. Primary non-oxidative metabolic pathways for JH in insects. (Halarnkar *et al.*, 1993)

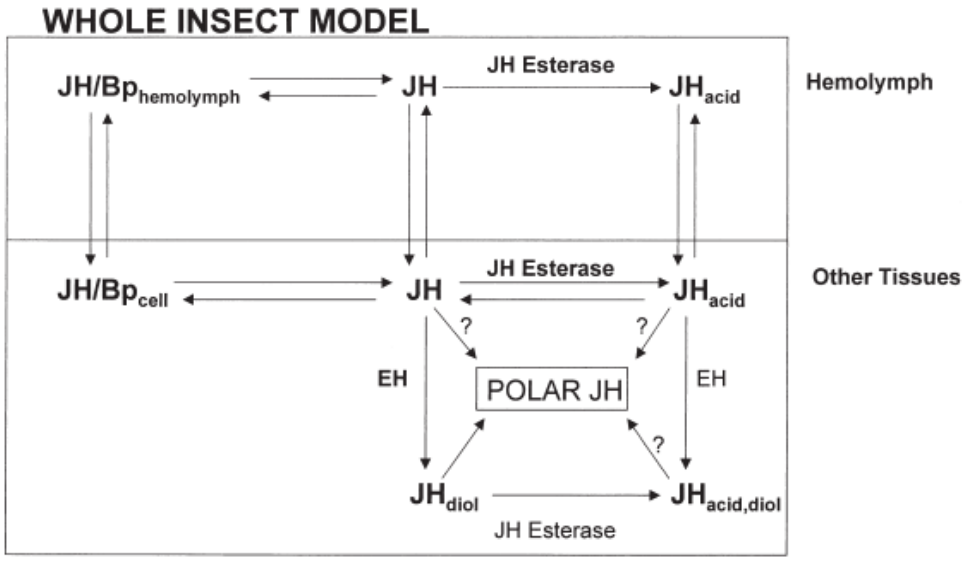

Fig 2. A model for JH metabolism. Bp, binding protein; EH: juvenile hormone epoxide hydrolase (Gilbert *et al.*, 2000).

JHE

Because of JHE's important role in regulation of the JH titer in insects as well as in the development of insecticides based on JHE, this enzyme has been extensively studied in many orders of insects. JHE proteins have been isolated and purified from *Trichoplusia ni* (Yuhas *et al.*, 1983; Hanzlik and Hammock, 1987; Rudnicka and Jones, 1987; Wozniak *et al.*, 1987), *Manduca sexta* (Coudron *et al.*, 1981; Venkatesh *et al.*, 1990), *Heliothesis virescens* (Hanzlik *et al.*, 1989), *Leptinotarsa decemlineata* (Vermunt *et al.*, 1997), *D. melanogaster* (Campbell *et al.*, 1992), *Tenebrio molitor* (Thomas *et al.*, 2000) and *Bombyx mori* (Shiotsuki *et al.*, 1994).

Study of the house cricket (*Acheta domesticus*) showed that JHE activity and alpha-naphthylacetate esterase activity were regulated in the hemolymph during the first reproductive cycle. Alpha-Naphthylacetate esterases increased during the first

gonotrophic cycle: peaks of their activity could be observed concomitant with peaks of JHE activity (Renucci *et al.*, 1984).

JHE is present in low quantities in insect hemolymph, but since it has a high affinity for JH, even a trace amount of JH can be rapidly degraded by JHE (Ward *et al.*, 1992). Renucci also reported the correlation between the fluctuations in JHE activity and those of hemolymph JH titers using *in vitro* methods (Renucci *et al.*, 1984).

JHE Genes

The JHE gene was first cloned from the tobacco budworm, *H. virescens* (Hanzlik *et al.*, 1989). This moth was used because JHE in this insect has fewest isoforms (Abdel-Aal and Hammock, 1986; Hanzlik and Hammock, 1987; Abdel-Aal *et al.*, 1988). In addition, the economic importance of this insect as an agricultural pest makes it a suitable model for the development of novel insecticides. JHE was purified and the NH_2-terminal end was sequenced. Several DNA probes were designed based on the sequence of the protein. The strategy used to isolate a cDNA clone of JHE mRNA was to screen a cDNA expression library initially with antisera and then to re-screen the positive clones by hybridization to a mixture of the 15-mer oligonucleotides complementary to the region of the mRNA transcript coding for the NH_2 terminus of JHE protein. This increased the likelihood of obtaining a full-length clone. The positive clones were sequenced. The complete sequence of one of the clones showed a 2989-base pair insert that fit the northern blot fragments (3.0 kb) by the 15-mer oligonucleotides. The clone had an open reading frame of 1714 base pairs with a predicted mature protein of 61kDa. The translated amino acid sequence fit the sequence of the protein well. Then the *H. virescens*

JHE gene was cloned. The enzyme was similar to other carboxylesterases in the NH_2-terminal half and in the active site, with an active site serine at position 201.

After the JHE gene had been cloned from *H. virescens* several other JHE genes were isolated from other insects. The *Choristoneura fumiferana* JHE gene was cloned by differential display of mRNAs to identify *C. fumiferana* genes that were induced by JH I. PCR products were then used to probe a cDNA library. The deduced amino acid sequence was similar to the *H. virescens* JHE sequence (Feng *et al.*, 1999). The JHE gene of *L. decemlineata* was cloned by RT-PCR and RACE. The RT-PCR primer was designed based on the amino acid sequence of JHE. The product was used to screen a cDNA library to get the whole DNA and sequence (Vermunt *et al.*, 1997). In *T. ni,* the partial sequence of a JHE gene has been reported (Venkataraman *et al.*, 1994), while a cDNA clone encoding a JHE-related protein has also been reported (Jones *et al.*, 1994). The JHE gene from *M. sexta* was cloned by RT-PCR using primers based on the amino acid sequence (Hinton and Hammock, 2001; Hinton and Hammock, 2003). *B. mori* JHE was cloned recently (Hirai *et al.*, 2002). The *H. virescens* JHE cDNA was inserted into the genome of *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) and active JHE produced by the recombinant virus (Hammock *et al.*, 1990).

JH in *D. melanogaster*

The JH titer was determined for whole-body extracts at different stages of the life cycle of *D. melanogaster* using combined gas chromatography/selected-ion mass spectroscopy (Bownes and Rembold, 1987). Only JHIII was detected in this study. JHIII

was present during all larval instars but absent from eggs. The JH titer in the first and second instar larvae was higher than that in third instars. A low titer of JH was detected in prepupae but JH was undetectable in pupae. However, there was an increase in JHIII just prior to eclosion for both males and females reaching a peak just after eclosion (Fig 3). In 1989 another form of JH, JHIII bisepoxide (JHB_3), was isolated from the ring gland of *D. melanogaster*. JHB_3 may be the main biologically active form of JH in the higher Diptera. The JHIII detected previously in *D. melanogaster* may have resulted from instability of JHB_3 (Richard *et al.*, 1989).

JH has multiple functions in *D. melanogaster*. The most well known effects of JH are on pre-adult development and metamorphosis (Riddiford and Ashburner, 1991). JH has an effect on initiation and continuation of vitellogenin uptake, oocyte development and ovarian maturation (Handler and Postlethwait, 1977; Ringo *et al.*, 2005). JH also functions in developing receptivity in females for mating, or in increasing receptivity once other factors have intiated this process (Manning, 1967; Ringo *et al.*, 2005). The antijuvenoid Precocene I slowed ovarian growth and markedly reduced oviposition (Ringo *et al.*, 2005).

JHE in *D. melanogaster*

D. melanogaster JHE was purified and characterized, and is highly selective for JHIII and JHIII bisepoxide (Campbell *et al.*, 1998). The JHE gene was identified in *D. melanogaster* by matching its peptide mass fingerprint with a sequence from the *Drosophila* genome project (Campbell *et al.*, 2001). JHE was purified and digested with trypsin. Only one predicted gene product (CG8425) from the *D. melanogaster* genome

matched the JHE tryptic fingerprint with high confidence. A cDNA encoding this JHE was isolated using 3' and 5' RACE. This sequence is in agreement with the *Drosophila* genome project's prediction except that the sixth predicted intron is not removed; instead there is a stop codon followed by a polyadenylation signal and a polyA tail (Campbell *et al.*, 2001). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of JHE showed that the expression level of JHE in *D. melanogaster* is regulated by both JH and 20-hydroxyecdysone (Kethidi *et al.*, 2005). Khlebodarova *et al* showed that both JHE and JHEH are related to changes in JH titer in *D. melanlogaster*. The high level of JH-hydrolyzing activity is determined by JHEH in adult flies (Khlebodarova *et al.*, 1996) (Fig 4A).

We determined the JHE activity in carefully staged *Drosophila* Oregon R prepupae and pupae. Three to five staged flies were ground in eppendorf tubes in PBS buffer on ice and centrifuged at 8000g at 4°C briefly to remove debris. The protein concentration was quantified by Bradford assay. JHE activity was measured in triplicate by a partition assay using ^{3}H-JH-III as substrate (Hammock and Sparks, 1977). The nanomoles of JH hydrolyzed per minute per mg protein were calculated. JHE activity reached a peak 11 hours after pupariation (Fig 4B). Our results are similar to the published data for expression of *D. melanogaster* JHE (Campbell *et al.*, 1992; Khlebodarova *et al.*, 1996). The peak JHE activity is consistent with an undetectable titer of JH in the pupal stage.

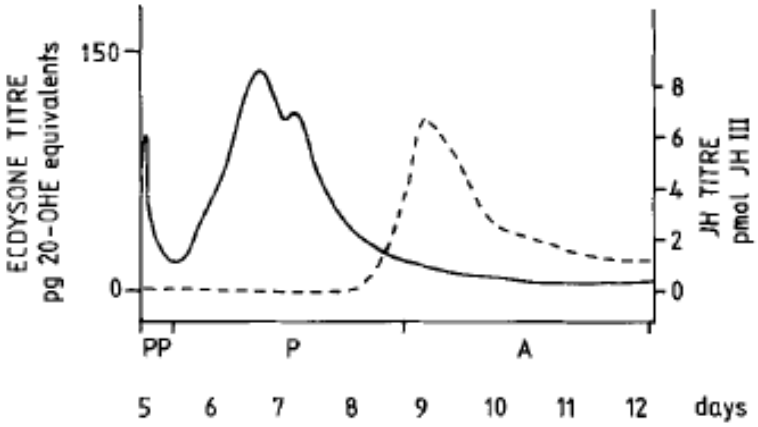

Fig 3. JH-III titer (- - - -) and ecdysteroid titer (-) during the life cycle of *D. melanogaster* (Hodgetts *et al.*, 1977; Bownes and Rembold, 1987).

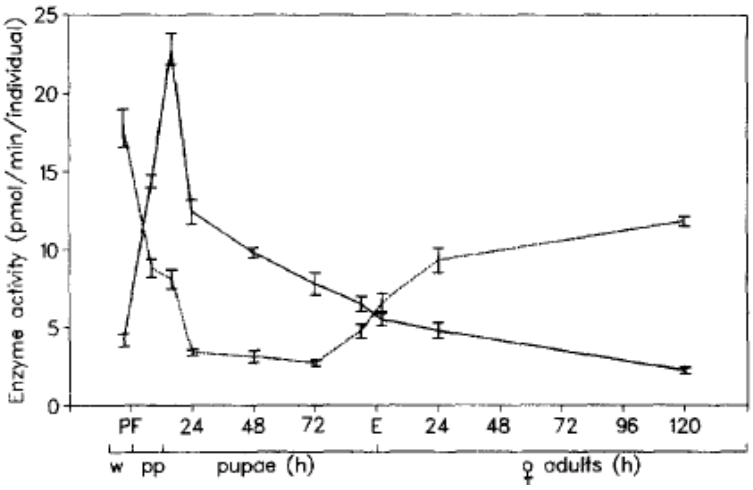

Figure 4 A. The activity of JHE and JHEH during pupal-adult development of *D. melanogaster* (Canton S. line). Continuous line=JHE activity, discontinuous line-JHEH activity (Khlebodarova *et al.*, 1996).

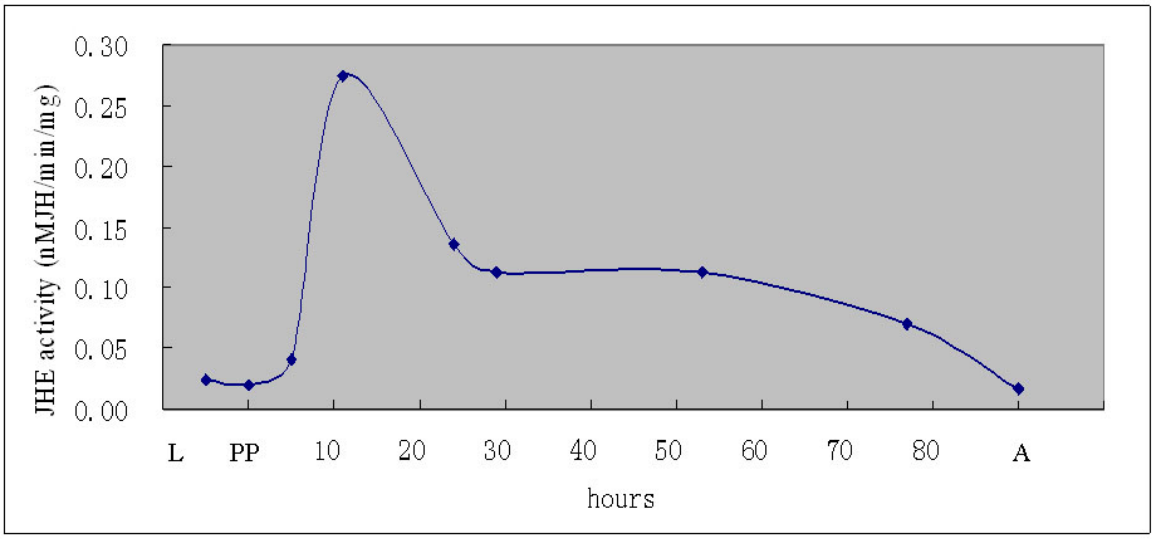

Fig 4B. JHE activity assay of staged *D. melanogaster* Oregon R (L: larvae; pp: prepupae; A: adult).

Nonspecific Carboxylesterases

Carboxylesterases can hydrolyze carboxylate esters and play an important role in insects. Some esterases such as JHE have highly specialized functions. However, most esterases are nonspecific and can catalyze different substrates, which enable them to protect the insect against foreign substances (Satoh *et al.*, 2002). The nonspecific carboxylesterases have broad and overlapping catalyzing activity against naturally occurring and xenobiotic esters, thioesters and amide esters (Heymann, 1982). Because of their lack of specificity, it is impossible to identify their true physiological substrates. The physiological function of the esterases may be detoxification, but there is a lack of firm evidence for this, except in the case of amplified esterases conferring resistance to organophosphorus (OP) insecticides (Long *et al.*, 1991). Carboxylesterases have different subcellular locations: some are secreted into the blood, while others are associated with

the cell membrane, cytoplasm and subcellular organelles (endoplasmic reticulum). No mitochondrial esterases have been reported and no mitochondrial esterases have been identified in *D. melanogaster*. In a previous study, the putative JHE binding protein P29 was shown to bind JHE in *M. sexta* (Shanmugavelu *et al.*, 2000). However, we found that P29 is predicted to be localized to the mitochondria. We aligned the esterases in *Drosophila* and found that α-E1 and cricklet which share 33% identity to JHE are predicted to be in mitochondria (MITOPROT). We hypothesize that P29 binds to α-E1 and/or cricklet in mitochondria and affects the function of α-E1 and/or cricklet.

α-esterases in *D. melanogaster*

D. melanogaster has over 40 esterases (Oakeshott *et al.*, 1993). Using naphthyl esters as substrates about 30 esterases have been detected by electophoretic assays (Healy *et al.*, 1991; Spackman *et al.*, 1994). α-esterase is so called because it can hydrolyze α-naphthyl ester. *D. melanogaster* has an α-esterase cluster which contains 10 active esterase genes (DmαE1 to DmαE10) and one pseudogene, dispersed over 60kb. The esterases encoded by the cluster have 37%-66% amino acid identity (Robin *et al.*, 2000). The α-esterase cluster has about 40% of the active esterases in the genome, therefore it might be expected to play an important role in esterase function (Campbell *et al.*, 2003). EST9 encoded by DmαE5 and EST23 encoded by DmαE7 are important esterases in *Drosophila*. It has been suggested they play a role in detoxification of xenobiotics or the digestion of esters (Oakeshott *et al.*, 1993; Russell *et al.*, 1996). This suggestion is based on the fact that EST9 and EST23 are abundant in digestive tissues and esterases are

important in OP insecticide resistance. α-esterase may also function in lipid metabolism because EST9 and EST23 are abundant in the fat bodies of larvae (Healy et al., 1991; Spackman et al., 1994). From screening of expressed cDNA libraries, DmαE1, DmαE2 and DmαE7 (EST23) appear to be highly expressed in the adult head, which conflicts with their role in the digestion of dietary esters (Campbell et al., 2003). *Culex* mosquito esterase ESTB1 has the most similar amino acid sequence to D. melanogaster αE1, which confers organophosphate resistance (Russell et al., 1996).

The Juvenile Hormone Esterase Binding Protein, P29 of *M. sexta*

Since JHE can degrade JH and affect insect metamorphosis, a study was conducted to determine whether a recombinant baculovirus containing the JHE gene would effectively kill Lepidoptera, based on the hypothesis that increased JHE titers would reduce JH titers at an inappropriate time and be toxic to insects. Baculoviruses infect insects primarily within the Lepidoptera. However, the recombinant baculovirus that expressed wild-type JHE had no noticeable effect on the survival time of infected larvae relative to the wild type, possibly because JHE is rapidly removed from the hemolymph. Three modified JHEs were produced with conservative changes to avoid disruption of the three-dimensional structure or the catalytic activity of JHE. Infection of larvae with a baculovirus expressing one of the mutated JHEs (JHE-KK) decreased feeding damage by 50% (Bonning *et al.*, 1997). JHE-KK had both Lys_{29} and Lys_{524} replaced with arginines. Analysis of pericardial cells exposed to either the wild-type JHE or the mutated form of JHE by electron microscopy showed that the mutation caused failure of the normal lysosomal targeting of JHE in pericardial cells (Bonning *et al.*,

1997). JHE-KK was scattered in pericardial cells, and accumulation of JHE-KK in the lysosomes was five-fold less than for wild type JHE. Uptake of JHE-KK from the hemolymph was not affected by the mutations made.

Pericardial cells take up JHE by endocytosis, which suggested that JHE was specifically binding to pericardial cells by receptors (Ichinose *et al.*, 1993; Mellman, 1996; Bonning *et al.*, 1997). To investigate possible binding proteins of JHE, a recombinant cDNA phage display library of *M. sexta* pericardial cells was screened and a sequence encoding a 29 kDa binding protein identified (GenBank database accession number AF153450) (Shanmugavelu *et al.*, 2000). P29 was confirmed to be present in the pericardial cells and fat body tissue of *M. sexta* larvae by western and northern blots. Interaction of P29 with recombinant *H. virescens*-derived JHE was confirmed *in vivo* and *in vitro* by immunoprecipitation with antisera that recognized both *H. virescens* JHE and *M. sexta* P29. Based on the previous result that JHE-KK had reduced targeting to lysosomes compared to wild type JHE and was insecticidal, the binding of P29 to JHE and JHE-KK was tested. P29 bound less effectively to JHE-KK than to wild type JHE (Shanmugavelu *et al.*, 2001). With this much data pointing to P29 as a significant potential intermediate in the degradation process of JHE, information about P29's pathway and the location of P29's interaction with JHE may provide a better understanding of how the titer of JHE can be manipulated.

Misexpression of proteins in *Drosophila*

A successful system for misexpression of specific proteins in *Drosophila* was introduced by Rørth (1996). A transposable element (EP element), which contains UAS

sites that can bind to the yeast transcription factor Gal4 and a promoter, was randomly inserted into the 5' untranslated region of genes. The insertion would affect expression of the EP tagged gene (the gene immediately downstream of the EP element). Furthermore, the EP element would allow for the gene immediately downstream to be overexpressed and misexpressed by crossing to lines with *gal4*-expressing insertions. Temporal control of gene expression in *Drosophila* has generally been accomplished by using a heat shock (hs) promoter; spatial control of gene expression has been accomplished by using tissue-specific promoters (Joseph, 2002).

D. melanogaster P29 Homolog

Following the release of the *D. melanogaster* genome sequence (Adams *et al.*, 2000), a P29 homolog was identified that allowed us to exploit the knowledge of *Drosophila* genetics and associated research tools to determine the role of P29. The gene product of CG3776 (Dm) shares 49% identity with *M. sexta* P29 (Ms) over 206 residues. MsP29 has 243 amino acids while CG3776 encodes a protein with 263 amino acids resulting in a calculated molecular mass of 30kD.

The *D. melanogaster* P29 gene CG3776 is located on the right arm of the second chromosome at position 60E10. This region is a gene-dense region. At the 5' end of CG3776 is the *RpL19* gene which encodes a ribosome protein, L19. At the 3' end of CG3776 is the *Phk-3* gene encoding a protein pherokine-3, which is a putative odor/pheromone binding protein (Sabatier *et al.*, 2003) (Fig 5). There are two *D. melanogaster* EP lines (EP835 and EP840) available at the Szeged *Drosophila* Stock Centre. Both of the EP lines are homozygous viable. EP835 has an EP element inserted

into the chromosome 35nt upstream of the CG3776 start site in the 5' untranslated region (Fig 5). The EP element of EP840 is inserted into the chromosome 2nt upstream from that of EP835 (i.e. 37 nt upstream of the start site). EP835 was used to make a double mutant with the *pnr* gene, which encodes a zinc-finger protein with homology to the vertebrate GATA transcription factors (Ramain *et al.*, 1993; Winick *et al.*, 1993). Hyperexpression of EP835 suppressed the *pnr*Gal4/+ phenotype, suggesting that P29 interacts with this transcription factor (Pena-Rangel *et al.*, 2002). However, no gene function studies have been conducted for CG3776 using this EP line. We used these lines to study gene function when hypoexpressed. It is estimated that more than two thirds of genes in *Drosophila* have no obvious loss of function phenotype, possibly due to functional redundancy (Miklos and Rubin, 1996). In such cases, overexpression of a gene can provide an indication of function (Rorth, 1996) (Fig 6). In addition, the EP line can be used to generate 'imprecise excisions' by remobilizing the P element, which may result in a complete null mutant.

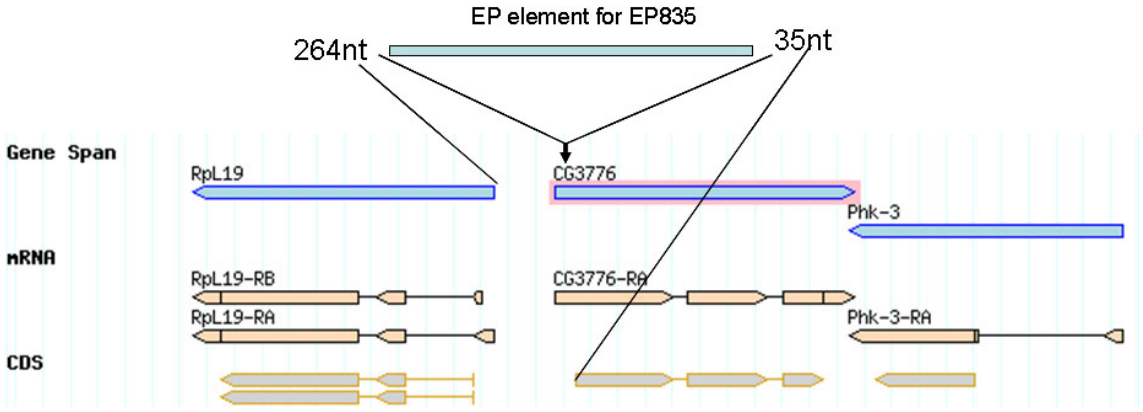

Fig 5. Diagram of CG3776 and its neighboring genes (From chromosome 2R: 20,476k to 20479.5k; adapted from flybase). The EP element insertion site of EP835 is indicated.

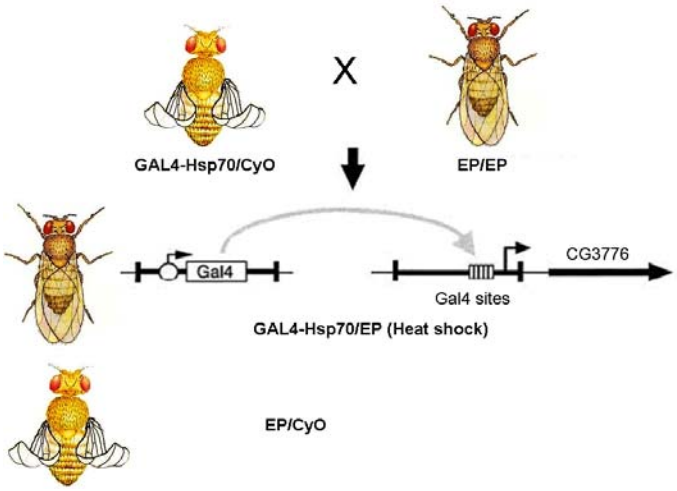

Fig 6. Outline of the overexpression screen. The EP line contains a single EP element inserted into the P29 5' untranslated region. When mated with GAL4 flies, progeny with straight wings will contain both the Gal4 and EP elements. This allows GAL4 to bind to GAL4 binding sites within the EP element, thereby inducing the EP promoter to transcribe the gene immediately adjacent to the element (P29).

Structural Analysis of *Drosophila* and *Manduca* P29

MITOP (mitochondria project) is a database for mitochondria-related proteins in selected species. MITOPROT is a program that can predict mitochondrial targeting sequences by analyzing the N-terminal sequence (Claros and Vincens, 1996) (http://ihg.gsf.de/ihg/mitoprot.html). Mitochondrial targeting peptides are known to be rich in arginine, alanine, and serine, while negatively charged amino acid residues (aspartic acid and glutamic acid) are rare (von Heijne *et al.*, 1989). Further, these residues are believed to form an amphiphilic α-helix important for import into mitochondria (Nakai and Kanehisa, 1992; Bannai *et al.*, 2002). The *Drosophila* P29 protein (DmP29) is predicted with 93% probability to be a mitochondrial protein and *Manduca* P29 (MsP29) has 99.6% probability to be a mitochondrial protein (Table 1). The predicted mitochondrial signal sequence of DmP29 is 54 residues. The signal peptide is therefore

~5.8kD. Therefore, the mature DmP29 should have a calculated molecular mass of 24.2kD.

This result made us review the former experiments in *M. sexta* (Shanmugavelu *et al.*, 2000). JHE and MsP29 can interact *in vitro* as detected by *in vitro* immunoprecipitation. In the *in vivo* immunoprecipitation experiment, biotinylated JHE was injected into *Manduca* larvae. Tissues were homogenized and precipitated with P29 antibody. If P29 is located in mitochondria it would be released by homogenization and then able to interact with JHE. Hence the data can be explained even if JHE and P29 are in different subcellular locations and do not bind *in vivo*.

Table 1. Predicted mitochondrial signal sequences of *D. melanogaster* and *M. sexta* P29 (MITOPROT)

Insect	Probability of export to mitochondria	Cleavage site	Predicted mitochondrial signal sequence
D. melanogaster	93.0%	55	MQHTLIRCLGMARISLMRLQPRP TVAASGGQEAGSISKPTQPVSR SFASLPQEQ
M. sexta	99.6%	44	MNLALRQVLTRQSFRLCDRYA HKNVAKQIPLTSQCSVIQYRKY

We also did amino acid alignment with CG3776. Other than *M. sexta*, we found five putative JHE binding proteins from five insect species. They all have putative mitochondrial targeting sequences (Table 2). The predicted N-terminal cleavage site is from 24 to 78 amino acids. *D. melanogaster* P29 has a putative conserved Tim-44 like domain. *M. sexta* and four of the five proteins also have this domain (Table 2).

Table 2. Sequence alignments with *D. melanogaster* CG3776 by Blast and their probability of transport to mitochondria as predicted by MITOPROT.

Species	Gene	Length (Amino acid)	Identity	Probability of export to mitochondria	Tim44-like domain
D. pseudoobscura	GA17681-PA	247	203/262 (77%)	98.6%	√
Anopheles gambiae str. PEST	EAA14399	285	115/204 (56%)	98.4%	√
Aedes aegypti	AET-4549	284	120/194 (61%)	92.0%	
Tribolium castaneum	LOC657974	260	102/196 (52%)	95.6%	√
Bombyx mori	DQ443374	243	97/211 (45%)	99.2%	√
M. sexta	AAD38067	243	102/199 (51%)	99.6%	√

Mitochondria

Mitochondria are subcellular organelles, which have an outer membrane, intermembrane space, inner membrane and matrix. The inner membrane is folded into cristae. The most important function of mitochondria is to generate ATP as a source of energy. In the Kreb's cycle, hydrogen ions (electrons) are donated to the two carrier molecules NAD or FAD. These electrons are carried to an electron transport chain in the mitochondrial cristae, which pump H^+ out of the matrix. This creates a gradient across the inner membrane with a higher concentration of hydrogen ions in the intermembrane space. The ATP synthase uses the energy of the hydrogen ion gradient to form ATP (Alberts *et al.*, 1994). Since mitochondria are so important for cells, misexpression of a gene encoding a mitochondrial protein could be lethal or have multiple effects on the organism (Beziat *et*

al., 1993; Walker and Benzer, 2004). Because mitochondria are responsible for energy generation and the site of formation of most reactive oxygen molecules, misexpression of mitochondrial proteins is often related to changes in life-span (Morrow *et al.*, 2004; Rand, 2005; Schriner *et al.*, 2005; Walker *et al.*, 2006).

Protein transport to mitochondria

Mitochondria have their own genome for expression of some mitochondrial proteins. However, the majority of mitochondrial proteins are encoded by the nuclear genome and have to be transported into mitochondria from the cytosol (Alberts *et al.*, 1994). Hence the membrane translocation machinery in the inner or outer membrane required for transport of nuclear-encoded proteins into the mitochondria is very important for mitochondrial function. Proteins translated in the cytosol are sorted to the four parts of the mitochondria. Both the outer and inner membrane translocation machineries are composed by multiple proteins. The multi-subunit protein complex in the outer membrane is called the TOM complex (translocase of the outer membrane), which is composed by signal receptors and the general import pore (GIP). The translocase of the inner membrane is called the TIM complex, which is composed by two complexes: TIM23 (the presequence translocase) and TIM22 (the protein insertion complex) (Rehling *et al.*, 2003). It is an exciting and fast developing field to identify the components of these complexes. Tim44 is an essential component of Tim23. Tim44 serves as a membrane anchor protein to anchor mitochondrial hsp70 to the inner membrane and is involved in import of proteins into the mitochondrial matrix (Moro *et al.*, 2002; Wiedemann *et al.*, 2004; Matsuoka *et al.*, 2005). There is a growing list of a

class of multifunctional and multicompartmental proteins that are initially targeted to mitochondria but then are exported to different compartments of the cell by an unknown mechanism. This list includes Hsp10, Hsp60, mHsp70, tumor necrosis factor receptor-associated protein-1, cytochrome c, gC1q-R/p33, and mitochondrial aspartate aminotransferase (Domanico *et al.*, 1993; Wadhwa *et al.*, 1993; Isola *et al.*, 1995; Soltys and Gupta, 1996; Cechetto and Gupta, 2000; Cechetto *et al.*, 2000; Ghebrehiwet *et al.*, 2001; Skanda *et al.*, 2001).

Dissertation Organization

Chapters 2, 3 and 4 of this dissertation contain manuscripts to be published. Chapter 2 deals with analysis of the subcellular location of P29. This manuscript has been accepted for publication in Insect Biochemistry and Molecular Biology. Chapter 3 presents data addressing the possible ligands of *D. melanogaster* P29. This manuscript has been submitted to Insect Biochemistry and Molecular Biology. Chapter 4 describes functional analysis of P29 by examination of phenotypes resulting from hypo- and hyperexpression of the gene. Chapter 5 consists of general conclusions, discussion of the project as a whole, and possible future research directions.

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CHAPTER 2

Localization of a *Drosophila melanogaster* homolog of the putative juvenile hormone esterase binding protein of *Manduca sexta*

Zhiyan Liu, Linda Ho and Bryony C. Bonning

Department of Entomology and Interdepartmental Program in Genetics, Iowa State University, Ames, IA 50011-3222, USA

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Abstract

A putative juvenile hormone esterase (JHE) binding protein, P29, was isolated from the tobacco hornworm *Manduca sexta* (J. Biol. Chem. 275(3): 1802-1806). A homolog of P29 was identified in *Drosophila melanogaster* by sequence alignment. This gene, CG3776 was cloned, recombinant DmP29 expressed in *E. coli* and two anti-DmP29 antisera raised. *In vitro* binding of the P29 homolog to *Drosophila* JHE was confirmed. P29 mRNA and an immunoreactive protein of 25 kDa were detected in *Drosophila* larvae, pupae and adults. The predicted size of the protein is 30kD. *Drosophila* P29 is predicted to localize to mitochondria (MitoProt; 93% probability) and has a 6kD N-terminal targeting sequence. Subcellular organelle fractionation and confocal microscopy of *Drosophila* S2 cells confirmed that the immunoreactive 25kD protein is present in mitochondria but not in the cytosol. Expression of P29 without the predicted N-terminal targeting sequence in High Five™ cells showed that the N-terminal targeting sequence is shorter than predicted, and that a second, internal mitochondrial targeting signal is also

present. An immunoreactive protein of 50 kDa in the hemolymph does not result from alternative splicing of CG3776 but may result from dimerization of P29. The function of P29 in mitochondria and the possible interaction with JHE are discussed.
Key Words: *Drosophila melanogaster*, juvenile hormone esterase binding protein, P29, *Manduca sexta*, mitochondria

1. Introduction

Juvenile hormone esterase (JHE: EC 3.1.1.1) plays a vital role in the regulation of the titer of juvenile hormone during insect development (Hammock, 1985). To exploit the role of JHE in the larval development of Lepidoptera for pest control purposes, recombinant baculoviruses expressing JHE were constructed on the basis that overexpression of JHE at an inappropriate time would reduce the titer of juvenile hormone and disrupt larval development (Hammock *et al.*, 1990; Bonning *et al.*, 1992; Eldridge *et al.*, 1992). However, overexpression of JHE had minimal effect, possibly because JHE is rapidly removed from the hemolymph by pericardial cells and degraded in lysosomes (Booth *et al.*, 1992; Ichinose *et al.*, 1992b) or because of the continued synthesis of juvenile hormone. A mutant form of JHE with disrupted lysosome targeting was insecticidal when delivered by a recombinant baculovirus (Bonning *et al.*, 1997).

To identify proteins that may function in the processing of JHE within the pericardial cells, we screened a pericardial cell cDNA phage display library derived from *Manduca sexta* by using recombinant *Heliothis virescens*-derived JHE as bait. The putative JHE binding protein P29 was identified (GenBank database accession number AF153450)

(Shanmugavelu *et al.*, 2000). Interaction of P29 with JHE was demonstrated *in vivo* and *in vitro* by immunoprecipitation with antisera that recognized both *H. virescens* JHE and *M. sexta* P29. Following release of the *D. melanogaster* genome sequence (Adams *et al.*, 2000), a homolog of P29 (DmP29) was identified, with a predicted size of 30 kDa (Fig. 1). *In vitro* binding of DmP29 to *D. melanogaster* JHE was confirmed (unpublished data). Using the extensive genetic resources associated with *D. melanogaster*, we sought to determine the function of P29.

Here we provide evidence that P29 is targeted to the mitochondria. The implications of this localization and detection of additional immunoreactive proteins in *Drosophila* hemolymph are discussed in relation to the biology of JHE.

2. Materials and methods

2.1 Expression and purification of recombinant P29

The cDNA of the *Drosophila melanogaster* gene CG3776, a homolog of *M. sexta* P29, was obtained by RT-PCR. mRNA was isolated from third instar larvae (73 mg) of *D. melanogaster* strain OregonR using the Micro Poly (A) Pure mRNA purification kit (Ambion Inc., Austin, TX) and cDNA synthesized by using a cDNA synthesis kit (Clontech, Mountain View, CA). PCR primers designed with reference to the published sequence were used: DrP29-2; 5'-GGGAATTC**ATG**CAGCACACGCTTATACGCTGCT-3' and DrP29-3; 5'-GGGGTACCATG**TTA**TATGGTCTCGTTAATAAGG-3' (start and stop sites are shown in bold), which contain *Eco*RI and *Kpn*I sites (underlined) for cloning, respectively. PCR

products (about 800 bp) were first cloned into the expression vector pRSET-JF (Shanmugavelu *et al.*, 2000), but no induced protein was detected from this vector. The insert was removed from pRSET-JF with *Eco*R I and *Hind* III and inserted into the plasmid pBAD/His (Invitrogen). The *E. coli* strain Top10 was transformed with the plasmid. The vector pBAD/His has an araBAD promoter and N-terminal polyHis and Xpress tags. Expression was optimized with different concentrations of arabinose and different temperatures. Recombinant protein expression was monitored by separation by SDS-PAGE and detection by western blot using the anti-Xpress antibody. The recombinant protein was expected to be 35.2 kD (30.3 + 4.9 kD) (Fig. 2). Because the majority of the recombinant protein was in inclusion bodies, the recombinant protein was purified by using a Ni-NTA column under denaturing conditions according to the manufacturer's directions (Qiagen, Valencia, CA). The 35kD protein band detected by SDS-PAGE with Coomassie blue R250 staining was cut from the gel and run on a second SDS-PAGE gel (12%). Proteins were transferred from this gel to Hybond-P membrane (Amersham Biosciences, Piscataway, NJ) for western blot analysis using anti-DmP29 (see below). The secondary antibody used for detection of primary antibody was HRP-conjugated anti-rabbit IgG. The HRP was detected by using the ECL detection system (Amersham Biosciences, Piscataway, NJ).

2.2 Production of anti-P29 antisera

Two anti-P29 antisera were generated for detection of DmP29 in New Zealand white rabbits by using standard procedures (Harlow and Lane, 1988). For the first antiserum, rabbits were injected with gel-purified recombinant P29 expressed in *E. coli* from the

pBAD/His vector described above. For the second antiserum, the antigen used was a 14 amino acid peptide sequence derived from the N-terminus of P29 (amino acids 51-64: PQEQDKKEQNARES). This sequence was selected based on hydrophilicity, surface-orientation and flexibility as determined by analysis with the Protean program from DNAStar. The first four amino acids (underlined) are part of the predicted mitochondrial localization signal of P29. Control blots were processed with preimmune serum. The two antisera recognized the same *D. melanogaster* proteins, which were not present on blots processed with the preimmune serum. On the basis that cross-reactivity was stronger for the antiserum raised against recombinant P29, this antiserum was used for subsequent experiments.

To assess the temporal and tissue distribution of P29, the antiserum was purified (Bassham and Raikhel, 1998) and used for detection of immunoreactive proteins in (1) homogenates of larvae, pupae and adults of *D. melanogaster*, (2) hemolymph, fat body and gut tissues of adult flies. For western analysis, proteins were separated by SDS-PAGE (12% gel), transferred to Hybond-P membrane and processed as described above.

2.3 Reverse transcription PCR and RACE

RT-PCR and Rapid Amplification of cDNA Ends (RACE) were used to determine whether CG3776 is alternatively spliced. RT-PCR was used for detection and analysis of P29 mRNA at different stages during development. mRNA was isolated from larvae, prepupae, pupae and adults as described above. RT-PCR was conducted using the primers DrP29-2 and DrP29-3 to amplify the full length P29 mRNA. 5'- and 3'-RACE

were conducted to determine whether CG3776 undergoes alternative splicing, using the SMART™ RACE cDNA amplification kit (Clontech) according to the manufacturer's directions. 5'-RACE was conducted on larval and adult mRNA, while 3'-RACE was conducted on larval mRNA only. The primer RACE5-2; 5'-GGTCTCGTTAATAAGGTCGATGGCCCTAAACTGG-3' (860 to 827 nt) was used for 5'-RACE, and primers RACE3-1; 5'-GGCCCGGATATCCCTGATGCGTTTGC-3' (53 to 78 nt) and RACE3-2; 5'-CCCTGGACAATCTTGTCTCGCCCGAGG-3' (412 to 438 nt) were used for 3'-RACE along with the SMART™ RACE universal primer. The cDNA products of the 5' and 3' RACE reactions were cloned into pCR-Blunt (Invitrogen; Zero Blunt PCR cloning kit) and sequenced. The full transcript of CG3776 is predicted to be 980 nt with the predicted 5' end 74 nt upstream of the ATG, and the 3' end 114 nt downstream of the TAA (FlyBase).

2.4 Subcellular organelle fractionation

By using the MITOPROT program, we determined that P29 in *M. sexta* and *D. melanogaster* both have predicted N-terminal mitochondrial localization signals (Table 1). To determine whether this prediction was correct, we adapted a protocol for isolation of mitochondria and cytosolic fractions for use with *Drosophila* S2 cells (Halarnkar *et al.*, 1986). The *Drosophila* embryonic cell line S2 (Schneider, 1972) was maintained in Shields and Sang M3 insect medium (Sigma-Aldrich) with 1% penicillin / streptomycin / amphotericin (PSA) and 10% serum at 27°C. Cultured S2 cells were detached from flasks by using a scraper and collected by centrifugation. The cells were suspended in hypotonic buffer (HEPES-KOH 20 mM, pH 7.4; EGTA 1mM) on ice for 10 minutes. Double-

isotonic buffer (hypotonic buffer with 0.5 M mannitol) was then added to restore normal osmolarity. Cells were homogenized in a prechilled porcelain mortar and pestle, and cell homogenate centrifuged twice at 500g for 10 minutes, at 4 ºC. The supernatant was centrifuged at 3,700g for 10 minutes at 4ºC. The resulting pellet was resuspended in isotonic buffer (hypotonic buffer with mannitol 0.25 M) and centrifuged at 7000g for 10 minutes at 4 ºC. The pellet (mitochondrial fraction) and cytosolic fraction (supernatant) were analyzed for the presence of P29 by western blot using the P29 antiserum. To confirm clean separation of the mitochondrial and cytosolic fractions, an anti- mouse Hsp60 monoclonal antibody (Stressgen Bioreagents, San Diego, CA), which cross-reacts with Hsp60 in the mitochondrial matrix, was used.

2.5 Expression of P29 in stably transformed HighFive^{TM} and S2 cells

The predicted N-terminal mitochondrial localization sequence of P29 will be cleaved during transport of P29 into the mitochondria. The cDNA sequences for P29 with (P29) and without (ΔLP29) the predicted mitochondrial leader sequence, were isolated for cloning into the pIE1/153A vector for expression in stably transformed insect cells (Farrell *et al.*, 1989; Farrell and Iatrou, 2004) as follows: First, the cDNAs for P29 and ΔLP29 were amplified by PCR using the primers DrP29-1; 5'-GGGAATTCGGG**ATG**CAGCACACGCTTATACGCTGCT-3' and P29-S2-2; 5'-CCCTCGAGTATGGTCTCGTTAATAAGGTCG-3' for P29, P29NL-1; 5'-GGGAATTC**ATG**GACAAGAAGGAGCAGAATGCCAGAG-3' and P29-S2-2 for ΔLP29. The PCR products were cloned into pMT/V5-His (Invitrogen) using *Eco*RI and *Xho*I restriction enyzme sites and the cDNA sequences confirmed by sequencing. The 5'

primers included ATG translation start sites, while primer P29-S2-2 did not include a stop codon. The inserts along with sequences encoding V5-His tags at the 3' end from the pMT/V5-His vector were removed with *Eco*RI and *Pme*I, blunted with Klenow (Promega), and cloned into pCR-Blunt (Invitrogen; Zero Blunt PCR cloning kit) to acquire appropriate restriction sites for cloning into the pIE1/153A vector. The sequences coding for P29 and ΔLP29 along with C-terminal V5-His tags were cut out with *Bam*HI and *Not*I and cloned into pIE1/153A for expression in stably transformed insect cells (Farrell *et al.*, 1998). The resulting plasmids pIE-P29 and pIE-ΔLP29 were amplified in Top 10 cells.

HighFive™ cells (Wickham *et al.*, 1992) were maintained in Ex-Cell 405 serum free medium. HighFive™ cells were transfected with pIE-P29 or pIE-ΔLP29 and pBmA.PAC, which confers puromycin resistance (Farrell *et al.*, 1998), by using cellfectin (Invitrogen) as transfection reagent. Stably transfected cell lines were acquired by selection with puromycin (5μg/ml). The expression of P29 and ΔLP29 in stably transformed HighFive™ cells was monitored by western blot analysis using anti-P29 and anti-V5 antisera. Localization of these recombinant proteins in the cytosolic and mitochondrial fractions of the stably transformed cells was assessed as described above.

2.6 Immunofluorescence microscopy

S2 cells (Schneider, 1972) were placed on Lab-Tek II chamber slides (Fisher Scientific) and incubated at 27ºC overnight. The cells were stained with 100nM Mitotracker Red CMXRos (Molecular Probes; Invitrogen) for 15 minutes, washed in fresh prewarmed

medium three times, and then fixed in 3.7% formaldehyde. The cells were rinsed with PBS, permeabilized with 0.2% Triton X-100 and then covered with blocking buffer (8% BSA in PBS) for one hour at room temperature to minimize non-specific binding of the antibodies. The cells were incubated with anti-P29 antibody for one hour. After two washes in PBS, cells were incubated with FITC-conjugated goat-anti-rabbit secondary antibody for one hour. Slides were washed with PBS and viewed under an inverted confocal fluorescence microscope (Nikon Eclipse 200) by using a TRITC filter.

3. Results

3.1 Drosophila CG3776 encodes a homolog of M. sexta P29

Following release of the *D. melanogaster* genome sequence (Adams *et al.*, 2000), a homolog of *M. sexta* P29 was identified. The putative *D. melanogaster* JHE binding protein encoded by the gene CG3776 is located on the right arm of the second chromosome at position 60E11. The gene product of CG3776 (Dm) shares 49% identity with *M. sexta* P29 (MsP29) over 206 residues (Fig. 1). MsP29 has 243 amino acids while CG3776 encodes a protein with 263 amino acids with a predicted molecular mass of 30kD. Homologs of *D. melanogaster* CG3776 are also present in *Drosophila pseudoobscura* (77% identity), *Anopheles gambiae* (56%), and *Tribolium castaneum* (52%).

Using the MITOPROT program, a predicted N-terminal mitochondrial targeting signal was identified (Table 1). A Tim44 (translocase of the inner mitochondrial membrane)-

like domain was also identified in CG3776 (amino acids 101 to 150) and in P29 homologs in *T. castaneum*, *D. pseudoobscura*, *An. gambiae* and *M. sexta* (Fig. 1).

3.2 Expression and purification of D. melanogaster P29

The 792 bp cDNA encoding *Drosophila* CG3776 was cloned into pBAD/His and optimal expression of recombinant P29 in *E. coli* Top10 cells induced with 0.2% arabinose at 37ºC. The expressed protein was 35kD, which includes the 4.9 kD N-terminal Xpress and polyHis tags. Induction of P29 expression was confirmed by western blot using the anti-Xpress antiserum (Fig. 3A). Because most of the P29 protein was in inclusion bodies, a denaturing method was used to solubilize recombinant P29 prior to purification using Ni-NTA agarose (Qiagen). A 35kD protein was detected by SDS-PAGE with Coomassie blue R250 staining (Fig. 3B). By cutting the purified 35 kDa recombinant DmP29 from an SDS-PAGE gel, running the protein on a second gel and then examining proteins by western blot with the P29 antiserum, we determined that DmP29 can dimerize under denaturing conditions (Fig. 3C). A band of approximately 65 kDa derived from the 35 kDa protein was detected in proteins from the second gel by western blot (Fig. 3C).

3.3 Detection of immunoreactive proteins in D. melanogaster

The two antisera raised against the P29 protein and N-terminal peptide sequence both recognized proteins of approximately 25, 35 and 50kD in larvae, pupae and adult flies (Fig. 4A). These proteins were not present on control blots conducted with the preimmune serum. Analysis of immnunoreactive proteins in different tissues revealed

tissue specificity of these three proteins: The 35 and 50kD proteins were only detected in the hymolymph (serum, excluding the hemocytes), while the 25 kD protein was detected in fat body and gut, but not in serum (Fig. 4B).

3.4 CG3776 is not alternatively spliced

RT-PCR confirmed the presence of P29 mRNA in larvae, prepupae, pupae and adults (Fig. 5A). The presence of a single band of the expected size (819 nt) also indicated that the P29 gene, which contains three exons, is not alternatively spliced. To assess whether alternative splicing of the P29 gene could account for the immunoreactive proteins of different sizes, 5'- and 3'- RACE reactions were also carried out (Fig. 5B and C). In all cases, reaction products were of the expected size: 934 nt with primer RACE5-2, 927 nt with RACE3-1 and 568 nt with RACE3-2. Sequencing of the cloned products confirmed that CG3776 is not alternatively spliced: Sequence from 21 to 73 nt upstream of the ATG was acquired for more than 10 clones of the 5'-RACE products derived from larvae and adults. Sequence from 26 to 109 nt downstream of the TAA was acquired from 17 clones of the 3'-RACE products from larvae.

3.4 Subcellular localization of P29 in S2 cells

Western blot analysis of mitochondrial and cytosolic fractions of S2 cells revealed a protein of approximately 26 kD in mitochondria recognized by the P29 antiserum. This protein was absent from the cytosolic fractions (Fig. 6). Clean separation of the two subcellular compartments was confirmed by detection of the 64 kD Hsp60 that localizes

to mitochondria. P29 does not appear to be secreted as it was not detected in the cell culture medium of S2 cells.

Localization of P29 to the mitochondria of S2 cells was confirmed by confocal microscopy (Fig. 7). P29 detected using the anti-P29 antiserum co-localized with the mitochondria-specific marker, Mitotracker. In contrast to the use of S2 cells, use of the HighFive™ cell line allowed for examination of *D. melanogaster* P29 in the absence of the native P29 in the cell line (below).

3.5 Subcellular localization of P29 in stably transformed HighFive™ cells

P29 with and without the mitochondrial signal peptide were detected in stably transformed cells by using the anti-P29 or anti-V5 antisera (Fig. 8). As found for native P29 in S2 cells, *D. melanogaster*-derived P29 localized to the mitochondria in stably transformed HighFive™ cells (Fig. 8). ΔLP29 localized primarily to the mitochondria but a small amount of ΔLP29 was also detected in the cytosolic fraction (Fig. 8). In addition, based on analysis of three western blots with reference to molecular mass markers, ΔLP29 was approximately 1.4 kD smaller than the 25.9 kD P29 that was naturally cleaved for removal of the N-terminal mitochondrial targeting signal (Fig. 2). The cleavage site for the mitochondrial targeting sequence was therefore estimated to be 13 amino acids upstream of the predicted cleavage site. Hence, the mitochondrial targeting sequence is estimated to be 41 amino acids in length: MQHTLIRCLGMARISLMRLQPRPTVAASGGQEAGSISKPTQ. The presence of this

shorter targeting sequence would result in a predicted molecular mass of 25.9 kD for the mature protein.

4. Discussion

The majority of mitochondrial proteins are encoded by the nuclear genome, synthesized as precursors and transported into mitochondria from the cytosol. Proteins are then transported to the inner or outer membrane, intermembrane space, or the matrix (Rehling *et al.*, 2003). The import of these proteins is achieved by different mechanisms for import of hydrophilic proteins with cleavable presequences, and hydrophobic proteins with internal sequences (Chacinska *et al.*, 2002). While a common translocase is used in the outer mitochondrial membrane (TOM), two distinct translocases of the inner mitochondrial membrane (TIM) are involved in translocation of proteins with N-terminal (TIM23 complex) and internal signals (TIM22 complex).

The precursor proteins typically consist of an N-terminal targeting sequence of 10 to 80 amino acids followed by the mature domain. The N –terminal sequence, which commonly form amphipathic α-helices with positive charges and hydroxylated amino acids, is cleaved by the matrix processing peptidase during or after import into the mitochondrion (Taylor *et al.*, 2001).

Tim44 is part of the TIM23 complex that functions in translocation of nuclear-encoded proteins across the inner mitochondrial membrane (Weiss *et al.*, 1999). Tim44 serves as a

membrane anchor protein to anchor mitochondrial hsp70 to the inner membrane and is involved in import of proteins into the mitochondrial matrix (Moro *et al.*, 2002; Wiedemann *et al.*, 2004; Matsuoka *et al.*, 2005). Tim44-like domains are also found in smaller bacterial proteins that are thought to function in protein transport. P29 is not Tim44, and the significance of a Tim44-like domain in P29 is unclear although it may suggest a role for P29 in protein transport.

Giot *et al*. examined *Drosophila* protein-protein interactions by using the yeast two hybrid system (Giot *et al.*, 2003). This analysis showed that the product of CG3776 interacts with three proteins from CG3978, CG30327 and CG15772 in this *in vitro* system, and could potentially interact with these proteins *in vivo*. The CG15772-encoded protein in turn interacts with 11 proteins including Tim9A which is part of the mitochondrial inner membrane pre-sequence translocase complex, i.e. the TIM23 complex. Hence, P29 could be a component of the *Drosophila* TIM23 complex.

The fact that removal of the predicted N-terminal mitochondrial targeting signal from P29 did not result in localization of all ΔLP29 within the cytoplasm, suggests that an internal mitochondrial targeting sequence may be present (Fig. 8). Such internal targeting sequences are poorly defined and hence cannot be predicted (Rehling *et al.*, 2003). The reduced size of ΔLP29 relative to the naturally cleaved mitochondrial P29 indicates that the predicted cleavage site for removal of the mitochondrial targeting signal is incorrect. The actual cleavage site appears to be approximately 13 amino acids upstream of the predicted site.

Three immunoreactive proteins were detected with both of the antisera (generated against recombinant P29 or a P29-derived peptide) in *D. melanogaster*. The 25 and 35 kD proteins were also detected in S2 cells (data not shown). The 35 kD protein was shown by MS-MS analysis to be tropomyosin (Liu, unpublished data). Although the identity of the 50 kD protein could not be confirmed by using this approach, the lack of effect of hyperexpression of P29 on levels of the 50 kD protein suggests that it is not P29 (Liu, unpublished data). Based on dimerization of recombinant P29 under denaturing conditions *in vitro* (Fig. 3), an alternative scenario is that the 50 kD protein is a dimer of P29 that is secreted into the hemolymph.

On the basis that differential splicing of *Drosophila* genes is common with about 40% of genes alternatively spliced (Stolc *et al.*, 2004), and that alternative splicing of the gene could account for immunoreactive proteins of different sizes, we conducted RT-PCR, 5'- and 3'-RACE on CG3776. We determined that the gene is not alternatively spliced. This result is consistent with a northern blot conducted on *M. sexta* mRNA which showed a single P29 mRNA band (Shanmugavelu *et al.*, 2000).

Although information on the JHE of D. melanogaster is limited (Campbell *et al.*, 1992; Campbell *et al.*, 1998; Campbell *et al.*, 2001), JHE has been well studied in the Lepidoptera. In lepidopterans, there are intracellular and extracellular pools of JHE (Vince and Gilbert, 1977; Mitsui *et al.*, 1979; Wroblewski *et al.*, 1990). JHE is synthesized by fat body and epidermal tissues and is secreted primarily by the fat body at

specific times during development into the hemolymph (Hammock, 1985; Wroblewski *et al.*, 1990). JHE is actively removed from the hemolymph by the pericardial cells by a saturable process hypothesized to be receptor-mediated endocytosis (Ichinose *et al.*, 1992a; Ichinose *et al.*, 1992b). JHE accumulates in the lysosomes of pericardial cells in the Lepidoptera, where it is presumed to be degraded (Booth *et al.*, 1992; Ichinose *et al.*, 1992b).

The discovery that the putative JHE binding protein, P29 is located in the mitochondria was unexpected, and raises the question of whether JHE is present in mitochondria. Juvenile hormone is known to impact mitochondria either directly or indirectly by increasing mitochondrial protein synthesis and cytochrome oxidase activity (Stepien *et al.*, 1988; Cotton and Anstee, 1991; Farkas and Sut'akova, 2001). There are also examples of proteins that are stored in mitochondria prior to secretion from the cell (Ghebrehiwet *et al.*, 2001). It is also possible that JHE and P29 do not colocalize *in vivo*. Tissues used to detect *in vivo* co-localization of JHE and P29 were homogenized prior to immunoprecipitation of the JHE-P29 complex, and this homogenization step would have been sufficient to break open mitochondria thereby releasing P29 (Shanmugavelu *et al.*, 2000). This result may provide a cautionary tale for methods used for analysis of protein-protein interactions (including the yeast two-hybrid system) that do not take the subcellular localization of the proteins into account. Alternatively, if the 50 kD immunoreactive protein proves to be a dimer of P29, this protein may interact with JHE in the hemolymph.

We are currently examining the effects of hypo- and hyperexpression of P29 in *D. melanogaster* mutant lines to elucidate the function of P29 and to ascertain whether or not P29 functions in the biology of JHE.

Acknowledgements

The authors thank Dr. Kostas Iatrou for provision of the pIE1/153A vector, Bob Doyle for assistance with fluorescence microscopy, Dr. Clark Coffman for guidance on maintenance of flies and critical reading of the manuscript. This work was funded by the National Science Foundation IBN 0090874. Linda Ho was also funded through the Iowa State University Program for Women in Science and Engineering. This journal paper of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 6657, was supported by Hatch Act and State of Iowa funds.

Figures

A

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Identities = 102/206 (49%), Positives = 141/206 (67%)
Ms: 42  KYSDNPTES-RRLPQLMEFPPVVWPSFIKFVKNWMFSNFIIRPYFDQEFSLGEFIEASKH 100
        K   N  ES  RLP+LM+FP +VWPS +  +KNW+   FIIRPYFD EF L +FI  +K
Dm: 56  KKEQNARESLNRLPRLMDFPEIVWPSALNSLKNWITIQFIIRPYFDSEFQLKDFIYGAKQ 115

Ms: 101 AVQVVSDALQQSDFKALEGLVEKDAIAALKTAVSKLSVSQRQLLAIDKEDIFYAFPYQVG 160
        A+QVVS  L   D  +L+ LV  +AIA L+  + KLS++QR+ L I + DI+ +FPYQVG
Dm: 116 ALQVVSSKLMGGDLDSLDNLVSPEAIAELRPVIQKLSMTQRRQLEIKESDIYLSFPYQVG 175

Ms: 161 VMFDDSD----KRWVEITMCYHVLRGLKHMKESGDLPPVSLGVQPEYQDNIFILNYRFIR 216
        +MFDD++    KR+VEITM +HV+RGL  M+E G+  P ++G  PEYQD +FI NYRF++
Dm: 176 IMFDDANDKLQKRFVEITMVFHVMRGLSEMRERGEEIPWNMGTLPEYQDKVFICNYRFVK 235

Ms: 217 EFTKGVEDSWWVNIVNHFQPHTIVKK 242
        EFT G +  W VN+ N F+   ++ +
Dm: 236 EFTAGHQSDWTVNVANQFRAIDLINE 261
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B

```
Tc    3  FIIKPYLDRDFNLPDFVVGSKKAVEVVSKKIAEGNVKALDGLVTEDILPALQRAVTLMSL  62
Ag   37  FII+PY DR+F+LP+F+ G+K+A++VVS  +A G VK L+GLV    L  L++++  MS+  96
Do   79  FII+PY D +F L DF+ G+K+A++VVS K+  G ++ALD LV+ D +  L+  +  +S+ 138
Dm   94  FII+PY D +F L DF+ G+K+A++VVS K+  G++ +LD LV+ + +  L+  +  +S+ 153
Ms   77  FII+PY D++F+L +F+  SK AV+VVS  + + + KAL+GLV +D + AL+ AV+ +S+ 136

Tc   63  SQREQIAVEVEDIYFSFPYQIGVMFNE--DNDQKRFVEITMVFHALKGL  109
Ag   97  ++R  I VE ED+YFSFPYQ+GVMF+E  D+ QKRFVEITMVFH L+GL  145
Do  139  SQR Q+ ++  DIY SFPYQ+G++F+E ND  QKR VEITMVFH ++GL+ 188
Dm  154  +QR Q+ ++  DIY SFPYQ+G+MF++ ND  QKRFVEITMVFH ++GL+ 203
Ms  137  SQR+ +A++ EDI+++FPYQ+GVMF    D+  KR+VEITM +H L+GL  183
```

Figure 1. A. *Comparison of M. sexta P29 with Drosophila melanogaster CG3776 gene product*. The gene product of CG3776 (Dm) shares 49% identity with *M. sexta* P29 (Ms) over 206 residues. P29 has 263 amino acids while CG3776 encodes a protein with 243 amino acids. B. Tim44-like domains in the homologs of *M. sexta* P29. Tc, *Tribolium castaneum*, Ag, *Anopheles gambiae*, Do, *Drosophila pseudoobscura*, Dm, *D. melanogaster*, Ms, *Manduca sexta*.

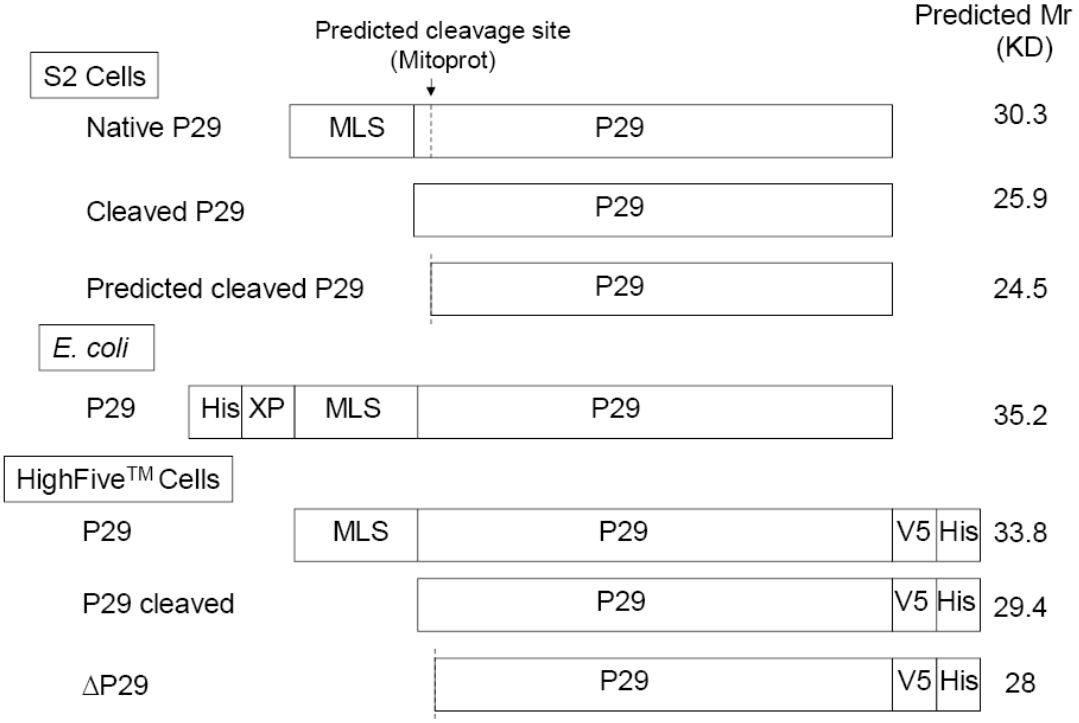

Figure 2. Schematic diagram of native P29 and recombinant P29 constructs for expression in *E. coli* and stably transformed HighFive™ cells. The relative positions of the predicted and actual cleavage sites of the mitochondrial leader sequence (MLS) are shown, and the predicted molecular mass of each protein is indicated. His, hexahistidine tag; XP, Xpress epitope tag; V5, V5 epitope tag.

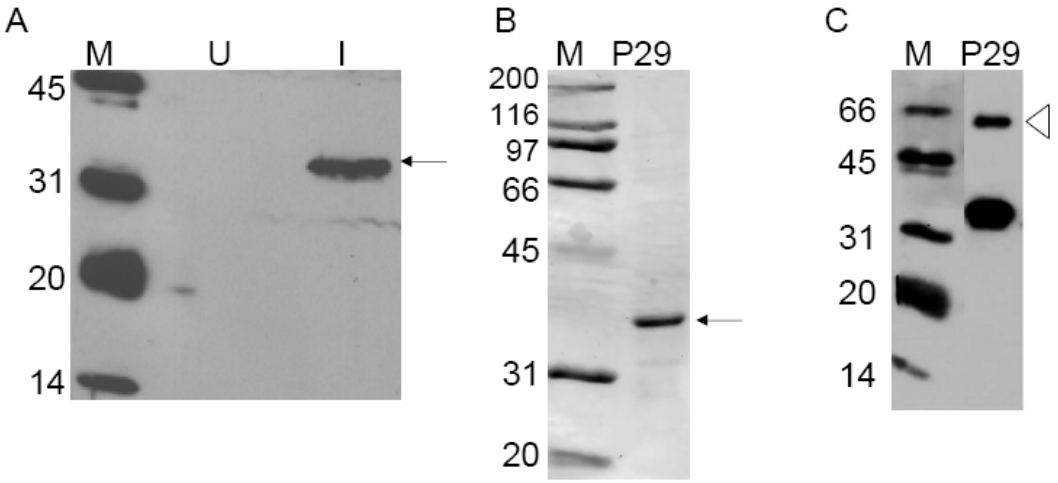

Figure 3. Analysis of recombinant *D. melanogaster* P29; P29 can dimerize *in vitro*. A. Western blot of recombinant P29 expressed in *E. coli.* The anti-Xpress monoclonal antibody was used for detection of P29. Twenty µg of total protein were loaded per lane for uninduced cells (U) and cells induced by addition of arabinose (I). Recombinant P29 is 35 kD (arrow). M, molecular size markers (kD). B. SDS-PAGE (12%) analysis of recombinant P29 purified by Qiagen Ni-agarose. Ten µg of purified protein was loaded (P29) and the gel stained with Coommassie Blue R-250. M, molecular size markers (kD). C. The 35 kD P29 band shown in B was cut and the protein run on a second SDS-PAGE gel and transferred to Hybond-P membrane. In addition to the 35 kD P29, a band running at approximately 65 kD (open arrow head) was detected by western blot with the anti-Xpress antibody. This result indicates that P29 can dimerize under denaturing conditions. M, molecular size markers (kD)

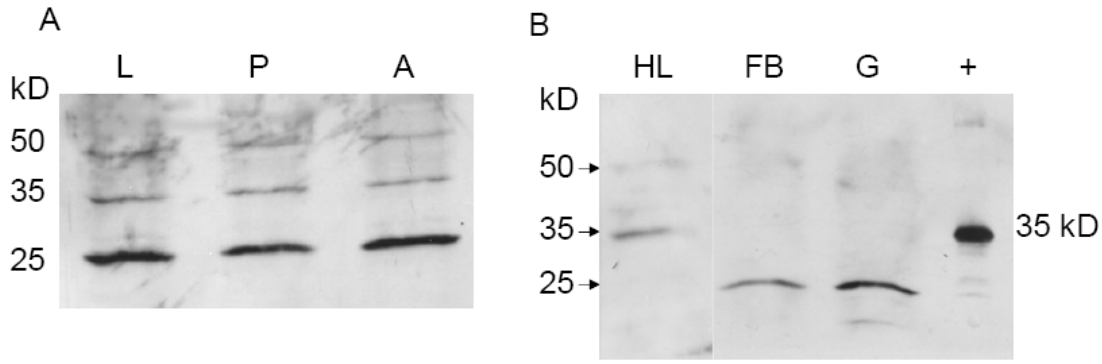

Figure 4. Analysis of *D. melanogaster* Oregon R P29. A. Western blot analysis using anti-P29 antiserum of total protein from one larva (third instar, L), one pupa (P) and one adult (A). B. Western blot analysis using anti-P29 antiserum of adult hemolymph (HL), fat body (FB), and gut (G) tissues. Fifteen µg protein was loaded per lane. +, 25 ng recombinant P29.

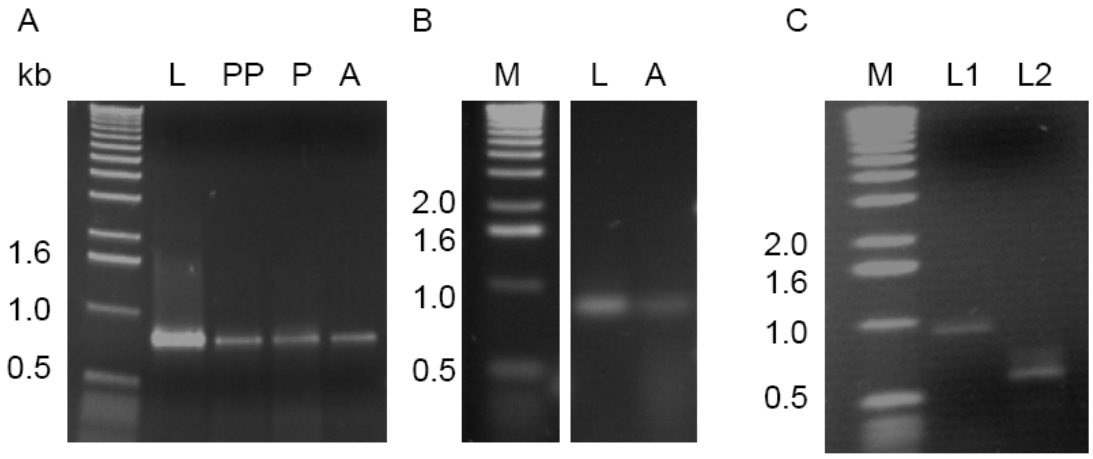

Figure 5. The CG3776 gene of *D. melanogaster* is not alternatively spliced. A. Reverse transcription PCR using primers DrP29-2 and DrP29-3 for detection of P29 mRNA in larvae (L), prepupae (PP), pupae (P) and adults (A). The presence of a single band of the expected size indicates that CG3776 is not alternatively spliced. B. 5' RACE for larvae (L) and adults (A) with primer RACE5-2 and the universal primer. C. 3' RACE for: L1, larvae with primer RACE3-1 and the universal primer; L2, larvae with primer RACE3-2 and the universal primer. The presence of single RACE products of the expected size and sequencing of these products confirmed that CG3776 is not alternatively spliced.

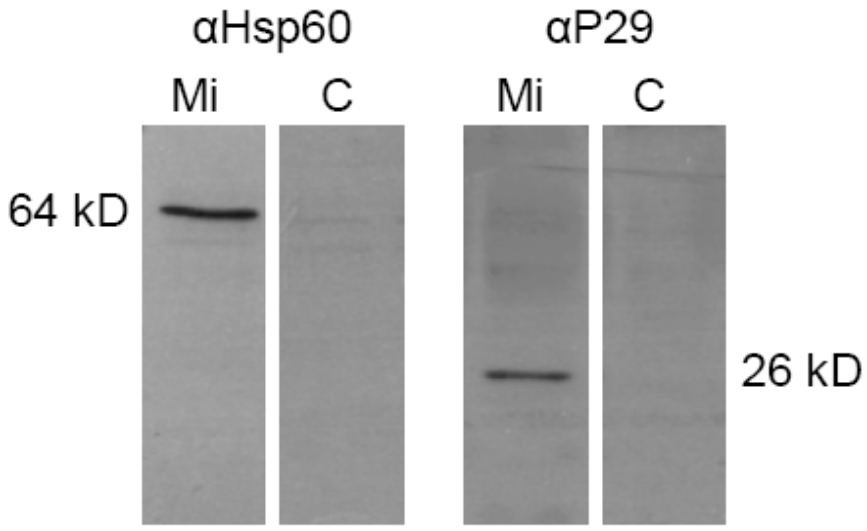

Figure 6. Detection of P29 in the mitochondria of S2 cells. Western blots of mitochondrial (Mi) and cytosolic (C) fractions by using A. anti-Hsp60 antiserum or B. anti-P29 antiserum. 20μg protein was loaded in each lane.

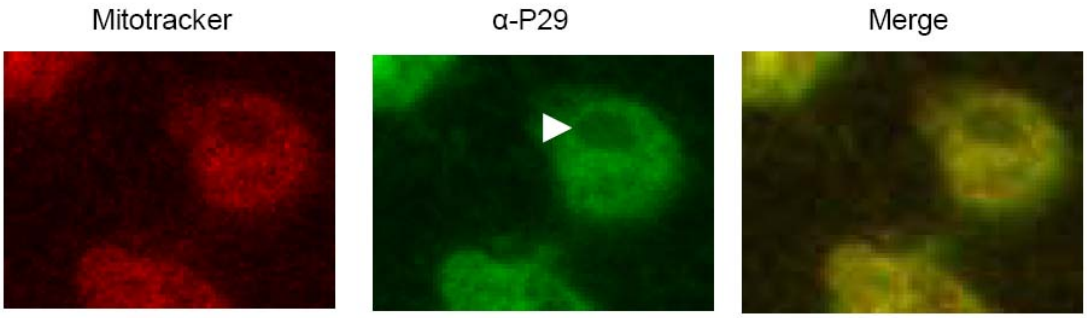

Figure 7. Analysis of subcellular location of P29 in S2 cells by confocal microscopy. S2 cells were stained with the mitochondrial fluorescent dye MitoTracker Red CMXRos (Invitrogen) and visualized using a TRITC filter. P29 was visualized by using anti-P29 antibody and goat anti-rabbit-FITC secondary antibody. Images were then merged to show colocalization of mitochondria and P29. The position of the nucleus is indicated (white arrowhead).

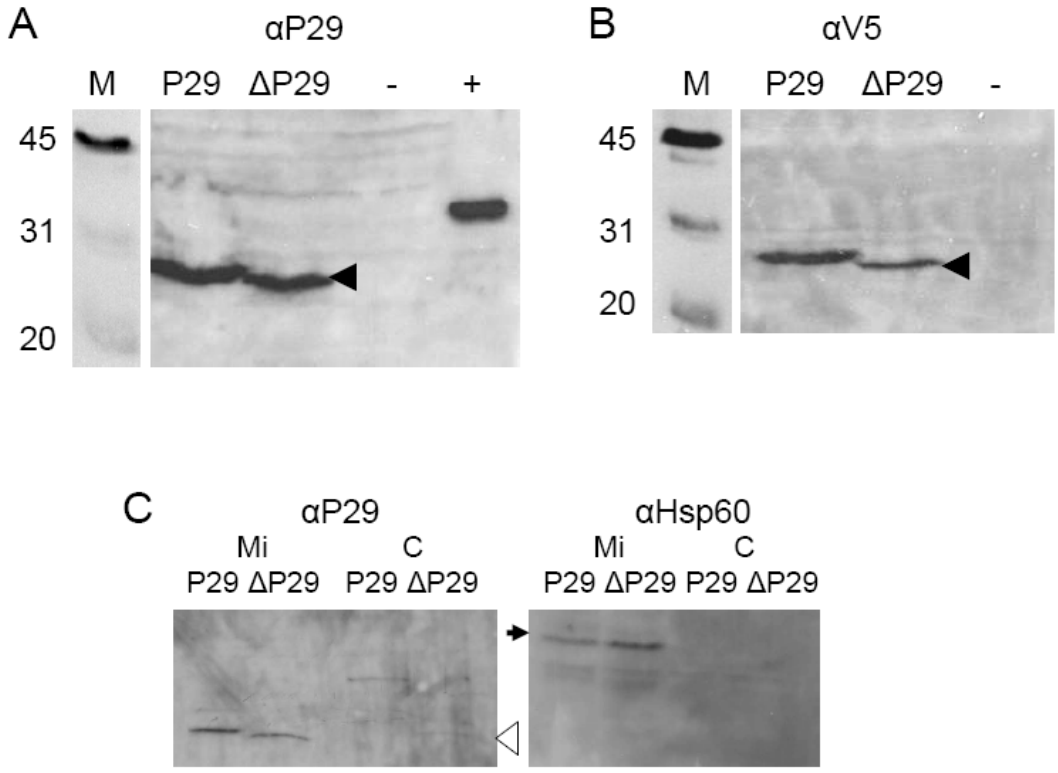

Figure 8. Expression of recombinant *D. melanogaster* P29 and ΔLP29 in stably transformed HighFive™ cells. Proteins were detected by western blot using (A) anti-P29 or (B) anti-V5 antisera. P29 and ΔLP29, HighFive™ cells transformed with pIE-P29 or pIE-ΔLP29 respectively; -, negative control, HighFive™ cells transformed with the pIE vector; +, positive control, 25 ng of *E.coli*-expressed P29 (35 kD). 20 µg protein was loaded per lane. The smaller protein produced by the ΔLP29 construct is indicated (arrow head). M, molecular size markers (kD). C. Analysis of mitochondrial (Mi) and cytosolic (C) fractions for the presence of P29 and ΔLP29 by western blot using the anti-P29 antiserum (left panel). Note that some ΔLP29 was detected in the cytosolic fraction (open arrow head). Some non-specific cross-reactive bands were also detected in the cytosolic fractions. Clean separation of mitochondria from cytosol was confirmed using the anti-Hsp60 antiserum (right panel). The 64kD Hsp60 is indicated by an arrow.

Tables

Table 1. Predicted mitochondrial targeting sequences of *D. melanogaster* and *M. sexta* P29 as determined by MITOPROT. The actual mitochondrial targeting sequence of *D. melanogaster* P29 was estimated to be 13 amino acids shorter than predicted. The 13 amino acids that are predicted by the current study to be excluded from the mitochondrial targeting sequence are underlined.

P29 Protein	Probability of export	Cleavage site	Predicted mitochondrial targeting sequence
Drosophila melanogaster	93.0%	55	MQHTLIRCLGMARISLMRLQPRPTVAASGGQE AGSISKPTQPVSRSFASLPQEQ
Manduca sexta	99.6%	44	MNLALRQVLTRQSFRLCDRYAHKNVAKQIPLT SQCSVIQYRKY

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CHAPTER 3

Ligands of the putative juvenile hormone esterase binding protein in *Drosophila melanogaster*

Zhiyan Liu, Narinder Pal and Bryony C. Bonning

Department of Entomology and Interdepartmental Program in Genetics, Iowa State University, Ames, IA 50011-3222, USA

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Dr. Narinder Pal contributed figures 6-9 to this manuscript.

Abstract

We identified a putative juvenile hormone esterase binding protein, DmP29 in *Drosophila melanogaster* based on homology to the P29 of *Manduca sexta*, and showed that DmP29 is located in the mitochondria. A second 50 kD protein, which may be a dimer of P29, is located primarily in the hemolymph (Chapter 2). To elucidate the function of DmP29, we investigated the potential ligands of DmP29 by testing three hypotheses: (i) DmP29 binds to *D. melanogaster* JHE: We produced a stably transformed insect cell line that expresses DmJHE and confirmed that DmP29 binds to *D. melanogaster* P29. DmJHE binds to both the 25 kD and 50 kD immunoreactive proteins. (ii) DmP29 binds other, non-specific esterases including two esterases predicted to be targeted to the mitochondria: We did not detect any interaction between DmP29 and non-specific esterases. (iii) DmP29 binds to other proteins in *D. melanogaster*: Ligand blot analysis, immunoprecipitation experiments and affinity binding experiments showed that

larval serum protein 1 binds the 25 kD P29. The potential role of P29 in the biology of JHE is discussed.

Keywords: juvenile hormone esterase binding protein; ligands; larval serum protein; juvenile hormone esterase.

Introduction

We identified a putative juvenile hormone esterase (JHE) binding protein called P29 in the tobacco hornworm *Manduca sexta* (Shanmugavelu *et al.*, 2000). Following the release of the *Drosophila melanogaster* genome sequence (Adams *et al.*, 2000) we identified a *D. melanogaster* homolog of this protein (DmP29; chapter 2; Liu et al, submitted). Our long term goal is to determine the function of DmP29 by using the wealth of genetic resources available for *Drosophila* research.

Localization studies indicated that three anti-P29 immunoreactive proteins are present in the larvae, pupae and adults of *D. melanogaster*: A 25 kD protein that localizes to the mitochondria, and 35 and 50 kD proteins that localize primarily to the hemolymph (chapter 2). The mitochondrial location of this putative JHE binding protein was unexpected based on current knowledge of the biology of JHE (EC 3.1.1.1). However, there is an intracellular pool of JHE (Mitsui *et al.*, 1979) and there is a growing list of a class of mitochondrial proteins that are initially targeted to mitochondria but then are exported to different compartments of the cell by an unknown mechanism (Ghebrehiwet *et al.*, 2001). The presence of a Tim44-like domain in P29 may suggest a role for P29 in protein transport.

Following localization studies, our next step was to identify potential ligands of P29 in *D. melanogaster*. Toward this end, we tested three hypotheses: (i) DmP29 binds to *D. melanogaster* JHE, (ii) DmP29 binds other, non-specific esterases (including two mitochondrial esterases) in *D. melanogaster*, and (iii) DmP29 binds to other proteins in *D. melanogaster*. Data generated in this study will facilitate interpretation of the phenotypes of mutant flies that are hypomorphic and hypermorphic for expression of DmP29.

Methods

Drosophila strains

EP835 was obtained from the Szeged *Drosophila* Stock Centre. EP835 is hypomorphic for expression of P29 (25 kD immunoreactive protein: chapter 2). In this line, the P element is inserted into chromosome 2R: 20,477,413, which is 35bp upstream of the CG3776 start site in the 5' untranslated region. Oregon R (wild type) and EP835 flies were maintained at 25°C in vials containing regular drosophila food (Roberts, 1998).

Cloning of D. melanogaster JHE

To determine whether DmP29 binds JHE, the cDNA of the *Drosophila melanogaster* JHE gene was obtained by RT-PCR. mRNA was isolated from third instar larvae (73 mg) of *D. melanogaster* strain OregonR using the Micro Poly (A) Pure mRNA purification kit (Ambion Inc., Austin, TX) and cDNA synthesized by using a cDNA synthesis kit (Clontech, Mountain View, CA). PCR primers designed with reference to the published sequence were used: JHE-IEF; 5'-GGGGGGATCC**ATG**CTACAACTGCTGCTTCTTGGA-3' and JHE-IER, 5'-

GGGGTCTAGA**TTA**CTTTTCGTTGAGTATATGCGACC-3' (start and stop sites are shown in bold), which contain *BamH*I and *Xba*I sites (underlined) for cloning, respectively. PCR products (about 1745 bp) were cloned into the expression vector pIE/153A. The cloned sequence was then confirmed by DNA sequencing.

Stable transformation of the High Five™ cell line for expression of DmJHE

The cDNA sequence for DmJHE was isolated for cloning into the pIE1/153A vector for expression in stably transformed insect cells (Farrell *et al.*, 1989; Farrell and Iatrou, 2004). Three JHE clones obtained were first screened for high level expression in High Five cells™ by transient transfection of cells. HighFive™ cells (Wickham *et al.*, 1992) were maintained in Ex-Cell 405 medium without serum. HighFive™ cells were transfected with pIE-JHE and pBmA.PAC, which confers puromycin resistance (Farrell *et al.*, 1998), by using cellfectin (Invitrogen) as transfection reagent. Medium was assayed for JHE activity using a highly sensitive radiochemical assay (Hammock and Sparks, 1977), five days after transfection with the pIE-JHE clones. On the basis of high JHE activity levels, one clone was selected for production of a stably transformed cell line. The cells transfected with this clone were used for selection of stably transformed cell lines. Stably transfected cell lines were acquired by selection with puromycin (5μg/ml). Cells were selected for one month using a six-well plate format. The colony with the highest JHE activity was then selected for future use. Transfected cells were maintained in 150 cm^2 flasks with 20ml medium with $1.8x10^8$ cells per flask harvested for analysis of JHE expression levels.

Biotin labeling of DmP29 and JHE

DmP29 was expressed in *E.coli* using the vector pBAD/His and purified by using a Ni-NTA column (Qiagen) as described previously (Liu et al., submitted; chapter 2). Purified P29 and recombinant *D. melanogaster* JHE from the medium of stably transformed HighFive^{TM} cells were labeled with biotin by using a biotin labeling kit (Roche). Biotin-labeled DmP29 and JHE were separated on a Sephadex G25 column. Biotinylated JHE was used in immunoprecipitation experiments with recombinant DmP29, while biotinylated Dm29 was used for ligand blotting, immunoprecipitation and affinity purification experiments.

Binding of recombinant P29 to DmJHE expressed in insect cells by ligand blot

Cells stably transformed to express DmJHE, or transformed with the vector pIE were harvested, concentrated in Centricon YM-30 column (Amicon, Millipore Corp.), and proteins separated by SDS-PAGE (12% gel)(20µg per lane). Proteins were then transferred to Hybond-P membrane. The blots were blocked with PBS with 5% milk and incubated overnight at 4°C with biotin-labeled P29 (1 mg/ml) in PBS. The membrane was then washed twice with PBS and 0.1% Tween 20. Biotin-labeled P29 was detected by using stretavidin-HRP.

Immunoprecipitation of recombinant JHE with recombinant DmP29

To determine whether recombinant DmP29 binds recombinant *D. melanogaster* JHE, immunoprecipitation experiments were conducted. Protein G beads (30µl)(Pierce, Illinois) were pre-incubated with the 30µl P29 antibody with IP buffer (0.1M Tris.HCl

PH 8.0). The bead-antibody complex was washed three times with IP buffer, 10 minutes per wash. Four µg of P29 and 8 µg of biotinylated JHE were rotated in 250µl IP buffer at 37°C for 1 hour. The mixture was added to beads and antibody complex and rotated at 4°C overnight. P29 only and JHE only were added to the beads and antibody complex as control samples in the presence or absence of DmP29. Precipitated proteins were separated by SDS-PAGE (12%) and transferred to Hybond-P. P29 was detected by using P29 antibody, and biotinylated JHE was detected by using streptavidin-HRP.

JHE binding to D. melanogaster proteins immunoprecipitated by the P29 antibody

Oregon R and EP835 pupae and larvae (150-200mg) were homogenized in IP buffer with 1mM PMSF. Imumoprecipitation was conducted with Protein G beads as described above. Beads with lysate only, was used as a negative control. Proteins were separated by SDS-PAGE and transferred to Hybond-P membrane. Blots were blocked with PBS with 5% milk and incubated overnight at 4°C with biotin-labeled JHE (2 mg/ml) in PBS to detect JHE binding to proteins precipitated by the P29 antibody. The membrane was then washed twice with PBS and 0.1% Tween 20 and binding of biotin labeled JHE detected with streptavidin-HRP.

Immunoprecipitation of DmP29 ligands and analysis by MALDI-MS: Method One

For identification of proteins bound to P29 and the 35 and 50 kD proteins recognized by P29 antisera, proteins from lysates of adult Oregon R or EP835 were immunoprecipitated with P29 antibody as described above and analyzed by western blot with P29 antibody. Precipitated proteins along with the 35 and 50 kD proteins detected

by western with the P29 antiserum, were excised from SDS-PAGE gels and were subjected to Matrix-assisted laser desorption/ionization, time-of-flight (MALDI-TOF) mass spectroscopy. To separate the 50 kD protein from IgG present in the precipitate, proteins were separated by SDS-PAGE for 2 hr at 200V.

In gel digestion of proteins and MALDI-TOF mass spectroscopy were conducted at the Iowa State University Proteomics Facility using standard procedures as follows: *In gel digestion:* The gel slices were cut into pieces and washed with 500 µl of 100 mM NH_4HCO_3 for one hour with agitation and then washed three times with 20 mM NH4HCO3/50% (v/v) acetonitrile. After dehydration in acetonitrile, the gel pieces were incubated with 10 mM DTT in 100 mM NH_4HCO_3 for 30 minutes at 60˚C, followed by reaction with 55 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 minutes in the dark at room temperature for reducing and alkylation. The gel pieces were washed with 20 mM NH_4HCO_3 and dehydrated with acetonitrile and the washing and dehydration procedure was repeated once. After drying at 37˚C for 30 minutes, the gel pieces were swollen in a digestion buffer containing 20mM NH_4HCO_3 and 10 ng/µl of tosylphenylalanylchloromethane (TPCK) treated sequence-grade trypsin (Promega, Madison, WI) at 4˚C. After 30 minutes, the supernatant was removed and replaced with 20 µl of 20mM NH_4HCO_3 to keep slices wet during overnight digestion at 37˚C. The supernatant was collected. The gel pieces were further extracted by two changes of 1% HAc in 50% acetonitrile. The solutions were combined with the supernatant and dried down. One µl of matrix solution (CHCA 10 mg/ml in 50% CH3CN/0.1% TFA) was used to dissolve the peptides and applied on a MALDI target plate.

MALDI Mass Spectrometry

MALDI-TOF MS/MS MS analyses were performed using a QSTAR XL quadrupole TOF mass spectrometer (AB/MDS Sciex, Toronto, Canada) equipped with an oMALDI ion source. The mass spectrometer was operated in the positive ion mode. Mass spectra for MS analysis were acquired over m/z 500 to 4000. After every regular MS acquisition, MS/MS acquisition was performed against most intensive ions. The molecular ions were selected by information dependent acquisition in the quadrupole analyzer and fragmented in the collision cell.

Data Analysis

All spectra were processed by a MASCOT (MatrixScience, London, UK) database search. Peak lists were generated by Analyst QS (AB/MDS Sciex, Toronto, Canada) and were used for MS/MS ion searches. Typical search parameters were as follows: Maximum missing cleavage is one, fixed modification carboxyamidomethyl cysteine, variable modification oxidation of Methionine. Peptide mass tolerances were +/- 100 ppm. Fragment mass tolerances were +/- 1 Da. No restrictions on protein molecular weight were applied. Protein identification was based on the probability based Mowse Score. The significance threshold p was set to less than 0.05.

Identification of non-specific esterases in mitochondria

To address whether esterases similar to JHE are present in mitochondria, we examined *D. melanogaster* esterases (Campbell *et al.*, 2003) for the presence of potential N-terminal mitochondrial targeting signals by using the MITOPROT program. Given the

mitochondrial localization of P29, it is conceivable that P29 interacts *in vivo* with proteins that are structurally similar to JHE (Ollis *et al.*, 1992).

Analysis of immunopreciptated proteins for non-specific esterase and JHE activity

To examine the proteins precipitated with the P29 antiserum for the presence of non-specific esterases, proteins from the pellet, supernatant and the original pupal extract (20 μg of each) were separated by native PAGE (10% gel) and stained for non-specific esterase activity following the method as described by Vernick et al (Vernick *et al.*, 1988). The gel was stained in 100ml of 0.1M sodium phosphate buffer pH 7.0 containing 0.5mM each of α and β napthyl acetate and 0.2% Fast Blue B. Gel staining was conducted in the dark at room temperature for 45 min followed by washing with distilled water and transfer to storage solution (35% methanol, 10% glacial acetic acid in distilled water).

The pellet of immunoprecipitated proteins from the pupal extract was also tested for JHE activity by radiochemical assay (Hammock and Sparks, 1977).

Does binding of P29 to JHE block JHE activity?

The peak of JHE activity in *D. melanogaster* occurs at the brown pupal stage, 6 – 12 hours after puparium formation (Campbell *et al.*, 1992). Oregon R pupae were ground in PBS buffer and centrifuged at 8000 g for 5 minutes at 4°C to remove debris. Two μl of 1 μg/μl recombinant P29 purified from *E. coli* was added to 40 μl of pupal lysate. Two μl of buffer was added to lysate for the control sample. Samples were analyzed in triplicate. The samples were incubated for 2 hours at room temperature. JHE activity was measured

by using a partition assay with ^{3}H-JH III as substrate (Hammock and Sparks, 1977). Ten µl of each homogenate sample was diluted 10-fold to test for JHE activity, with each sample assayed in triplicate. JHE activity was calculated according the nanomoles of JH hydrolyzed per minute per ml of lysate.

Ligand blotting with DmP29 to identify other potential ligands

To identify other *Drosophila* proteins that may interact with DmP29, adult flies and pupae were homogenized in buffer (20mM Tris base, 150mM NaCl, 1% Triton X-100, 1mM PMSF, 10mM EDTA) pH 6.8 and the homogenate centrifuged at 18,000g for 15 minutes to pellet the debris. The protein concentration of the supernatant was determined by Bradford assay (Bradford, 1976). Ten-20µg of the protein extract from adult flies and pupae were separated on 10% SDS PAGE gels and blotted onto PVDF membrane. The membrane was processed for identification of P29 binding proteins using biotin-labeled P29 as described above for ligand blot with biotin-labeled JHE. Control treatments of adult and pupal protein extracts were run in the absence of biotin-labeled DmP29 to identify native biotin proteins.

Purification of mitochondria

Mitochondria from *D. melanogaster* adults were prepared according to Halarnkar (Halarnkar *et al.*, 1986). Adults were crushed batchwise in a prechilled porcelain mortar and pestle containing homogenizing buffer (HEPES-KOH 20 mM, pH 7.4; EGTA 1mM). The released material was suspended and separated from the rest of insect debris by passing through glass wool. The total insect debris was resuspended in homogenization

buffer, rehomogenized in the mortar and pestle and filtered. The total suspension of material was centrifuged by uniform acceleration to 12,000g over a period of 60 second and held at 12,000g for 5 seconds. The supernatant was carefully decanted and centrifuged as rapidly as possible to 46,000g with immediate deceleration by breaking. The mitochondria were washed once with homogenizing buffer.

Assay of mitochondria for non-specific esterases

The purified mitochondria were homogenized in 200 µl of 20mM Na-phosphate buffer, pH 7.2 containing phenylthiocarbamide (PTC) 1mM. The homogenates were centrifuged at 14,000 g for 10 min at 4°C. Two aliquots of 10 µl of supernatant were transferred to different wells of a microtiter plate. Two hundred µl of α-naphthyl acetate (α –NA) or β-naphthyl-acetate (β –NA; 30 mM in 20 mM Na-phosphate buffer, pH 7.2 for both) were added to the wells and the plates incubated at 30^{o}C. After 30 min, 50 µl of fast blue solution (45 mg Fast blue B in 9.75 ml of distilled water plus 5.25 ml of 10% SDS) was added and the color was detected by recording absorbance at 595 nm for α –NA and 520 nm for β –NA (Brogdon and Dickinson, 1983). The total protein in each fraction was determined by Bradford protein assay.

JHE activity assay

The purified mitochondria were homogenized in 200 µl PBS buffer. The homogenates were centrifuged at 14,000 g for 10 min at 4°C. Mitochondria and the cytosol protein were qualified by Bradford protein assay. JHE activity was measured by a partition assay using ^{3}H-JH-III as substrate (Hammock and Sparks, 1977). Ten µl of the

homogenate was diluted to 100 µl to test for JHE activity. Each sample was assayed in triplicate. The nanomols of JH hydrolyzed per minute per fly were calculated. To test whether JH degradation from mitochondria is from JHE other than non-specific esterase, 1µl JHE inhibitor 3-Octylthio-1,1,1-trifluoro-2-propanone (OTFP diluted in ethanol; from Dr. Bruce D. Hammock University of California, Davis, CA) was added to 100µl of the mitochondria or cytosol solution (final OTFP concentration 10^{-4}). Controls with same amount of proteins were added 1µl ethonal. All the treatments were incubated in 30 ^{o}C waterbath for 10 minutes. JHE activity was measured and the percentage of JHE activity inhibited was calculated compared to control.

Immunoprecipitation of DmP29 ligands: Method Two

Ten adults or pupae were homogenized in IP buffer (10mM Tris pH 7.5, 150mM NaCl, 0.1% Triton X-100, 1mM PMSF). 100µg of protein extract was incubated with 40µg of recombinant DmP29 at room temperature for 2 hrs and then mixed with Affigel protein A (Biorad) complexed with anti-Xpress antiserum (10µl). The mixture of lysate plus DmP29 plus Affigel protein A was allowed to incubate overnight at 4ºC and then centrifuged at 3000 g for 5 min. to pellet the immune complexes. The pellet was washed twice with IP buffer and 1% Triton X-100, twice with IP buffer and 500mM NaCl, and twice with IP buffer only. Proteins were eluted by resuspending the pellet in Laemmli sample buffer (Laemmli, 1970), heated at 95ºC for 5 min. and centrifuged at 12000 g for 1 min. Proteins in the supernatant were separated in a 10% SDS-PAGE gel and stained with Coomassie Brilliant Blue R-250. Proteins immunoprecipitated along with recombinant DmP29 were excised from the gel and processed for in-gel digestion by

trypsin. MALDI-TOF mass spectroscopy was conducted on the precipitated proteins at the Iowa State University Protein Facility as described above.

Identification of DmP29 ligands using Dynal beads

As an alternative approach to isolation of DmP29 interacting partners, we used an affinity binding method using Streptavidin-coupled M-280 dynal beads (Dynal Biotech, New York, USA). One mg of beads in binding buffer (20 mM Tris pH 7.2, 150 mM NaCl, 1 mM CaCl2, 0.1% Triton X-100) were incubated with 10µg biotin labeled-P29 for 4 hr at room temperature. Beads were then washed twice with the binding buffer according to the manufacterer's protocol. The eppendorf tube containing beads was placed in a magnetic holder for 2min. which caused the beads to align themselves to the sides of the tube. The solution containing unbound P29 was then pipetted out. The P29-biotin-streptavidin-magnetic bead complex was redissolved in 100µl binding buffer and incubated with 100µg of protein extracts from pupae or adults with gentle mixing overnight at 4˚. The dynal beads were then washed three times with the binding buffer using the magnetic holder and the solution containing unbound proteins was pipetted out. Bound proteins were eluted by incubating the beads with 20µl of elution buffer (10 mM Tris pH 7.2, 50 mM DTT, 1% SDS) at 30˚ for 15min. 20µl of the eluted protein mixture was mixed with 5µl of 4X protein loading dye and the entire mixture was boiled for 5min., separated on 10% SDS-PAGE gel and visualized by staining with Coomassie Brilliant Blue R-250. Selected protein bands were cut from the gel for analysis by MALDI-TOF at the Iowa State University Protein Facility.

Immunoprecipitation of LSP1 ligands

Magnabind Protein G beads (Pierce, IL, USA) were used according to the manufacterer's protocol. 100µl of magnabind beads were resuspended in 100µl of binding buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.5% Triton X-100, 1mM PMSF). 15µl of these magnabind beads were incubated with 15µl of LSP1 antiserum for 4 hours at 4˚ with gentle mixing followed by magnetic separation of the beads using a magnetic holder and removal of unbound antibody. The bead antibody mixture was then washed twice with the binding buffer, resuspended in 100µl of binding buffer and incubated with 100µl of adult lysate (1µg/µl) at 4˚ overnight. After washing the beads three times with binding buffer using the magnetic holder, the unbound proteins were pipetted out. The bound proteins were then eluted by adding 30µl of 0.1M glycine buffer (pH 2-3). The low pH of the eluent was neutralized by adding 1.5µl of 1M Tris pH 7.5 – 9. In the control experiments 15µl each of magnabind beads were incubated with 15µl of LSP1 antiserum or 100µl of adult lysate (1µg/µl) at 4˚ for 4 hours. The bound complexes were eluted in the same way as described above. The eluted proteins (30µl) were mixed with protein loading dye, boiled for 5 min., separated on 12% SDS-PAGE gel and then transferred to a PVDF membrane. After blocking in 5% non-fat dry milk, the blot was incubated with DmP29 antiserum (1:1000) in wash buffer (1XTBS, 0.1% Tween-20, 1% non-fat dry milk) for one hour at room temp. Blots were then washed two times with the wash buffer each for 10 min. and incubated with goat anti-rabbit IgG coupled with HRP (Sigma) at 1:2500 dilution for one hour at room temp. After washing the blots 2X with wash buffer followed by 2X with TBS, proteins were detected by using detection reagents 1 and 2 of the ECL western blotting kit (Amersham Biosciences).

Results

Cloning and expression of D. melanogaster JHE

Three clones of *D. melanogaster* JHE were acquired, each with one amino acid substitution, namely L360V, D537Y and F446S. Each cDNA was cloned into the pIE/153A vector for expression in transiently transfected insect cells. JHE-L360V gave the highest level of expression of active enzyme. This clone was then used to produce stably transfected insect cells. The JHE activity from the stably transfected colony with the highest level of JHE expression was about 4.7 nmol JH hydrolyzed/min/ml medium.

Interaction of DmP29 with JHE

Total protein from the medium of transfected HighFive^{TM} cells was loaded on to an SDS-PAGE gel and a ligand blot conducted with biotinylated P29 followed by detection with streptavidin-HRP. A 66kD protein was detected in the medium of cells containing pIE-JHE (Fig 1A). Immunoprecipitation was also conducted to test for interaction between the two proteins. Biotinylated JHE was pulled down by the P29 antibody in the presence, but not in the absence, of P29 (Fig 1B).

Examination of proteins precipitated with the P29 antiserum by ligand blot analysis with biotin-labeled JHE showed that JHE binds the 25 and 50 kD proteins (but not the 35 kD protein) that are recognized by the P29 antiserum (Fig 2). Examination of proteins precipitated with the P29 antiserum (Fig 3A) by peptide analysis showed that JHE was precipitated with P29 from homogenates of EP835 (Figure 3B). Peptides

derived from P29 but not from JHE were detected in the precipitates from OregonR extracts.

We use MALDI mass spectrometry to identify the 35kD and 50kD proteins. The bands were excised from the gel (Figure 4) and digested with trypsin. MALDI-TOF mass spectrometry showed that the 35kD protein was Tropomyosin 1, isoforms 9A/A/B (also known as Tropomyosin II: Cytoskeletal tropomyosin) (NCBI No. P06754) with more than 50% amino acid coverage (Fig. 5). Five peaks were selected to do MALDI-TOF MS/MS. The amino acid sequences matched the sequence of the protein (Fig. 5). The 50kD protein was identified as rabbit antibody Ig gamma chain C region (P01870) with four peaks matching the amino acid sequence. We also found that one peak matched (Q9VT08) putative odorant receptor 67a sequence (47kD). The presence of antibody in this band restricted our ability to identify the 50 kD protein by MS/MS.

Identification of mitochondrial esterases

We identified two *Drosophila* esterases with putative mitochondrial localization sequences that are structurally similar to JHE, which may be ligands of P29. These esterases are αE1 and Cricklet (Shirras and Bownes, 1989).

Analysis of immunopreciptated proteins for non-specific esterase and JHE activity

Proteins immunoprecipitated with the P29 antibody from pupal extracts were tested for non-specific esterase and JHE activity. Staining of a native polyacrylamide gel for non-specific esterase activity indicated that while non-specific esterases were abundant in the supernatant, there were no non-specific esterases present among proteins

precipitated with P29 (Fig. 6). Analysis of precipitated proteins using a sensitive radiochemical assay for JHE activity failed to detect the presence of JHE.

We addressed whether binding of P29 to JHE resulted in loss of enzyme activity: The JHE activity of pupal lysates did not decrease following addition of denatured recombinant P29 when compared to control samples with buffer added in place of recombinant P29. The activity with P29 added was 0.677- compared to 0.669- nm JH hydrolyzed/min/ml for the control sample (no significant difference: ANOVA, Student's t-test).

Detection of non-specific esterase activity in mitochondria

Purified mitochondria were tested for the presence of non-specific esterase activity. Activity was also detected in the mitochondria and in the supernatant which contained soluble enzyme and microsomes. The esterase activity in mitochondria was much higher than in the supernatant (Table 1).

Identification of other potential ligands of DmP29

Ligand blot analysis (Fig. 7), immunoprecipitation experiments (Fig 8A) and affinity binding experiments (Fig 8B) showed that an 85 kDa *D. melanogaster* protein binds DmP29 in adults and in pupae. Three bands were cut from the gel shown in figure 8 for analysis by MALDI-TOF (band 1, 85 kD; band 2 and 3, 60-70 kD). When the resulting peptide mass fingerprint for band 1 was used to screen *Drosophila* proteins, the 85kD band isolated by both immunprecipitation and by dynal bead-mediated affinity

isolation, was identified as Larval serum protein 1 (LSP1). Band 2 was identified as LSP precursor protein. Band 3 could not be identified.

Binding of P29 to LSP1

To further examine the potential interaction of P29 and LSP1, we conducted immunoprecipitation experiments from OregonR adults with the LSP1 antiserum. Precipitated proteins were separated by SDS-PAGE and examined by western blot with the P29 antiserum. Both the 25 and 35 kDa proteins were precipitated with the LSP1 antiserum (Fig 9).

Discussion

In order to identify potential ligands of DmP29, we tested three hypotheses: **(i) DmP29 binds *D. melanogaster* JHE** Immunoprecipitation experiments and ligand blot analyses confirmed that recombinant DmP29 binds recombinant *D. melanogaster* JHE. JHE binds the 25 and 50 kD proteins that are recognized by the P29 antiserum. Immunoprecipitation of *Drosophila* proteins with the P29 antibody and subsequent peptide analysis of precipitated proteins confirmed the presence of both P29 and JHE in the precipitate. The fact that JHE was detected on precipitation from the EP835 line, which is hypomorphic for expression of the 25 kDa P29, may indicate that JHE interacts primarily with the 50 kD protein which is located primarily in the hemolymph (chapter 2). Only P29-derived peptides were detected in the precipitate from adult OregonR flies, which may result from the higher amount of P29 masking the presence of the P29-JHE complex. The fact that P29 can also dimerize may also contribute to this result. The

amount of JHE-P29 complex is expected to be low given the relatively low titers of JHE in adult flies.

Analysis of the 35 kD protein that is recognized by the P29 antiserum showed that this protein is tropomyosin 1. Tropomyosins bind to actin filaments in muscle and nonmuscle cells (Hanke and Storti, 1988). The identity of the 50 kD protein could not be confirmed in part because of the presence of IgG which is of a similar size.

(ii) DmP29 binds non-specific esterases of *D. melanogaster* By examining the non-specific esterase sequences of *D. melanogaster* (Campbell et al., 2003), we found that two of these esterases contain putative mitochondrial localization signals. On the basis of structural similarity to JHE, we hypothesized that P29 interacts with mitochondrial esterases. However, we did not detect non-specific esterase activity on examination of proteins precipitated with the P29 antiserum from pupal extracts (Fig. 6). This result suggests that P29 does not bind non-specific esterases. We also failed to detect JHE activity in these precipitates. It is conceivable that binding of P29 to JHE blocked the catalytic activity of JHE. Addition of denatured recombinant P29 purified from *E. coli* did not reduce the JHE activity of pupal lysates. Because recombinant P29 could not be purified under non-denaturing conditions (chapter 2), we do not know whether native P29 binding blocks JHE activity. This would provide one possible explanation for the failure to detect JHE activity in the P29 immunoprecipitate. The JHE assay could be modified for detection of very low activity levels of JHE (Ward *et al.*, 1992).

Both non-specific esterase and JHE activities were detected in mitochondria purified from adult flies. Detection of JHE activity in mitochondria is notable. To

confirm this result, we plan to repeat the assays with the JHE-specific inhibitor OTFP (Abdel-Aal and Hammock, 1986), and to use immuno-electron microscopy to look for JHE in mitochondria. The presence of JHE in mitochondria raises the following questions: Does JHE function in mitochondria to regulate JH-mediated transcription of mitochondrial genes? Is JHE stored and then released from mitochondria, as has been shown for other proteins (Ghebrehiwet *et al.*, 2001)? It is also possible that in vivo interaction between JHE and P29 occurs in the hemolymph, if indeed the 50 kD protein is a dimer of P29. However, the lack of effect of hyper- and hypo- expression of P29 on 50 kD protein levels suggests that this protein may not be P29 (see Chapter 4).

(iii) DmP29 binds to other proteins in *D. melanogaster* Several different methods were used to examine the potential partners of P29 from *Drosophila* homogenates. Ligand blot analysis, affinity binding and immunoprecipitation experiments all showed that P29 interacts with LSP1. Larval serum proteins (LSPs) or hexamerins, are storage proteins produced by the fat body during the third instar to provide energy and amino acids during metamorphosis. In *D. melanogaster*, two LSPs have been identified, LSP1 consists of three different subunits (α, β and γ), while LSP2 is a hexamer of identical subunits. LSPs are exported into the hemolymph and subsequently taken up at the end of the third (final) larval instar into the fat body in response to a sharp increase in the titer of 20-hydroxy ecdysone. LSP1 uptake is mediated by the receptor, Fat Body Protein 1 (Burmester *et al.*, 1999). The LSPs are stored in protein storage granules.

LSP1 appeared to bind both the 25 kD and 35 kD proteins that are recognized by the P29 antiserum. LSP1 may also bind the 50 kDa protein, but this band in Figure 9 may

have been masked by the presence of IgG at approximately 53 kD. The fact that LSP1 binds to the 35 kD protein which has been identified as tropomyosin 1, suggests that binding of LSP1 to P29 in these experiments may have resulted from the high abundance of LSP1 in the insect and the sticky nature of this protein.

Relatively little research has been conducted on the JHE of *D. melanogaster* (Campbell *et al.*, 1992; Campbell *et al.*, 1998; Campbell *et al.*, 2001), especially with respect to tissue localization. JHE has been studied fairly extensively in other insects however, particularly in the Lepidoptera; From these studies, it has been determined that JHE is synthesized by the fat body, epidermis, and various other tissues (Wing *et al.*, 1981; Wroblewski *et al.*, 1990; Stoltzman *et al.*, 2000; Elliot *et al.*, 2006) and in conjunction with JH epoxide hydrolase serves to remove juvenile hormone (JH). Juvenile hormone is a transcriptional regulator that in conjunction with ecdysteriods has widespread action (Flatt *et al.*, 2005; Glaser *et al.*, 2005). Reduction of the JH titer by degradative enzymes is required for molting or pupation (Khlebodarova *et al.*, 1996). The fate of JHE appears to be differentially regulated in a tissue-specific fashion: JHE in the integument primarily remains within the tissue, while the fat body appears to serve as the major source of hemolymph JHE at specific times during development (Vince and Gilbert, 1977; Mitsui *et al.*, 1979; Wroblewski *et al.*, 1990). JHE is efficiently removed from the hemolymph by the pericardial cells (athrocytes) by a receptor-mediated process and is subsequently degraded in lysosomes (Ichinose *et al.*, 1992; Ichinose *et al.*, 1992).

Common features of the two potential ligands of DmP29, JHE and LSP1 are (1) synthesis by the fat body, (2) an intracellular pool of the protein, (3) secretion from the

fat body, (4) presence at high titers within the hemolymph, (5) receptor-mediated removal from the hemolymph. Based on the localization and ligand data for P29, we hypothesize that P29 interacts with JHE in the mitochondria. Binding to LSP1 may be a non-specific interaction.

In summary, we (i) confirmed that DmP29 binds to *D. melanogaster* JHE by using both *in vivo* and *in vitro* approaches, (ii) did not detect any interaction between P29 and non-specific esterases, and (iii) identified LSP1 as a second potential binding partner of P29. By examining the phenotypes of flies that are hyper- or hypomorphic for expression of P29, we plan to elucidate whether interaction of P29 with JHE and/or LSP1 occurs *in vivo*. We will also determine whether interaction between these proteins occurs within the mitochondria, and what role P29 plays in the biology of JHE and LSP1.

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Figures

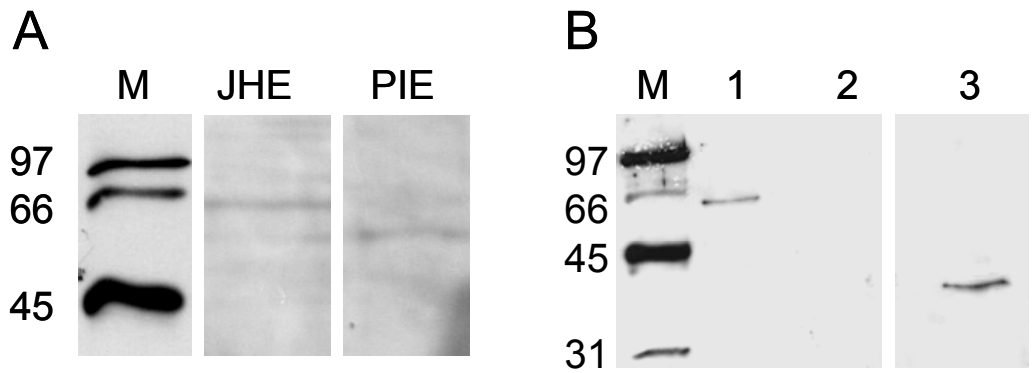


Figure 1.
Binding of recombinant DmP29 to recombinant DmJHE
A. Biotin-labeled DmP29 binds DmJHE expressed in insect cells. Cells stably transformed with pIE-JHE (JHE) or pIE (IE, negative control), were homogenized and proteins separated by SDS-PAGE followed by transfer to Hybond-P membrane for ligand blot analysis with biotin-labeled P29. M, size markers (kD). B. Immunoprecipitation of recombinant DmP29 with recombinant DmJHE. Biotin-labeled JHE was immunoprecipitated with DmP29 by the P29 antibody. Lane 1, P29 and biotin-labeled JHE precipitated by the P29 antibody and protein G beads. Precipitated, biotin-labeled JHE was detected by streptevidin-HRP. Lane 2, biotin labeled JHE precipitated with P29 antibody and protein G beads (no recombinant P29), followed by detection with streptevidin-HRP. Lane 3, P29 precipitated with P29 antibody and protein G beads and detected by western blot with the P29 antibody.

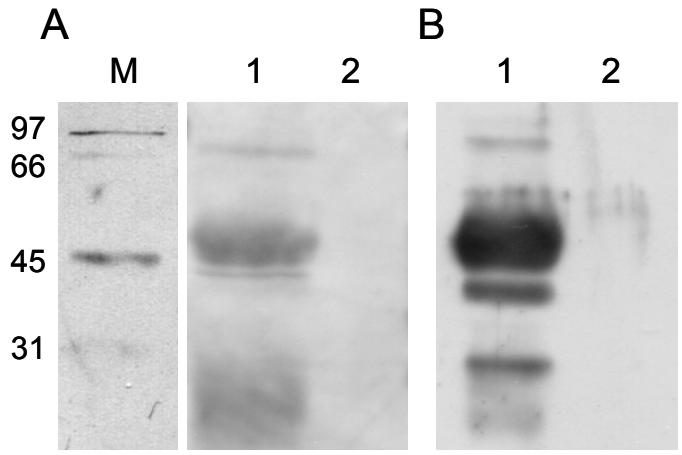
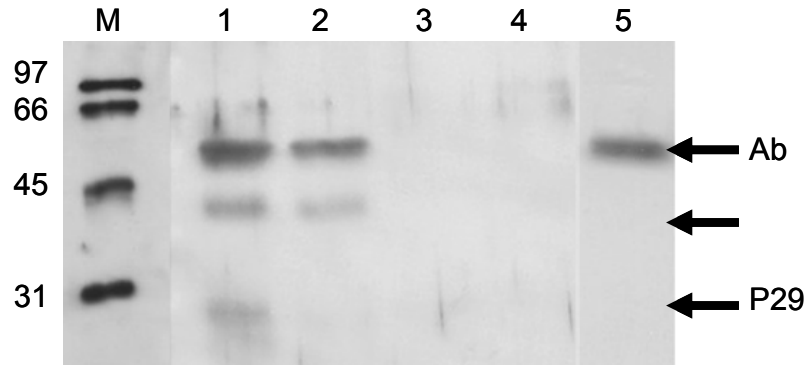


Figure 2
Binding of DmJHE to pupal proteins precipitated with P29 antibody.
Pupal proteins immunoprecipitated with (lane 1) or without (lane 2) the P29 antiserum, plus Protein G beads (lanes 1) were separated by SDS-PAGE, transferred to Hybond-P and analyzed by A. ligand blot with biotin-labeled JHE, or B. western blot with the P29 antiserum.

A



B

D. melanogaster JHE

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   1 MLQLLLLGQL LAGPGPFCAA LATVDQLTVC PPSVGCLKGT NLQGYQSERF
  51 EAFMGIPYAL PPIGDLRFSN PKVMPKLLGM YDASAPKMDC IQKNYLLPTP
 101 VVYGDEDCLY LNVYRPEIRK SALPVMVYIH GGGFFGGSAG PGVTGPEYFM
 151 DSGEVILVTM AYRLGPFGFL STQDAVMSGN FGLKDQNLAL RWVQRNIRFF
 201 GGDPQRVTIF GQSAGGVAAH MHLLSPRSHG LFHRVISMSG TANVPFAIAE
 251 QPLEQARLLA EFADVPDARN LSTVKLTKAL RRINATKLLN AGDGLKYWDV
 301 DHMTNFRPVV EEGLEVDAFL NAHPMDMLAQ GMPTSIPLLL GTVPGEGAVR
 351 VVNILGNETL RQSFNLRFDE LLQELLEFPA SFSQDRREKM MDLLVEVYFQ
 401 GQHEVNELTV QGFMNLISDR GFKQPLYNTI HKNVCHTPNP VYLYSFNYQG
 451 PLSYASAYTS ANVTGKYGVV HCDDLLYLFR SPLLFPDFQR NSTEAKVIHS
 501 FVDYFVHFAK FGKPRNSVLL TPCSIEVLQS RPDGICDYHE FANAPDAYQG
 551 FEVHVASEFQ TDRVNLWSHI LNEK
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D. melanogaster P29

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   1 MQHTLIRCLG MARISLMRLQ PRPTVAASGG QEAGSISKPT QPVSRSFASL
  51 PQEQDKKEQN ARESLNRLPR LMDFPEIVWP SALNSLKNWI TIQFIIRPYF
 101 DSEFQLKDFI YGAKQALQVV SSKLMGGDLD SLDNLVSPEA IAELRPVIQK
 151 LSMTQRRQLE IKESDIYLSF PYQVGIMFDD ANDKLQKRFV EITMVFHVMR
 201 GLSEMRERGE EIPWNMGTLP EYQDKVFICN YRFVKEFTAG HQSDWTVNVA
```

Figure 3

A. Immunoprecipitation of proteins from lysates of adult *Drosophila* with the P29 antibody. Immunoprecipitated proteins were separated on an SDS-PAGE gel, transferred to PVDF membrane and visualized by western blot with the P29 antibody. Lane 1, OregonR; Lane 2, EP835; Lane 3, negative control: lysate of OregonR with beads, no antibody; Lane 4, negative control: lysate of EP835 with beads, no antibody; Lane 5, negative control: P29 antibody with beads, no lysate. B. Proteomic analysis of immunoprecipitated proteins shown in A. Peptides derived from proteins

immunoprecipitated from adult EP835 lysate observed by MALDI-MS matched the sequence of *D. melanogaster* JHE. These peptides covered 22% of the JHE sequence. Peptides matched to the JHE sequence are shown in bold. Peptides derived from proteins immunoprecipitated from adult OregonR lysate observed by MALDI-MS matched the sequence of DmP29. These peptides covered 11% of the P29 sequence. Peptides matched to the DmP29 sequence are shown in bold.

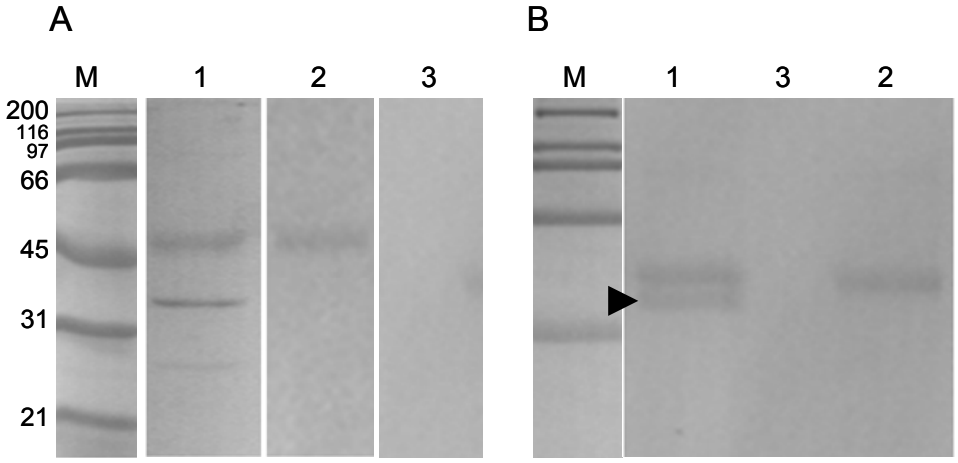
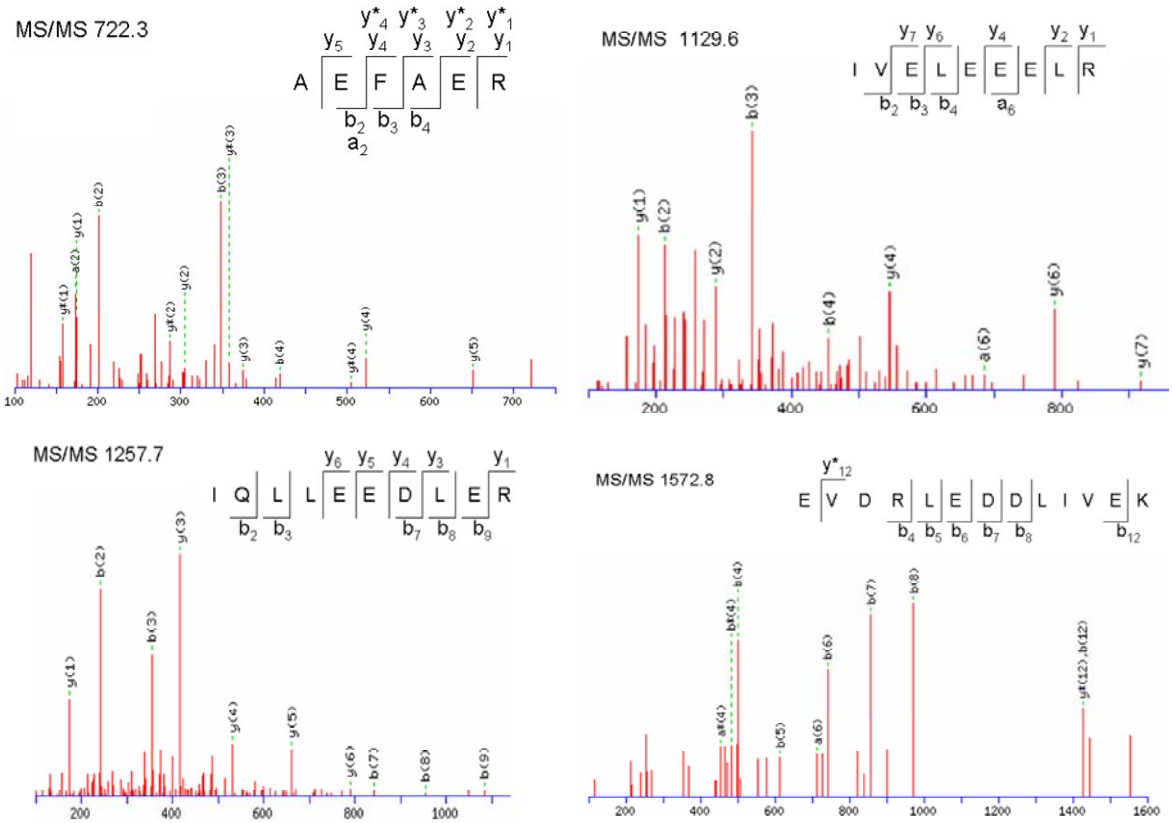


Figure 4
Separation of 50 kD P29 immunoreactive protein for Maldi-Tof analysis. Proteins immunoprecipitated from Oregon R were separated for (A) 1 hr and (B) 2 hr at 200 V and stained with Coomassie Blue. M, size markers (kD); lane 1, P29 antibody, beads and lysate; lane 2, P29 antobody and beads; lane 3, beads and lysate. The lower band in B, lane 1 (arrow head) was excised for MALDI-TOF MS/MS analysis

A



B Tropomyosin 1

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  1 MDAIKKKMQA MKVDKDGALE RALVCEQEAR DANTRAEKAE EEARQLQKKI
 51 QTVENELDQT QEALTLVTGK LEEKNKALQN KKKTTKMTTS IPQGTLLDVL
101 KKKMRQTKEE MEKYKDECEE FHKRLQLEVV RREEAESEVA ALNRRIQLLE
151 EDLERSEERL GSATAKLSEA SQAADESERA RKILENRALA DEERMDALEN
201 QLKEARFLAE EADKKYDEVA RKLAMVEADL ERAEERAEQG ENKIVELEEE
251 LRVVGNNLKS LEVSEEKANQ REEEYKNQIK TLNTRLKEAE ARAEFAERSV
301 QKLQKEVDRL EDDLVLEKER YKDIGDDLDT AFVELILKE
```

Figure 5.
The 35kD, anti-P29 immunoreactive protein is tropomyosin 1. Peptides were derived from the 35kD protein immunoprecipitated from Oregon R lysate for analysis by MALDI-TOF MS/MS.

A. The amino acid sequence and y ion and b ion detected are shown for four of the peptides analyzed. B. Peptides derived from the 35kD protein immunoprecipitated from Oregon R lysate observed by MALDI-MS matched the sequence of *D. melanogaster* Tropomyosin 1, isoforms 9A/A/B. These peptides covered 83% of the protein sequence. Peptides matched to the protein sequence are shown in bold.

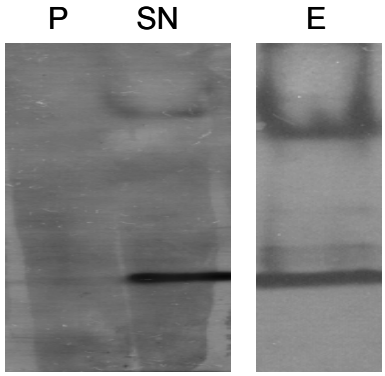

Figure 6
Examination of proteins immunoprecipitated by the P29 antibody for non-specific esterase activity. Proteins precipitated with DmP29 (P), in the pupal extract (E) or remaining in the supernatant (SN) were separated in a 10% native polyacrylamide gel and stained for non-specific esterase activity. Non-specific esterases remained in the supernatant with no esterase activity detected in the pellet.

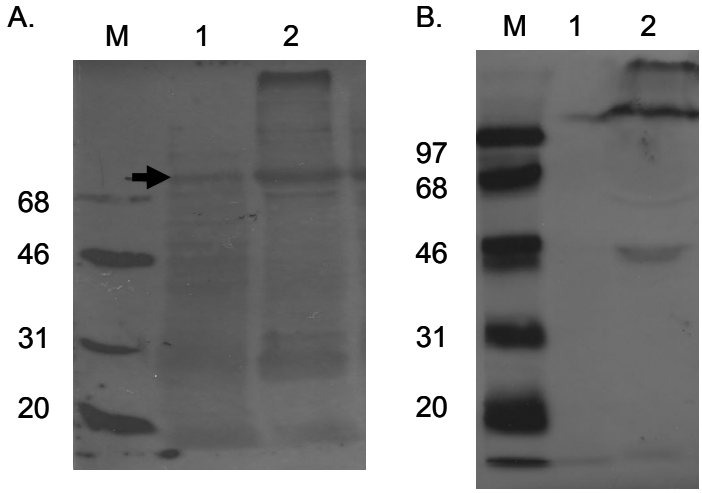

Figure 7

Ligand blot analysis of adult and pupal *D. melanogaster* proteins that bind P29 in the presence (A) and absence (B) of biotinylated P29. M, ECL protein markers (kD). Lane 1, Protein extract from adult flies. Lane 2, protein extract from pupae. An 85 kD protein that binds biotinylated P29 in adult and pupal lysates is indicated (arrow).

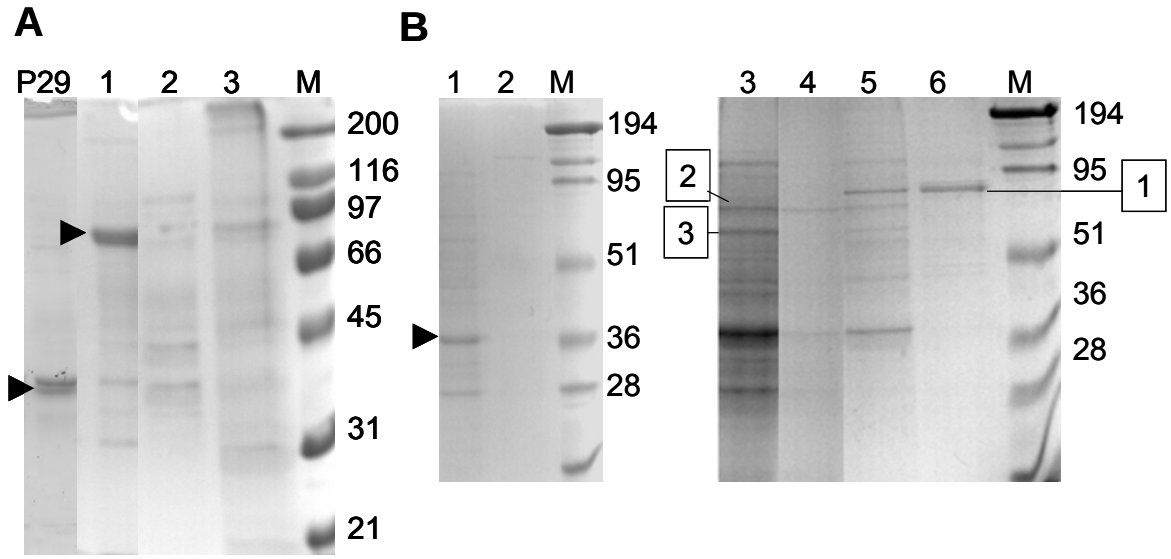

Figure 8
A. SDS PAGE analysis of proteins immunoprecipitated with recombinant P29 from pupal and adult homogenates of *D. melanogaster* OregonR. P29, 35 kDa recombinant P29. Lane 1, proteins immunoprecipitated from pupae (Affigel Protein A, anti-Xpress antiserum, recombinant P29 and pupal lysate). Lane 2, negative control (Affigel Protein A, anti-Xpress antiserum, recombinant P29 and no pupal lysate). Lane 3, negative control (Affigel Protein A, anti-Xpress antiserum, and pupal lysate, no recombinant P29). M, protein markers (kD). The positions of recombinant P29 and the 85 kDa protein are indicated (arrow heads).
B. SDS-PAGE analysis of proteins isolated from pupal and adult homogenates by affinity binding to P29-dynal bead complex. Lane 1, negative control (eluent from P29 only, no lysate). The position of recombinant DmP29 is shown (arrow head). Lane 2, negative control (eluent from adult lysate, no P29). Lanes 3 and 4, first and second eluents of proteins bound from adult lysate. Lanes 5 and 6, first and second eluents of proteins bound from pupal lysate. M, protein markers (kD). Bands 1 – 3 cut from the gel for analysis by MALDI-TOF are indicated.

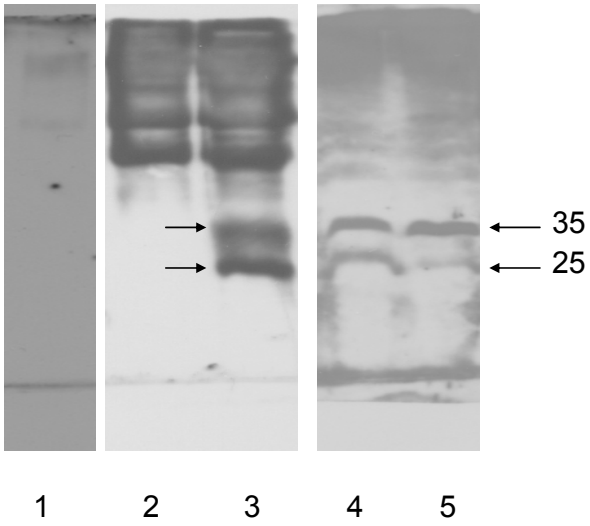

Figure 9
Analysis of proteins immunoprecipitated by the LSP1 antiserum by western blot with the P29 antiserum. Lane 1, negative control: protein G beads with adult lysate, no antibody. Lane 2, negative control: protein G beads with antibody, no lysate. Lane 3, proteins immunoprecipitated from adult Oregon R lysate with LSP1 antibody and protein G beads. Western blot with P29 antibody of protein extract from adult Oregon R flies (lane 4) and adult EP835 flies (lane 5) are shown for comparison. The 25 and 35 kDa proteins that are precipitated with the LSP1 antiserum and that cross-react with the anti-P29 antiserum are indicated.

Table 1. Detection of JHE activity and non-specific esterase activity in purified *D. melanogaster* mitochondria.

Cellular fraction	JHE activity (nmolJH/min/mg)	non-specific esterase activity		% JHE activity inhibited by OTFP
		α–NA (OD/mg)	β–NA (OD/mg)	
Mitochondria	0.48	2.942	2.105	97%
Serum and cytosol	0.72	0.041	0.036	99%

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CHAPTER 4

Misexpression of a putative juvenile hormone esterase binding protein in *Drosophila melanogaster*

Zhiyan Liu, Xiuli Li, Russell Jurenka and Bryony C. Bonning

Department of Entomology and Interdepartmental Program in Genetics, Iowa State University, Ames, IA 50011-3222, USA

(GC and GC-MS examination of cuticular hydrocarbons was conducted by Dr. Russell Jurenka. Parts of the longevity study were conducted by Xiuli Li.)

ABSTRACT

A putative juvenile hormone esterase (JHE) binding protein P29 has been identified in *Drosophila melanogaster*. P29 was localized to mitochondria by confocal microscopy and subcellular fractionation. *Drosophila* JHE and P29 can bind in vitro. JHE activity was detected in purified mitochondria. In this paper we used P29 hypo- and hyper-expression mutants to elucidate the function of P29 and the potential interaction of P29 with JHE. Hyperexpression of P29 during the early larval stages was lethal, while hyperexpression during the third instar resulted in reduced size of adult flies. These phenotypes showed that overexpression of P29 interfered with insect development. Hyperexpression in newly eclosed but not in old females resulted in reduced fecundity,

which suggested that P29 affected ovarian development. Fecundity was not affected by P29 hyperexpression in the male. Hypermorphic adults exhibited male-male courtship behavior. Hypermorphic females showed reduced receptivity to mating. Hypermorphic females had decreased courtship pheromone, cis, cis-7, 11-hepta cosadiene, which resulted in male flies being unable to locate female flies. Hyperexpression of P29 in males resulted in decreased production of the aggregation pheromone, cis-vaccenyl acetate. For EP835, the hypomorphic mutant, the JHE titer was significantly lower than in wild type flyies. For EP835/Gal4, the hypermorphic mutant, all hyperexpression phenotypes were consistent with a reduced JH titer in *Drosophila,* which may have resulted from an increased titer of JHE. Therefore, the titer of P29 appeared to be positively correlated with the titer of JHE and negatively correlated with the titer of JH. We also found that flies with both hypo- and hyper- expression of P29 had a significantly shorter lifespan. The fact that hypermorphic flies were hyperactive and hypomorphic females laid more eggs, likely contributed to the reduced longevity. Together with the observation that JHE activity was detected in mitochondria, we hypothesize that JHE is stored in mitochondria and that P29 helps to transport JHE to the cytosol.

INTRODUCTION

Juvenile hormone (JH) in combination with ecdysteroids plays a vital role in insect development (Hammock, 1985). Multiple functions of JH have been found in *D. melanogaster*. The most well known effects of JH are in larval-pupal development and

metamorphosis (Riddiford and Ashburner, 1991). JH has an effect on initiation and continuation of vitellogenin uptake, oocyte development and ovarian maturation (Handler and Postlethwait, 1977; Ringo *et al.*, 2005). JH functions in developing female primary receptivity to mating or increasing receptivity (Manning, 1967; Ringo *et al.*, 2005). The antijuvenoid Precocene I slowed ovarian growth and markedly reduced oviposition (Ringo *et al.*, 2005). *D. melanogaster* JHE was purified and characterized, which is highly selective for JHIII and JHIII bisepoxide, which is the predominant form of JH in *Drosophila* (Campbell *et al.*, 1998). The JHE gene was identified in *D. melanogaster* by matching its peptide mass fingerprint with a sequence from the *Drosophila* genome (Campbell *et al.*, 2001). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of JHE showed that the expression level of JHE in *D. melanogaster* is regulated by both JH and 20E (Kethidi *et al.*, 2005). The JHE titer in insect hemolymph is regulated by biosynthesis in the fat body tissue (Renucci *et al.*, 1984) and the removal by pericardial cells in the hemolymph (Locke and Russell, 1998). Pericardial cell uptake of JHE from the hemolymph is by receptor-mediated endocytosis, and JHE goes to the lysosomes where it is degraded (Booth *et al.*, 1992; Ichinose *et al.*, 1992; Ichinose *et al.*, 1992; Bonning *et al.*, 1997). Other than hemolymph and pericardial cells, JHE was also detected in the epidermal cells of *Manduca sexta* (Mitsui *et al.*, 1979). Lysosomes were the only subcellular organelle in which JHE was detected (Bonning *et al.*, 1997).

A putative JHE binding protein P29 was identified in *M. sexta* by screening a

phage display library for proteins that bind to JHE (Shanmugavelu *et al.*, 2000). Binding of P29 to JHE was confirmed by immunoprecipitation and ligand blot (Shanmugavelu *et al.*, 2001). A putative P29 homolog was found in *D. melanogaster*. Both *M. sexta* and *D. melanogaster* P29s are predicted to localize to the mitochondria (Chapter 2). We have shown that *D. melanogaster* P29 is located in mitochondria using confocal microscopy and subcellular fractionation (Chapter 2). Binding of *D. melanogaster* P29 and JHE was confirmed by immunoprecipitation and ligand blot (Chapter 3). Based on the finding that P29 is located in mitochondria, we used *D. melanogaster* mutants to determine the function of P29 and whether it interacts with JHE in vivo.

The *D. melanogaster* P29 gene CG3776 is located on the right arm of the second chromosome at position 60E10. There are two *D. melanogaster* EP lines (EP835 and EP840) available at the Szeged *Drosophila* Stock Centre. Both of the EP lines are homozygous viable. P{lacW}l(2)k03704[k03704] which is homozygous lethal is also listed as a stock of CG3776, but the EP insertion is 322bp upstream of the CG3776 start codon, which puts the EP insertion in the 5' UTR of the neighboring gene, RpL19 (ribosomal protein L19) (Lynn Crosby, personal communication). EP835 and EP840 were used in this study and a hypermorphic mutant was also generated to study the function of P29. EP835 was used to make a double mutant with the *pnr* gene, which encodes a zinc-finger protein with homology to vertebrate GATA transcription factors (Ramain *et al.*, 1993; Winick *et al.*, 1993). Hyperexpression of EP835 suppressed the *pnr*Gal4/+

phenotype, which suggests that P29 interacts with this transcription factor (Pena-Rangel *et al.*, 2002). However, the gene function of CG3776 has not been studied using this EP line. From study of the hypermorphic and hypomorphic mutants of P29 we found that P29 affects JHE activity, development, fecundity, receptivity, ovarian development, sex pheromone production and life span. All phenotypes suggest that P29 hyperexpression results in a decreased titer of JH. Hypomorphic flies had reduced JHE activity. Interaction of P29 with JHE may result in a reduction of the JH titer. Here we discuss hypotheses to account for these observations.

MATERIALS AND METHODS

***Drosophila* strains** The EP lines EP835 and EP840 were obtained from the Szeged *Drosophila* Stock Centre (Rorth, 1996). In EP835 the EP element is inserted into chromosome 2R: 20,477,413, which is 35bp upstream of the CG3776 start site in the 5' untranslated region. In EP840 the EP element is inserted into chromosome 2R: 20,477,413, which is 37bp upstream of the CG3776 start site. GAL4-HSP70/CyO (Bloomington *Drosophila* Stock Center, stock# 2077**)** expresses GAL4 in all tissues under control of the Hsp70 promoter. All flies were maintained at 25°C with a 12:12 L:D cycle in vials containing regular *Drosophila* food (Roberts, 1998).

Fly crosses and test for hypo- and hyper- expression of P29 Virgin females and males were collected within 6 hours of eclosion. GAL4-HSP70/CyO virgin females were

mated to EP835 males. Five pairs of flies were maintained in food vials. F1 progeny were heat shocked at different stages for P29 hyperexpression. Two genotypes EP835/GAL4-HSP70 and EP835/CyO were generated from the EP835 and GAL4-HSP70/CyO cross. Control flies (EP835/CyO) had curly wings and experimental flies (EP835/GAL4-HSP70) had normal, straight wings.

For heat shock in adults, flies were sorted by genotype and gender within 6 hours of eclosion, and then heat shocked at 37°C for one hour. Flies were then frozen at different times after heat shock. To test for P29 overexpression, proteins were extracted, quantified by Bradford assay (Bio-Rad Protein Assay Kit) and separated by SDS-PAGE (12% gel). After transfer to Hybond-P membrane (Amersham Biosciences, Piscataway, NJ) proteins were detected by using purified P29 antibody as described in chapter 2. To test whether the two EP lines are hypomorphic in P29 expression proteins from EP835, EP840 and Oregon R were also extracted. SDS-PAGE and western blot were conducted to test the expression of P29.

Juvenile hormone esterase activity assay JHE activity was tested using staged hypomorphic flies. Prepupae and pupae of EP835, EP840 and Oregon R were collected 11 hour after pupariation and frozen in -80°C. Individual staged pupae were ground in eppendorf tubes with 60µl PBS on ice and centrifuged at 8000g at 4°C briefly to remove debris. JHE activity was measured by a partition assay using ^{3}H-JH-III as substrate (Hammock and Sparks, 1977). Ten µl of the homogenate was diluted to 100 µl to test for

JHE activity. Each sample was assayed in triplicate. The nanomols of JH hydrolyzed per minute per fly were calculated.

Purification of mitochondria Mitochondria were purified as described previously (Chapter 2), and further purification using percoll-sucrose gradient centrifugation was conducted. Polycarbonate tubes were filled with percoll-sucrose mixture (55% percoll-sucrose 0.55M, 45% isotonic extraction medium (HEPES-KOH 20mM; EGTA 1mM; mannitol 0.25M)). The gradient was formed by centrifugation for 1 hour at 92,000g at 4°C. The crude mitochondrial fraction was laid on to the gradient and centrifuged at 100,000g at 4°C for 1 minute. The band corresponding to mitochondria was removed by syringe and needle and diluted into 5 volumes of the isotonic extraction medium. A final centrifugation step (10,000g; 5 min; 4°C) was used to wash out the residual percoll (Stepien *et al.*, 1988). The protein concentration of all samples was determined using the Bradford method. JHE activity of the fractions was also tested.

Fertility assays Newly eclosed *Drosophila* adults from the cross of EP835 and GAL4-HSP70/CyO were sorted according to gender and genotype and then heat shocked at 37˚C for one hour. Flies were maintained for 1 day in food vials to allow for P29 expression and then crossed. Female flies with overexpression of P29 (EP/Gal4♀) were crossed with hypermorphic males (EP/Gal4♂) or control males (EP/CyO♂). Control females (EP/CyO♀) were crossed with hypermorphic males (EP/Gal4♂) or control males (EP/CyO♂). Four pairs of flies were crossed in vials or apple juice plates with yeast. The

progeny adults were counted for the crosses in vials. Eggs laid per day were counted for the crosses in apple juice plates (80 pairs of flies were used). To see whether overexpression of P29 affects fecundity of older females, newly eclosed flies were sorted and maintained separately in vials for 7 days. The flies were heat shocked at 37˚C for one hour and maintained at 25˚C for 1 day. The flies crossed as above and eggs laid per day were counted (60 pairs of flies were used). To see whether ovary development was affected by overexpression of P29 and whether ovary development could be recovered by application of the JH analog, methoprene, one microgram of methoprene in 0.2 µl acetone was applied to the abdomen of anesthetized flies 1 day after heat shock (control, acetone only). Ovaries were dissected under a light microscope. Heat shocked EP/CyO flies were used as controls.

A fertility assay was also conducted for hypomorphic flies. To make the data comparable to hyperexpressed flies, hypomorphic (EP835) and control (Oregon R) flies were also heat shocked at 37˚C for one hour and maintained at 25˚C for 1 day before being crossed. EP835 females were crossed with EP835 or Oregon R males. Oregon R females were crossed with Oregon R or EP835 males. Four pairs of flies were crossed in apple juice plates with yeast. One hundred and twenty pairs of flies were used and eggs laid were counted daily for the first three days.

Overexpression of P29 in larval stages Five pairs of GAL4-HSP70/CyO virgin females and EP835 males were crossed in vials with *Drosophila* food. Adult flies were

removed 4 days later. Flies were reared at 25°C with a 12:12 L:D cycle. The vials with eggs and larvae were heat shocked at 37˚C for one hour immediately after the adult flies were removed. Progeny adults were counted daily.

Mating behavior Newly eclosed EP/Gal4 were heat shocked and incubated at 25°C for 3 or 5 days. Mating was observed for one hour when 5 pairs of flies were put into a tube with fly food. Crosses were the same as for the fertility study.

Pheromone assay Newly eclosed EP/Gal4 and EP/CyO were heat shocked at 37°C for 1 hour and kept at 25°C. Cuticular hydrocarbons were extracted 1 day after heat shock. Five flies were immersed in 100μl hexane for 10 minutes to extract hydrocarbons. The hexane was dried and subjected to GC and GC/MS. Analyses were conducted by capillary GC-MS using a Hewlett-Packard 5890 GC equipped with a DB-1 column (30 mX0.25 mm). The GC was interfaced with a Hewlett-Packard 5972 Mass Selective Detector operated in scan mode. Separations were conducted in splitless mode with temperature programming at 80°C for 1 min, then 10°C /min to 32°C (Choi *et al.*, 2005).

To see whether production of pheromone could be recovered by application of the JH analog, methoprene, one microgram of methoprene in 0.2 μl acetone was applied to the abdomen of anesthetized flies at 1 day after heat shock (control, acetone only). Pheromone was extracted 1 day after methoprene application (two days after eclosion). EP/CyO was used as control to see whether methoprene affects the pheromone

production of normal flies.

Longevity study of flies with hyper- and hypo- expression of P29 EP/Gal4 and EP835 were used to study whether expression of P29 affects life span with EP/CyO and Oregon R as controls. Males and females were separated in this experiment. One hundred flies were kept in 32 oz transparent plastic food containers with adapter tubes with *Drosophila* food at 25°C with a 12:12 L:D cycle (Spencer *et al.*, 2003). Fresh food vials were provided and flies were scored for survival every two to three days. The starting population for each genotype and gender was 300. Flies were heat shocked at 37°C for one hour every day. Initially some flies got trapped on the food when it liquefied during heat shock. These flies were not counted. Food vials were subsequently replaced with vials that contained filter paper soaked with 1% sucrose solution during heat shock. The longevity experiments were conducted in two replicates.

Statistical analysis Analysis of variance was run using JMP 6 (SAS institute) and SAS to test for differences among treatments. F-test was used to test significance among treatments. All pairwise comparisons were made using Tukey's HSD method. For the lifespan assay, pairwise comparisons among genotypes for age-specific survival were conducted with Kaplan-Meier survival analyses (log-rank test and wilcoxon test) using PROC LIFETEST in SAS with significance corrected for multiple tests (Bonferroni's method).

RESULTS

Determine whether the mutant P29 allele is hypomorphic The EP lines EP835 and EP840 (EP/EP) have an EP element inserted upstream of the start site of P29. Western blot with purified P29 antibody was conducted to determine whether the two EP lines are hypomorphic. The predicted size of P29 is 30kD, but as it is transferred to mitochondria a 4.4kD N-terminal targeting sequence is cleaved. Hence the mature P29 in *Drosophila* is 25.9kD (Chapter 2). Western blot using purified P29 antibody showed a faint 25.9 kD band for EP835 compared to a strong band in Oregon R, which confirmed that EP835 is hypomorphic for P29 expression. However, even though the EP element insertion site of EP840 was only 2 base pairs upstream of the EP element insertion site of EP835 there was no significant decrease in the amount of P29 protein in EP840 (Fig 1A). Therefore EP835 was used for examination of the effects of hypoexpression of P29. EP835 was also used to generate flies with overexpressed P29.

Determine whether heat shock results in overexpression of P29 Two genotypes EP835/GAL4-HSP70 and EP835/CyO were generated from the EP835 and GAL4-HSP70/CyO cross and P29 expression examined by western blot. Heat shock for overexpression of P29 in the hypermorphic *Drosophila* mutant resulted in appearance of a 30 kD protein with peak expression at 24hr after heat shock (Fig 1B). This 30kD protein is the size of unprocessed P29, which was present until 48hr after heat shock. The

amount of 35kD protein was not affected by heat shock. In a previous study we determined that this 35kD protein is tropomyosin (Chapter 3). The 50kD protein band was weak, and the amount of 50kD protein was not affected in hyperexpressed and hypoexpressed flies compared to control. Therefore, it is unlikely that this 50kD protein is P29.

Determine whether hypoexpression of P29 affects JHE activity JHE activity was analyzed for prepupae of hypomorphic flies (EP835) with Oregon R as control. Individual flies were used to test JHE activity. There was a statistically significant difference in JHE activity for the three genotypes Oregon R, EP835 and EP840 (ANOVA, df = 26, $F = 3.98$, $P < 0.05$). The JHE activity in prepupae was significantly lower in the EP835 line than in the wild type flies. The JHE activity of Oregon R flies was not significantly different from that of EP840 (Table 1). Based on peak JHE activity in Oregon R at 11 hours after pupariation (Chapter 1), we also tested JHE activity of flies at this stage. There was also a statistically significant difference in JHE activity for the three genotypes at this stage (ANOVA, df = 14, $F = 8.74$, $P < 0.005$). The JHE activity of EP835 was significantly lower than that of Oregon R (Table 1), as was the case for prepupae.

Determine whether JHE is present in mitochondria Since P29 is in mitochondria and P29 binds to JHE we conducted cellular fractionation to determine whether JHE is present in the mitochondria. JHE activity assay was used to detect JHE in

different cellular fractions. Percoll-sucrose gradient was conducted to separate mitochondria (density = 1.075 to 1.085 g/ml) from contaminants such as lysosomes (density = 1.05 to 1.06). We detected JHE activity in mitochondria which was similar to the activity of the whole insect lysate, but lower than the JHE activity detected in the serum and cytosol (Table 2).

Hypermorphic females have reduced fecundity and hypomorphic females have increased fecundity. One day old hypermorphic flies were mated and allowed to lay eggs for 3 days. Overexpression of P29 in newly eclosed female flies resulted in a reduced number of adult progeny flies. Even when hypermorphic females were crossed with normal males, they still had significantly fewer offspring than normal flies. Hyperexpression of P29 in males did not affect the number of adult progeny (Fig 2). To determine whether female flies laid fewer eggs, apple juice plates were used to collect eggs. Eggs were counted every 24 hours. Control flies on apple juice plates laid fewer eggs than on cornmeal-yeast diet, indicating the apple juice diet was inferior. Again, hyperexpression of P29 in females resulted in significantly fewer eggs than those laid by control females. P29 hyperexpression in the males did not affect fecundity (Table 3A). Dissection of ovaries from four day old females showed that hypermorphic females had fewer eggs than control females. Application of the JH analog methoprene to hypermorphic females recovered egg development (Fig 4). Fecundity was not affected when flies were heat shocked on the 7th day after eclosion (Table 3A). Fecundity assay

was also conducted for hypomorphic flies. Hypo-expression of P29 in females resulted in significantly more eggs than those laid by control females (Oregon R). Hypo-expression of P29 in the males did not affect fecundity (Table 3B).

Overexpression of P29 in larvae *D. melanogaster* were maintained at 25 °C with a photoperiod at 12-h light/12-h dark cycle. Under these rearing conditions, embryonic and larval stages were completed in 24 and 96 h, respectively. We heat shocked flies 4 days after crossing at which time the vial contained larvae from 1st to 3rd instar. In heat shocked vials, both EP/Gal4 and EP/CyO adults eclosed in the first two days (Table 4), but EP/Gal4 had a smaller body size than EP/CyO (Fig 5), resulting from heat shock of the third instar larvae. When larvae were not heat shocked, EP/Gal4 flies had a similar body size to that of EP/CyO (Fig 5). After 2 days, no more flies with overexpressed P29 (EP/Gal4) eclosed, while control flies eclosed normally (EP/CyO). These flies were heat shocked as first and second instar larvae. In the control vial without heat shock, both EP/Gal4 and EP/CyO flies eclosed during the five days and the population size from the third to the fifth day was twice that of the heat shocked vial (Table 4). This experiment was repeated three times. In conclusion, hyperexpression of P29 (EP/Gal4) during the 1st and 2nd instar was lethal. Hyperexpression of P29 during the third instar resulted in eclosion of adults of smaller size.

Mating behavior and pheromone production of hypermorphic flies Five virgin females and males were put into a food vial to observe mating behavior. Males

with hyperexpressed P29 (EP/Gal4) had a high frequency of mating behavior and showed male - male courtship behavior (tapping, singing and attempting) (Amrein, 2004) when mating with female (Table 5). EP/Gal4 flies courted both male and female EP/Gal4. The frequency of courtship to male and female was similar. Sometimes the male flies formed chains of 3 males with one female, with each male courting the fly in front and being courted by the fly behind. We observed that EP/Gal4 males were tapping, singing and attempting and formed a line in the beginning of the longevity study with 100 male flies in the 32oz plastic container. No such behavior was observed in Oregon R, EP/CyO or EP835/EP835 flies. When mating with EP/CyO female, EP/Gal4 males did not have a high frequency of mating behavior as with EP/Gal4 females. The possible reason for this observation is that (1) EP/Gal4 females were less receptive than EP/CyO and escaped from the chasing males; (2) EP/Gal4 females were more attractive to EP/Gal4 males. Males court moving females more vigorously than they do immobile females (Tompkins *et al.*, 1982). Mating behavior was also observed for flies five days after heat shock. The crosses were the same as those for three days after heat shock. No such behavior was found in EP/Gal4 crosses. Most flies mated successfully within 1 hour of being introduced to the vials. A possible reason for this result is that P29 was not overexpressed after 5 days.

Hydrocarbons were extracted from the fly cuticle and GC-MS used to identify pheromones. Hyperexpression of P29 in males resulted in decreased production of the

aggregation pheromone, cis-vaccenyl acetate compared to control flies (EP/CyO). Hyperexpression of P29 in females (EP/Gal4) resulted in decreased production of courtship pheromone cis, cis-7, 11 -heptacosadiene compared to control flies (EP/CyO) (Fig 6). Pheromone extraction was also conducted using 2 day old flies following addition of methoprene (1 day after heat shock followed with 1 day after methoprene application). Again, EP/CyO had higher female courtship pheromone cis, cis-7, 11-heptacosadiene and cis, cis-7, 11-nonacosadiene than EP/Gal4. However, no obvious recovery of pheromone production was detected by methoprene application.

Effect of expression of P29 on life span We observed that flies with overexpression of P29 were super active. We hypothesized that P29 may affect life span. Hypermorphic fly EP835/Gal4 and hypomorphic fly EP835 were used to study whether expression of P29 affects life span with EP835/CyO and Oregon R as controls. Pairwise comparisons among genotypes for age-specific survival were conducted with Kaplan-Meier survival analyses (log-rank test and wilcoxon test) using PROC LIFETEST in SAS with significance corrected for multiple tests (Bonferroni's method). Among males and females, relative to the wild type Oregon R and control fly EP835/CyO, the age-specific survival of hypomorphic fly EP835/EP835 and hyermorphic fly EP835/Gal4 is reduced in both log-rank test and wilcoxon test ($P<0.001$); survival of EP835 and EP835/Gal4 does not differ in wilcoxon test (male: $P=0.98$, female: $P=1$) but they do have difference in log-rank test (male: $P<0.001$, female: $P=0.027$). Wilcoxon test has more power at early

stage comparison but is less sensitive to the differences at later stage than log-rank test. The difference of the two tests means that overall they have difference showed in log-rank test but no significant difference in early stage showed in wilcoxon test (Fig 7). The mean ages of phenotypes were compared using Tukey-Kramer HSD (alpha=0.05; JMP 6; SAS institute). The mean ages of hypomorphic fly EP835 and hyermorphic fly EP835/Gal4 were significantly lower than those of wild type fly Oregon R and control fly EP835/CyO in both male and female. However, the mean ages of hypomorphic fly EP835 and hyermorphic fly EP835/Gal4 did not differ significantly (Table 6).

DISCUSSION

M. sexta P29 was first identified as a putative JHE binding protein because it was found to bind to JHE by immunoprecipitation and ligand blot (Shanmugavelu *et al.*, 2000). *Drosophila* P29 was also shown to bind to *Drosophila* JHE in vitro by immunoprecipitation and ligand blot (Chapter 3). P29 is located in mitochondria (Chapter 2). The P29 antibody can detect 25.9kD, 35kD and 50kD proteins with 35kD and 50kD proteins detected in the hemolymph. We determined that the 35kD protein is not P29 (Chapter 3). Although we do not know the identity of the 50kD protein, it is unlikely to be P29. When P29 is hypoexpressed in EP835, the titer of the 50kD protein was not affected. Therefore, there is a good chance that P29 is only located in the mitochondria. Based on this, along with the fact that we also find JHE activity in purified mitochondria, we hypothesize that P29 interacts with JHE in mitochondria. There is a putative

conserved Tim 44 motif in P29 (Blast; Chapter 2). The presence of a Tim44-like domain in P29 suggests a role for P29 in protein transport. We hypothesize that P29 may be involved in transferring JHE through the mitochondrial membrane.

gC1q-R/p33 is a well studied human protein, which is stored in mitochondria prior to secretion from the cell (Ghebrehiwet *et al.*, 2001). There is a growing list of a class of mitochondrial proteins (Hsp10, Hsp60, mHsp70, tumor necrosis factor receptor-associated protein-1, cytochrome c, gC1q-R/p33, mitochondrial aspartate aminotransferase etc.) that are initially targeted to mitochondria but then are exported to different compartments of the cell by an unknown mechanism (Domanico *et al.*, 1993; Wadhwa *et al.*, 1993; Isola *et al.*, 1995; Soltys and Gupta, 1996; Cechetto and Gupta, 2000; Cechetto *et al.*, 2000; Ghebrehiwet *et al.*, 2001; Skanda *et al.*, 2001). JHE may be stored in mitochondria and secreted into the cytosol and/or hemolymph as needed. P29 may help to transport JHE to the hemolymph, which would explain the phenotype we observed in hypomorphic flies: reduced expression of P29 results in reduced JHE activity. JHE is a glycosylated protein with five potential glycosylation sites (Eldridge *et al.*, 1992). There is a report that the N-acetylglucosami-nyl-transferase involved in the direct transfer of N-acetylglucosamine is oriented outside of the outer membrane of mitochondria (Levrat *et al.*, 1989). We hypothesize that JHE can follow two pathways. One is through the Golgi where it is glycosylated and into the hemolymph; the other pathway is that JHE is glycosylated in the mitochondrial outer membrane and stored in

mitochondria. In a previous study, immuno-EM was conducted to look for JHE in lysosomes in *M. sexta* and *Trichoplusia ni* (Bonning et al, 1997; Booth et al, 1992). There was no evidence that JHE was present in mitochondria. There are the two possible reasons: (1) JHE may not be abundant in mitochondria. (2) The authors were looking for JHE in lysosomes and may have overooked JHE in mitochondria. Once we have a *Drosophila* JHE antibody we will conduct immuno-EM to see how much JHE is in the mitochondria and to which part of mitochondria JHE is localized.

Multiple functions of JH have been found in *D. melanogaster*. EP/Gal4 shows multiple phenotypes related to decreased JH. Overexpression of P29 in the first and second larval instar was lethal. Hyperexpression during the third instar was not lethal but resulted in reduced size of adult flies. This phenotype is consistent with the function of JH which is essential to insect metamorphosis (Riddiford and Ashburner, 1991). Overexpression of P29 in newly eclosed females resulted in reduced fecundity, but this was not the case for older females (Table 3), which is consistent with the function of JH in ovarian maturation (Handler and Postlethwait, 1977; Ringo *et al.*, 2005). Females with overexpressed P29 were less receptive, similar to wild type flies treated with the antijuvenoid precocene I (Manning, 1967; Ringo *et al.*, 2005). We also found that males with overexpressed P29 had less aggregation pheromone. A role for JH in *Drosophila* pheromone production has not been studied, but studies in other insects suggest JH is involved in pheromone biosynthesis (McNeil, 1987; Cusson *et al.*, 1990; Ignell *et al.*,

2001). Flies with hypoexpressed P29 had a shorter life span and higher reproduction, which is consistent with an increased titer of JH. All of the above phenotypes indicate that the titer of P29 is positively correlated with that of JHE and negatively correlated with that of JH. This result is consistent with the hypothesis that P29 functions in JHE transport. However, flies with overexpressed P29 differ from flies with reduced JH synthesis. Reducing insulin-like peptides (*InR* mutant) increases the life-span of *Drosophila* by decreasing JH synthesis and the extension of lifespan can be restored by applying methoprene (Tatar *et al.*, 2003; Tu *et al.*, 2005). The theory that reproduction and lifespan are two trade-off phenotypes is not the case in hypermorphic flies, which had lower fecundity and shorter lifespan. Considering that P29 is in mitochondria and has a Tim-44 like domain, it would not be surprising to see that P29 transfers other proteins in addition to JHE. Flies with both hypo- and hyper- expression of P29 had a shorter lifespan, which suggests that P29 is an important protein in the insect. The most important function of mitochondria is to generate ATP by oxidative phosphorylation (Darley-Umar *et al.*, 1987). It has been reported that mitochondrial function is affected by steroids and thyroid hormones in mice and rats (Cornwall *et al.*, 1992; Fernandez *et al.*, 1994; Starkov *et al.*, 1997). JH is a sesquiterpenoid hormone which is a small lipophilic molecule. JH was also reported to affect mitochondrial function (Stepien *et al.*, 1988; Farkaš and Šutáková, 2001). JH acted to uncouple oxidative phosphorylation with resulted in swelling of the mitochondria (Farkaš and Šutáková, 2001). JH was also found

to stimulate mitochondrial protein synthesis and cytochrome oxidase activity in *D. melanogaster* cells. JH does have an active influence on energy metabolism (Stepien *et al.*, 1988). Considering that JH has multiple functions in mitochondria, JHE may also function in mitochondria to degrade JH.

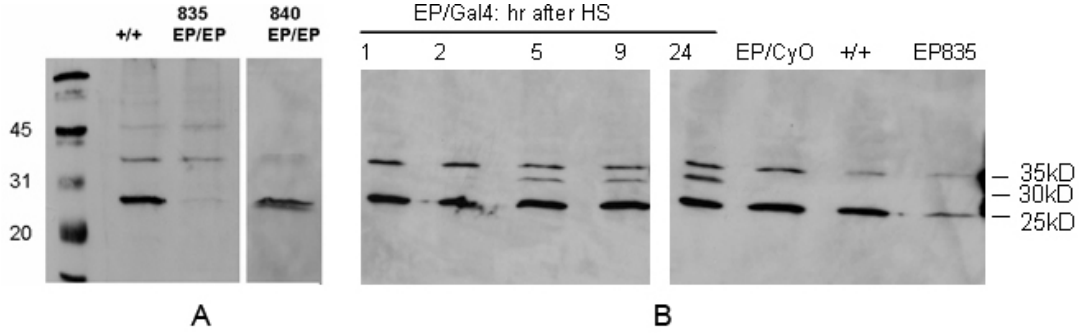

Fig 1. Expression of P29 in hypomorphic and hypermorphic flies. A. Western blot of protein from Oregon R (++), EP835 and EP840. B. Western blot of protein from the offspring of EP835 and GAL4-HSP70/CyO. Newly eclosed adults were heat shocked at 37˚C for 1 hour and maintained in tubes with *Drosophila* food at 25˚C. Flies were collected at different times after heat shock (hr after HS). EP/CyO was also heat shocked and maintained for 24 hours. Twenty µl of protein were loaded per lane for both gels. Purified P29 antibody was used.

Table 1. Comparison of JHE activity among prepupae and pupae. Different letters within a column show statistically different JHE activity (Tukey HSD**,** Alpha=0.050)

Strain	JHE activity ± SE (pmol JH hydrolyzed/min/fly)			
	Prepupae		Pupae	
Oregon R	11.64±0.84	A	195.10 ± 13.21	A
EP840	9.84±1.26	A B	175.60 ± 9.47	A
EP835	8.1±0.48	B	125.01 ± 13.59	B

Table 2. JHE activity of different cellular fractions of *D.melanogaster* (Oregon R) pupae

Cellular fraction	JHE activity (nmol JH hydrolyzed /min/mg)
Mitochondria	0.48
Serum and cytosol	0.72
Whole insect lysate	0.47

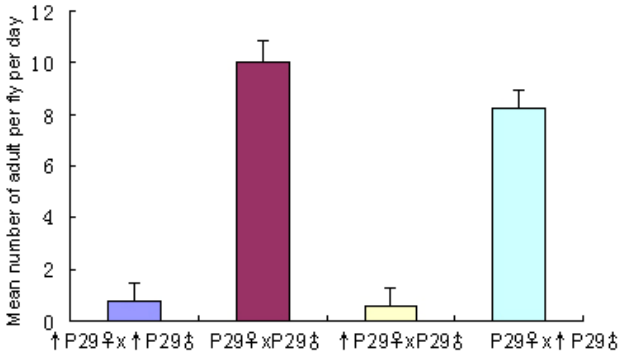

Fig 2. Mean number of adults eclosed per pair of flies per day. Newly eclosed flies were sorted and heat shocked, then kept in vials for one day to allow for full expression of P29. They were crossed in vials with *Drosophila* food and laid eggs for 3 days. Mean number of adult eclosed were calculated. Standard deviation is given in the figure. Twenty pairs of flies were used per cross. P29: EP/CyO; ↑P29: EP/Gal4.

Table 3. A. Comparison of eggs laid for the first 3 days for hyperexpressed flies. Different letters within a column show statistically different egg production (Tukey HSD**,** Alpha=0.05). P29: EP/CyO; ↑P29: EP/Gal4.

Cross	Mean eggs laid / fly / day ± SE (first 3 d)	
	Newly eclosed fly	7 day old fly
P29♀xP29♂	4.83 ± 0.68 A	24.87 ± 5.10 A
P29♀ x↑P29♂	4.77 ± 1.30 A	20.50 ± 0.88 A
↑P29♀xP29♂	0.53 ± 0.32 B	21.54 ± 1.89 A
↑P29♀x↑P29♂	0.52 ± 0.31 B	24.15 ± 1.37 A

B. Comparison of the eggs laid for the first 3 days for hypoexpressed flies. Different letters show statistically different egg production (Tukey HSD**,** Alpha=0.05). P29: Oregon R; ↓P29: EP835.

Cross	Mean eggs laid / fly / day ± SE (first 3 d)
↓P29♀x↓P29♂	13.70 ± 1.32 A
↓P29♀ xP29♂	13.44 ± 1.49 A
P29♀x↓P29♂	6.43 ± 1.40 B
P29♀xP29♂	4.50± 1.61 B

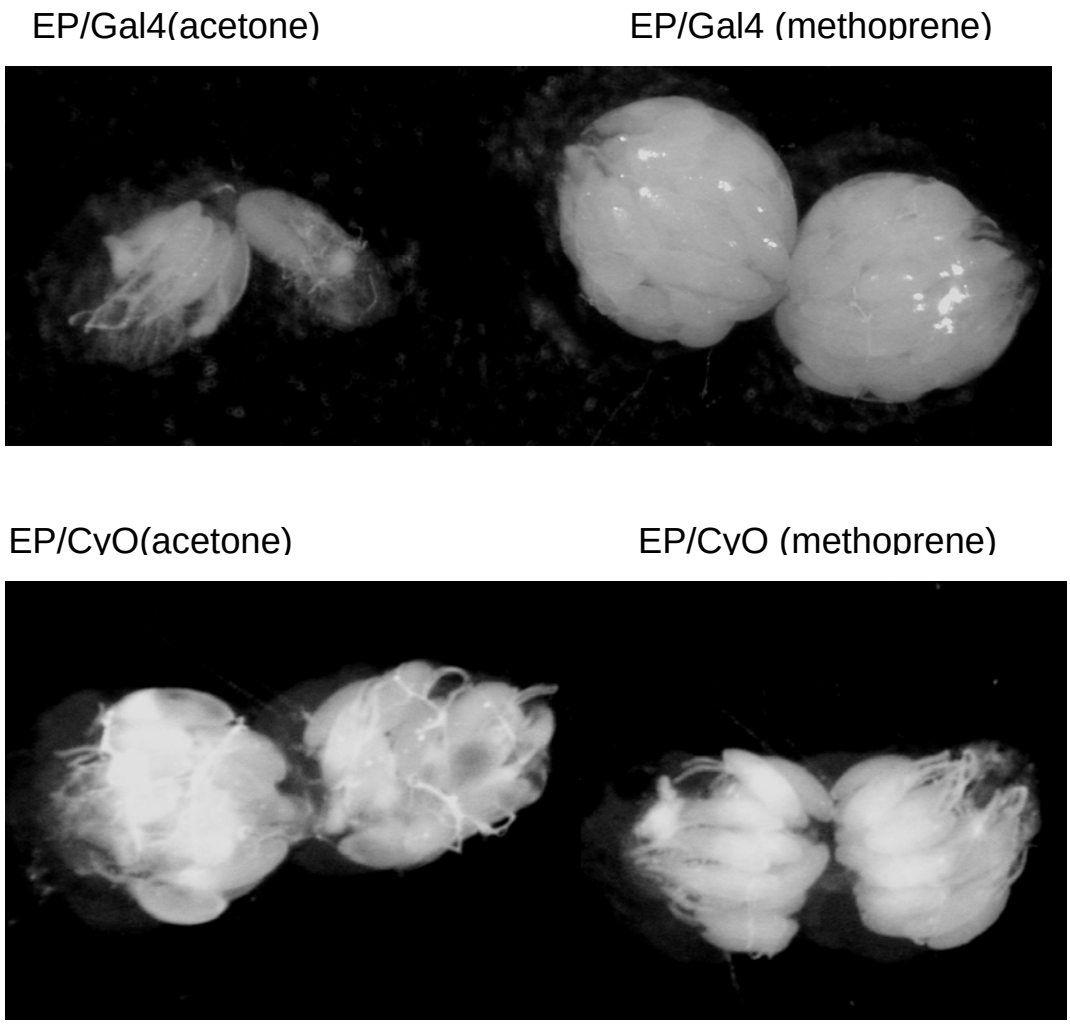

Fig 4. Ovaries of P29 hypermorphic and wild type flies. Newly eclosed flies were heat shocked at 37°C for 1 hour and kept in 25°C. One microgram of methoprene in 0.2 µl acetone was added to the abdomen of anesthetized flies at 1 day after heat shock (control, acetone only). Dissect ovaries 11 days after methoprene application.

Table 4. Number of adults eclosed over five days. Heat shock was conducted fours days after parental flies laid eggs.

	Heat shock		No heat shock	
Day	EP/Gal4 (↑P29)	EP/CyO (P29)	EP/Gal4 (P29)	EP/CyO (P29)
1	6	3	3	2
2	7	2	9	12
3	0	25	38	24
4	0	32	30	17
5	0	21	12	13

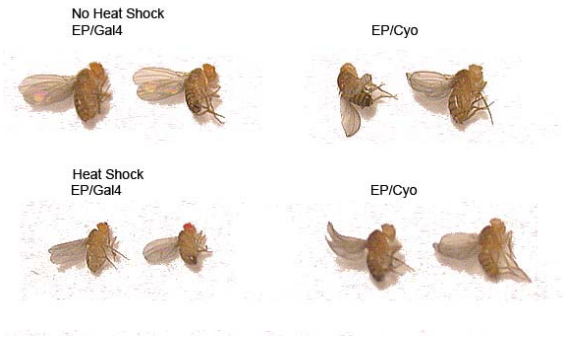

Fig 5. Hyperexpression of P29 during the third instar larvae results in reduced size of adult flies.

Table 5. Mating behavior of flies with overexpression of P29. The table shows the number of times courtship behavior was exhibited within a 1 hour period. Five pairs of flies were put into a vial with food 3 days after heat shock. P29: EP/CyO; ↑P29: EP/Gal4.

		↑P29♀x↑P29♂	P29♀ x↑P29♂	P29♀xP29♂	↑P29♀xP29♂
Mating behavior in 1 hr	♀♂	28	2	0	0
	♂♂	15	3	0	0
	♀♂♂	8	0	0	0
	♀♂♂♂	3	0	0	0
Mate in 1hr		0	1	0	0
Mate in 2 hr		1	2	0	0

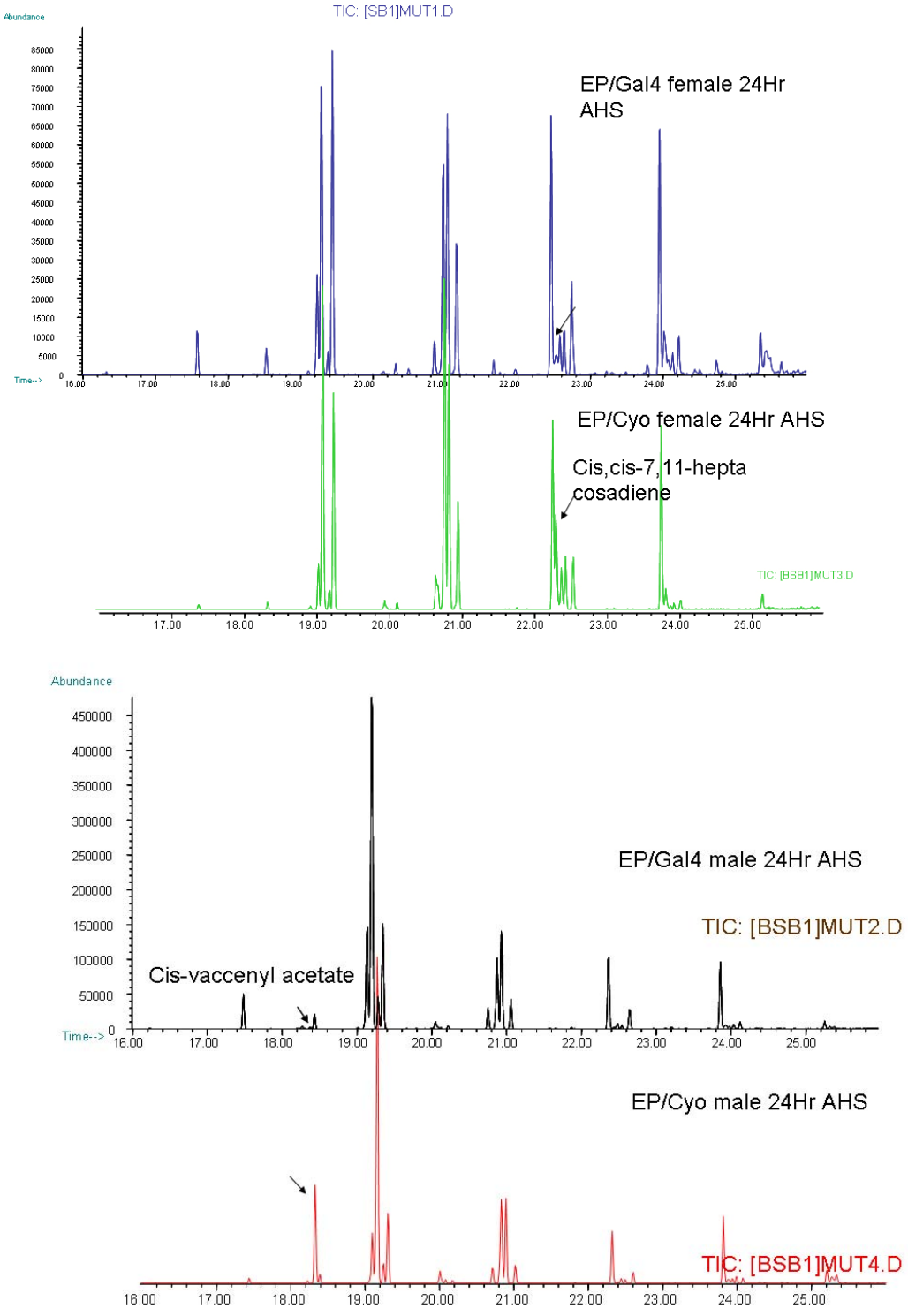

Fig 6. Chromatograms of hydrocarbons from the cuticle of flies. The hydrocarbons were purified and analyzed as described in Materials and Methods. AHS, after heat shock.

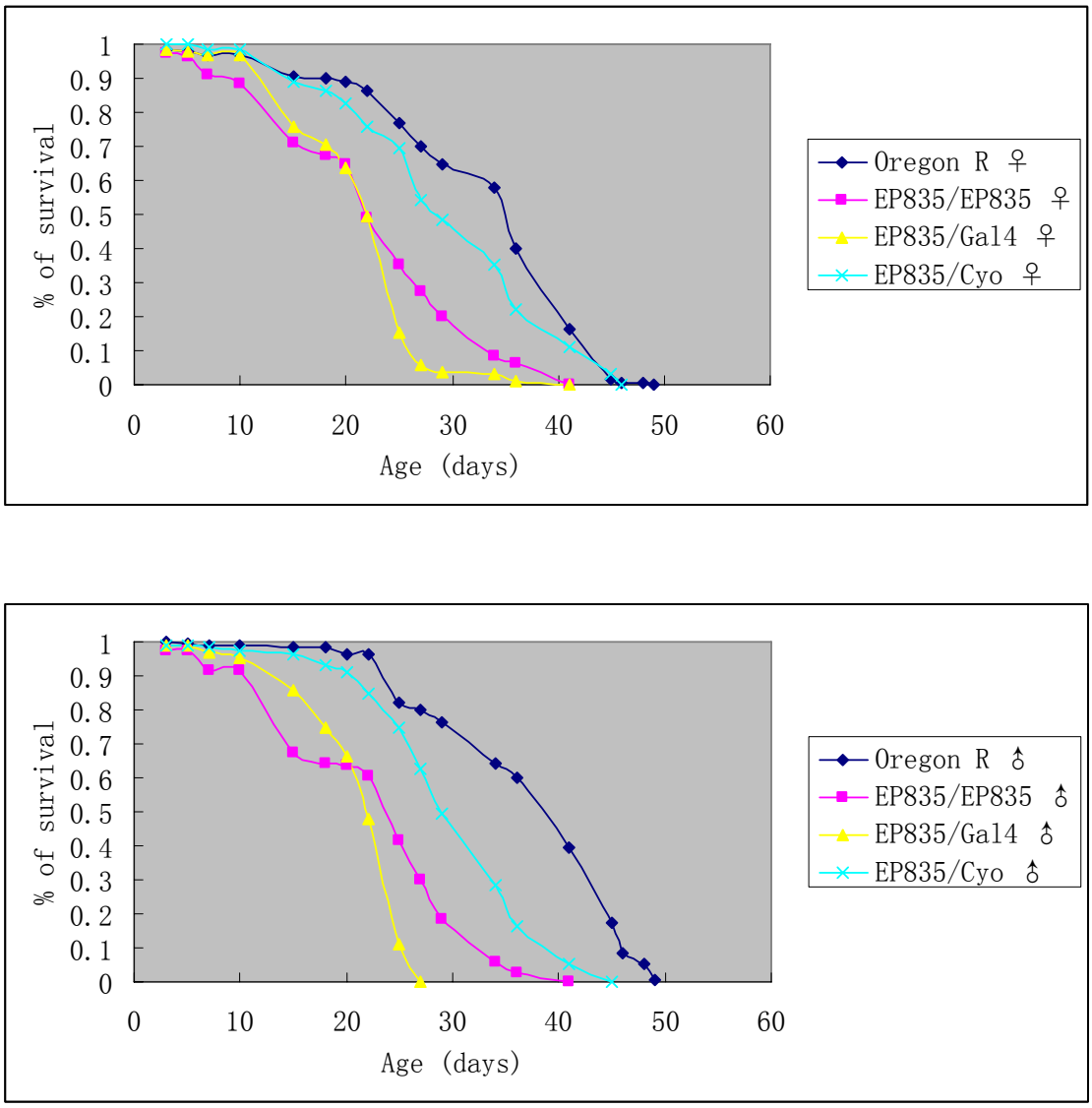

Fig 7. Survival of P29 mutant genotypes (hypomorphic fly EP835/EP835 and hyermorphic fly EP835/Gal4) relative to the wild-type Oregon R and control fly EP835/CyO. Results are expressed as percentage of surviving flies on each day.

Genotype	Ages (days) Mean±SE	
	Male	Female
Oregon R	37.86±0.60 A	33.59±0.67 A
EP835/Cyo	30.70±0.56 B	30.34±0.74 B
EP835/EP835	23.18±0.76 C	22.98±0.83 C
EP835/Gal4	21.74±0.61 C	21.85±0.61 C

Table 6. Mean ages of different phenotypes. The mean ages of phenotypes were compared using Tukey-Kramer HSD (alpha=0.05; JMP 6; SAS institute). Different letters within a column show statistically different ages.

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CHAPTER 5

General conclusions

Summary

The *Drosophila melanogaster* putative juvenile hormone esterase (JHE) binding protein, P29, was confirmed to localize in mitochondria by subcellular organelle fractionation and confocal microscopy of *Drosophila* S2 cells. P29 mRNA and an immunoreactive protein of 25 kDa were detected in *Drosophila* larvae, pupae and adults. Expression of P29 without the predicted N-terminal targeting sequence in High Five™ cells showed that the N-terminal targeting sequence is shorter than predicted, and that a second, internal mitochondrial targeting signal is also present. P29 has a conserved Tim-44 like domain which suggested it may involve in protein transport.

We investigated the potential ligands of DmP29 by testing three hypotheses: (i) DmP29 binds to *D. melanogaster* JHE: We produced a stably transformed insect cell line that expresses DmJHE and confirmed that DmP29 binds to *D. melanogaster* P29 by using both *in vivo* and *in vitro* approaches. DmJHE binds to both the 25 kD and 50 kD immunoreactive proteins. (ii) DmP29 binds other, non-specific esterases including two esterases predicted to be targeted to the mitochondria: We did not detect any interaction between DmP29 and non-specific esterases. (iii) DmP29 binds to other proteins in *D. melanogaster*: Ligand blot analysis, immunoprecipitation experiments and affinity

binding experiments showed that larval serum protein 1 binds the 25 kD P29. Based on binding of LSP1 to an unrelated protein, the interaction of P29 with JHE may be a non-specific association.

We used P29 hypo- and hyper- expression mutants to elucidate the function of P29 and the potential interaction of P29 with JHE. The hypomorphic mutant of P29 had reduced JHE activity. Many, if not all of the phenotypes resulting from hyperexpression of *Drosophila* P29 are consistent with the effects of a reduced juvenile hormone (JH) titer: Hyperexpression of P29 during the early larval stages was lethal, while hyperexpression during the third instar resulted in reduced size of adult flies. Hyperexpression of P29 in adult flies resulted in hyperactivity and reduced longevity. Hyperexpression in females resulted in reduced fecundity and decreased production of courtship pheromone, cis,cis-7,11-hepta cosadiene. Hyperexpression of P29 in males resulted in male-male courtship behavior and in decreased production of the aggregation pheromone, cis-vaccenyl acetate. In addition, hypoexpression of P29 resulted in increased egg production and reduced longevity, which is consistent with increased JH titer. Hence, the titer of P29 was positively correlated with the titer of JHE and negatively correlated with the titer of JH. Based on the collective phenotypes and detection of JHE activity in mitochondria, we hypothesize that JHE is stored in mitochondria and that P29 functions in transport of JHE to the cytosol.

Discussion

We detected JHE activity in mitochondria, which was unexpected. To confirm this observation, we will use a second method to demonstrate the presence of JHE in mitochondria. First, we will test for JHE activity again using the specific JHE transition-state inhibitor OTFP to eliminate the possibility of nonspecific JH degradation (Abdel-Aal and Hammock, 1986). Second, we will make a DmJHE antibody and use this for detection of JHE in mitochondria by immunotransmission electron microscopy (IEM).

P29 has a Tim-44 like domain suggestive of a role in protein transport and the phenotype of flies of hypo- and hyper- expressed P29 suggested that the titer of P29 is positively correlated with the titer of JHE. Therefore we hypothesize that P29 functions in JHE transport from the mitochondria to the cytosol. To test this we will use IEM to see whether P29 and JHE co-localize in the mitochondria using the P29 and JHE antibodies, along with secondary antibodies from different species labeled with different sized gold particles. We hypothesize that P29 localizes to the mitochondrial inner membrane and that JHE localizes to the mitochondrial matrix. We hypothesize that JH will activate P29 transport of JHE from the mitochondria. To test this we will apply methoprene to the flies, and look for a change in distrubution of JHE in the mitochondrial matrix and inner

membrane, and cytosol by IEM and subcellular fractionation.

If the presence of JHE in mitochondria is confirmed, JHE may either be stored in the mitochondria prior to release into the cytosol, or it may function to degrade JH in mitochondria. While JH is known to affect regulation of mitochondrial genes, the impact of JH may be an indirect effect rather than direct regulation of the transcription of mitochondrial genes (Charniaux-cotton, 1965; Stepien *et al.*, 1988; Farkas and Sut'akova, 2001).

As JH affects *Drosophila* mitochondria and the titer of JH appeared to change in flies with hypo- or hyper- expressed P29 we will also examine the mitochondrial morphology of flies with hypo- or hyper- expressed P29 by TEM. In a previous study, swelling of mitochondria was detected in S2 cells following addition of the JH homolog methoprene (Farkaš and Šutáková, 2001). We expect that mitochondria will be enlarged in hypomorphic flies, relative to control treatments. However, the titer of methoprene used to induce swelling of mitochondria was much higher than in vivo titers of JH (Farkaš and Šutáková, 2001).

Although the presence of an intracellular pool of JHE has been noted in the Lepidoptera (Vince and Gilbert, 1977; Mitsui *et al.*, 1979; Wroblewski *et al.*, 1990), the

location, function, and regulation of the intracellular pool of JHE have not been addressed. The function of P29 in relation to JHE may increase our understanding of the biology of intracellular JHE.

A direct JH assay using an antibody to JH will also be conducted for flies with hypo- and hyper- expressed P29 along with appropriate controls, in collaboration with Dr. David Borst of the University of Central Florida (Hunnicutt *et al.*, 1989; Huang *et al.*, 1994). However, while the antibody will detect JH III in the flies, it is estimated that the majority of JH in *D. melanogaster* (perhaps 90%) is JH bis-epoxide, which is not recognized by the JH antiserum (Richard *et al.*, 1989; Alteratz *et al.*, 1991). Hence, large numbers of flies are needed for this experiment for adequate detection of JH III, and liquid chromatography purification of material will be required to eliminate lipids that interfere with the radioimmunoassay analysis. We will also continue work on reversal of the apparent anti-JH effects that result from hyperexpression of P29 (such as the decreased production of aggregation and courtship pheromones), by addition of methoprene to flies.

Based on the lack of impact of hypo- or hyperexpression of CG3776 on the 50 kD protein recognized by the P29 antisera, we do not think that this protein is P29. However, we will continue work on identification of the 50 kD protein to eliminate the possibility

that P29 and JHE interact in the hemolymph.

In conclusion, P29, as a mitochondrial protein and a potential binding protein of JHE, which results in multiple phenotypes when overexpressed, is an important protein in insects. The flies that hypo- and hyper- express P29 will provide valuable tools for elucidation of the function of P29 in mitochondria and the relationship of P29 to JHE.

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APPENDIX 1

Expression of the mitochondrial non-specific esterases α-E1 and cricklet in insect cells

Additional data relevant to Chapter 3.

Introduction

We tested the hypothesis that P29 binds non-specific esterases, including two esterases that are predicted to be targeted to the mitochondria (chapter 3). Toward this end, we constructed stably transformed insect cell lines for expression of α-E1 and cricklet for specific analysis of their interaction with P29. This work was not completed, but is included here for reference.

Methods

Cloning of genes encoding α-E1 and cricklet

α-E1 and cricklet both share 32% identity to DmJHE. The genes encoding α-E1 and cricklet were isolated by RT-PCR from larval mRNA using primers AE1-IEF 5'-CCCCCCCGGGATGGAGATCCGAGTGGGAGT-3' (*Sma* I site underlined), and AE1-IER 5'-CCCCTCTAGACTAAAACAAGTCGTTTTTATCGTATAGTC-3' (*Xba* I site underlined) for α-E1, and Cri-IEF 5'-GGGGATCCATGCTTCTTCGTCCGTGTGGA-3' (*Bam*H I site underlined) and Cri-IER 5'-GGTCTAGATTAAAGTTTAGCTCTCACAGCTTCGAAA-3' (*Xba* I site underlined) for cricklet. The PCR products for α-E1 and cricklet (1718 bp and 1705bp respectively)

were cloned into the expression vector pIE/153A to create pIE.α-E1 and pIE.cricklet. The sequences were confirmed by DNA sequencing. The recombinant constructs were transfected into High Five™ cells using cellfectin (Invitrogen), and the pIE vector alone was transfected as a control treatment. Esterase expression was checked by using an in gel esterase assay.

PAGE and in gel esterase assay

Proteins in the lysate of transfected cells were separated by SDS-PAGE (10%) followed by staining with Coomassie Blue for examination of protein bands. Protiens were also separated by native PAGE and the gel was stained for general esterase activity (Vernick *et al.*, 1988). Electrophoresis was carried out under nondenaturing conditions with a 10% polyacryamide separating gel and 4% stacking gel, both with 0.1% Triton X-100. The separating gel was buffered with 0.375 M Tris-HCl pH 8.8. The stacking gel buffer was 0.13 M Tris-HCl, pH 6.3. Samples were homogenized in 20 µl of sample buffer (0.375 M Tris, 8% sucrose, 1% Triton X-100, 0.1% bromphenol blue dye). Electrophoresis was performed in the following buffer: 0.09 Tris, 0.08 M boric acid, pH 8.2, at 4°C with a constant current of 50-100mA per gel, until the dye front was about 1 cm from the bottom of the gel. Immediately after electrophoresis, esterase bands were visualized. Gels were incubated in fresh substrate solution in the dark at 37°C with rocking for 15 min before addition of fast blue B salt (Sigma) and rocking for another 20-25 min. Gels were then transferred to storage solution (35% methanol, 10% glacial acetic acid) in which they can be kept indefinitely at room temperature.

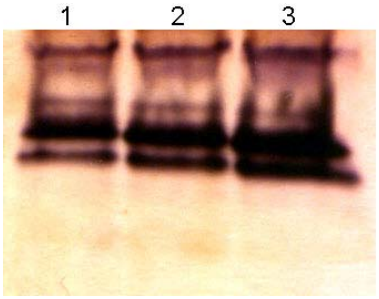
Results

Proteins from cells transfected with pIE.α-E1 and pIE.cricklet were run on an SDS-PAGE gel and stained by Coommassie blue. No additional bands were detected when compared to the control cells transfected with pIE. A native PAGE gel was also run and stained for esterase activity. Because the High Five™ cells contain native esterases of similar size to α-E1 and cricklet, no additional bands were detected for pIE.cricket and pIE.α-E1 when compared with pIE.

Discussion

Esterase activity was not detected in proteins precipitated with the P29 antibody (chapter 3). However, to test whether P29 specifically interacts with α-E1 and cricklet, we produced cell lines that express α-E1 and cricklet. However, because the pIE vector does not contain an epitope tag, and because native esterases were present in the cell line employed, we were unable to determine whether the recombinant esterases were expressed. This work could be continued by addition of an epitope tag such as V5, of hexahistidine, to the pIE vector. Immunoprecipitation assays could then be conducted with recombinant α-E1 or cricklet and P29 to test whether these proteins are able to interact.

Figure 1.
Analysis of cell lines transfected to express cricklet and α-E1. In gel esterase assay of transfected insect cells separated by native gel electrophoresis. Cells transfected with Lane 1, pIE.cricklet; lane 2, pIE.α-E1; lane 3 pIE (negative control).



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APPENDIX 2

Null mutant generation

Additional data relevant to Chapter 4.

This work was conducted by Dr. Narinder Pal.

Introduction

To see the effect of P29 in null mutant, the EP line can be used to generate 'imprecise excisions' by remobilizing the P element, which may result in a complete null mutant. This work was not completed, but is included here for reference.

Materials and methods

Null Mutant Generation Three stocks EP835, the transposase line 3164 and Deficiency line 3157 were used for generation of the null mutant flies. All stocks were purchased from the Szeged *Drosophila* Stock Centre or Bloomington Stock Center, IL. Males from stock EP835 were crossed with females from a second and third chromosome balancer stock (yw/yw; SP/CyOy+; TM2Ubx/TM6SbTbe) and then flies heterozygous for the P element and third chromosome balancer (EP/CyOy+; +/TM2Ubx) were selected and crossed to produce a homozygous EP stock with third chromosome balancers TM2Ubx/TM6SbTbe (Fig 1A).

The transposase stock 3164 with PΔ2-3wc on chromosome 3 was crossed with a second

and third chromosome balancer stock (SP/CyOy+; TM2Ubx/TM6SbTbe) to produce a transposase stock with second and third chromosome balancers (Fig 1B).

The heterozygous deficiency stock 3157 with a deficiency on the right arm of chromosome 2 (regions 60E6; 60F1-2) was crossed with the yellow body, white eye mutant stock with second and third chromosome balancers to produce a yellow body, white eye mutant heterozygous deficiency stock (Fig 1C).

For null mutant generation (Fig 1D), males from the homozygous EP stock with third chromosome balancers were crossed with females from the transposase stock with second chromosome balancers generated as described above. From this cross, variegated eye dysgenic males with curly wing, ultrabithorax and stubble markers were selected and then crossed with the second chromosome balancer (SP/CyOy+) stock. White eye, curly wing potential revertant males were selected in the next generation and each individual male was separately crossed with three heterozygous deficiency yellow body, white eye, curly wing females. Potential null mutant revertant flies with heterozygous deficiency on the right arm of chromosome 2 were then selected and tested for the presence of the P29 allele by PCR and western blot analysis.

PCR Analysis DNA used for PCR was prepared by single fly DNA preparation method. Briefly a single fly was mashed using 50µl of squishing buffer (10mM Tris HCL pH 8.2, 1mM EDTA, 25mM NaCL, 200µg/ml Proteinase K), then incubated at 37° C for 30 min. The sample was then heated to 95° C for 1-2 min to inactivate proteinase K and stored at

4° C. 2µl of the DNA prep was used in a 25µl PCR reaction volume using gene specific primers. The primers used were: P29F-2 (at 225 nt upstream of P element insertion in CG3776 ORF/P29 gene) 5' AACGTAACTCGGGGGTCTG 3'; P29R-2 (at 7 nt upstream of the P element insertion site) 5' GCTGCAACTGGGCTAAAAAG 3'; P29F-1 (at 12 nt upstream of the P element insertion site) 5' AACAACTTTTTAGCCCAGTTGC 3'; P29R-1 (at 198 nt downstream of the P element insertion site in CG3776) 5' GCTCTCTCTGGCATTCTGCT 3'; P29S2-2 (the 3' end of CG3776 coding sequence, Chapter 2). The PCR conditions used were: 95° C for 50 sec., 55° C for 1 min., 72° C for 1 min. or 3 min. (depending on the primer combination being used) for 30 cycles.

Western Blot Analysis A single fly was ground in 30 µl SDS-PAGE buffer, boiled for 5 minutes, cooled on ice, centrifuged briefly and run on 10% SDS-PAGE gel at 120 volts. The proteins were then transferred onto Hybond P PVDF membrane (Amersham Biosciences) and blocked in TBS/0.1%Tween-20/5% dry milk for 2 hours. The membrane was then incubated with 1:1000 dilution of anti-P29 antiserum for one hour followed by washing twice each for 10 min. After incubation with 1:2500 dilution of goat anti-rabbit IgG-HRP for 1 hour, the membrane was washed twice with TBS-Tween and twice with TBS each time for 10 min. Detection was done using detection reagents 1 and 2 from ECL western blotting detection kit (Amersham Biosciences).

Results

A total of 94 white eye revertant males were selected and each of these males were separately crossed with 3 Df(2R)/CyOy+ females. In 42 out of 94 crosses (45%), the revertant males died within a few days without producing any progeny. Movement of the P element in these males might have led to lethality making these males unhealthy and sterile. Further western blot analysis (Figure 2D) showed the absence of P29 protein in these males (11, 3, 28).

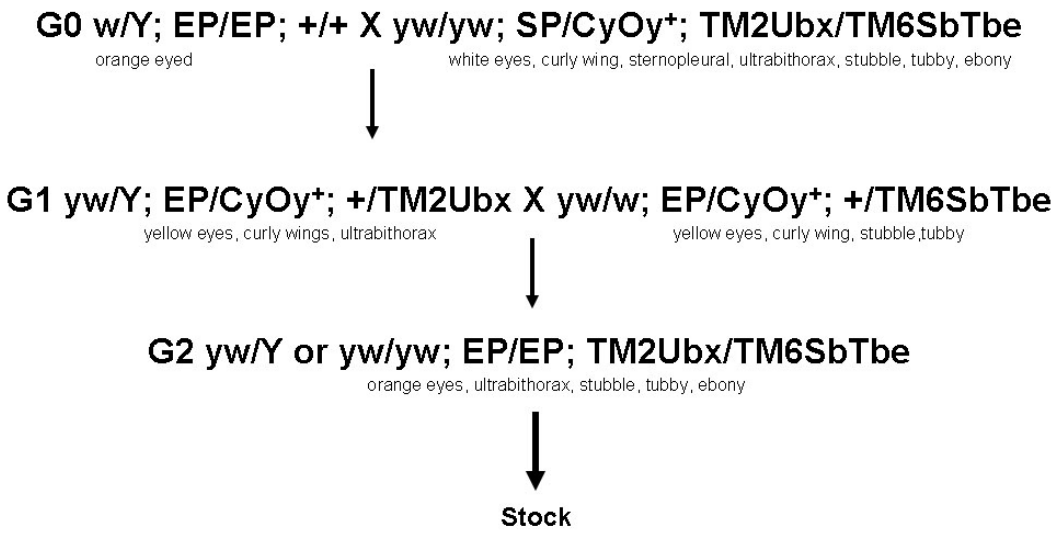
In four (9, 32, 33, 34 in Figure 2D) of the 52 EP(R)XDf(2R) crosses that produced progeny the P29 25kD protein product was much lower as compared to that in Oregon R flies. PCR analysis on these flies using primers P29F-2 and P29R-2 or P29F-2 and P29S2-2 failed to give PCR product suggesting upstream deletion of CG3776 ORF with the movement of P element (Fig 2B, C). In one (26) of the EP(R)XDf(2R) cross, the progeny flies were very small in size and fewer in number compared to other crosses. PCR analysis of these progeny flies failed to produce any amplification product with primers P29F-1 and P29R-1 or P29F-1 and P29S2-2, but produced the right size product with primer Inter P2 (at 479 nt downstream of the ATG start codon of CG3776) suggesting that movement of the P element might have resulted in partial loss of the CG3776 ORF. Western blot analysis on these flies also did not show the presence of the P29 protein.

Discussion

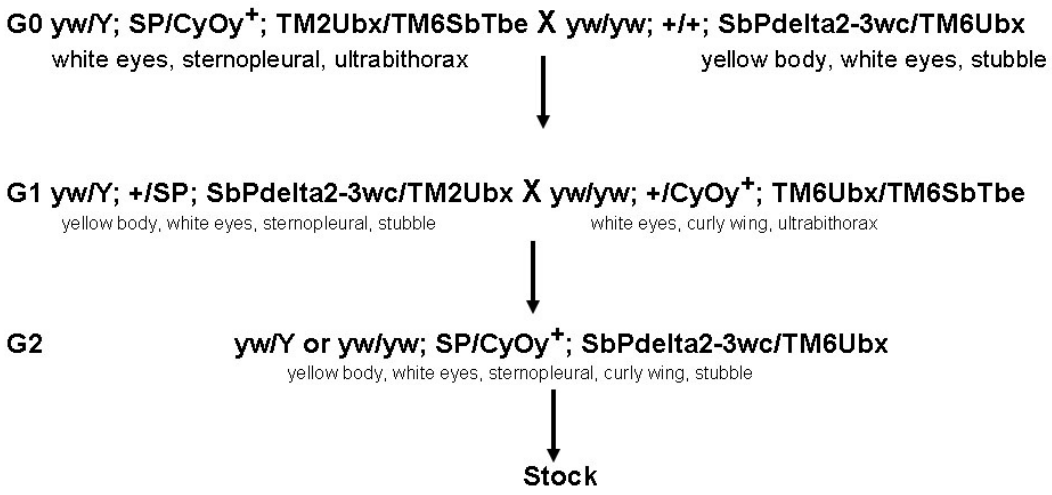
As CG3776 is in a gene-dense region, PCR should be conducted for its neighboring genes when the P element is remobilized. If the P element carried nucleotides more than 264 from its 5' end it will affect expression of RpL19 expression, which is homozygous lethal; if the P element carried nucleotides more than 1127 from its 3' end it will knock out the expression of the gene Phk-3 (pherokine-3) by destroying the stop codon of Phk-3, which also has multiple effects on flies (Fig 2A) (Sabatier *et al.*, 2003). One of the chromosomes of this potential null mutant is from the deficiency mutant, which already has the Phk-3 and RpL19 missing. Because no PCR was conducted to see whether the P element carried the DNA from these two genes we can not draw any conclusion from this potential null mutant. The testing of 33 other lines is ongoing.

Fig 1. Null mutant generation.

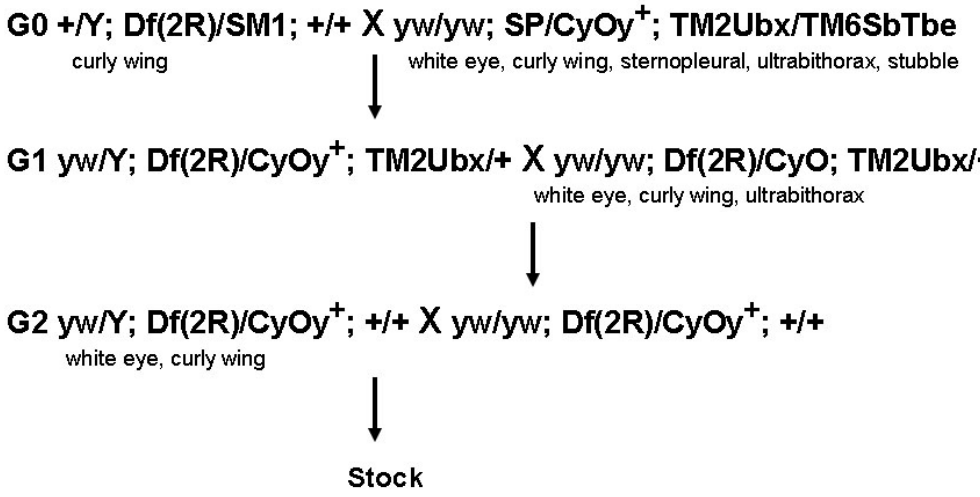
A. Produce EP stock with third chromosome balancers TM2Ubx/TM6SbTbe



B. Produce transposase stock with second and third chromosome balancers



C. Produce deficiency stock with second and third chromosome balancers



D. Produce null mutant.

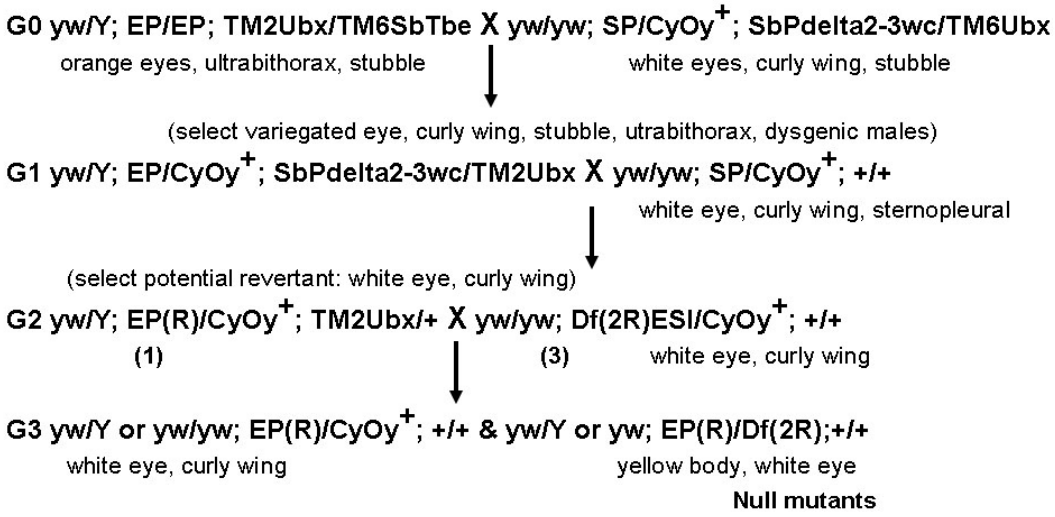
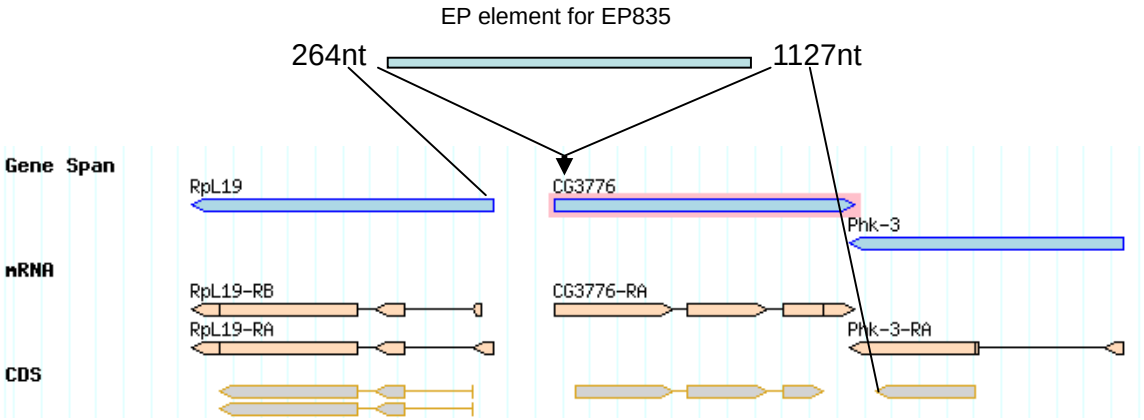
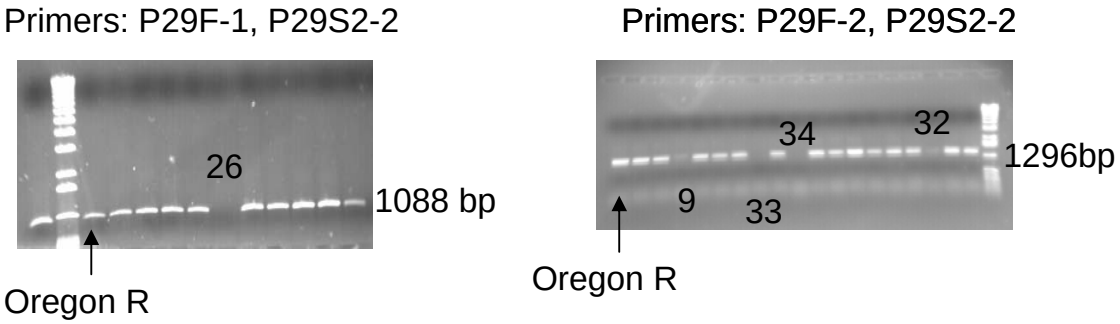


Fig 2. PCR and western blot to test for potential null mutants. A. CG3776 and neighboring genes. B, C, PCR products using different primers. D, Western blot using P29 antibody (see text for details).

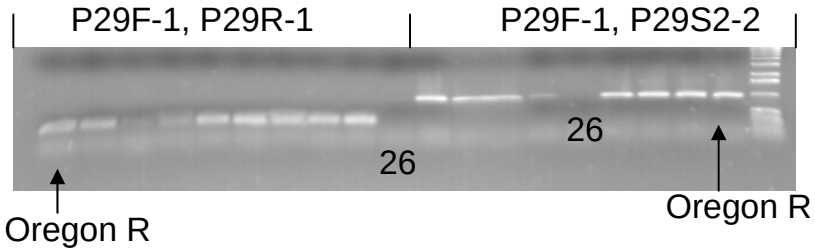
A



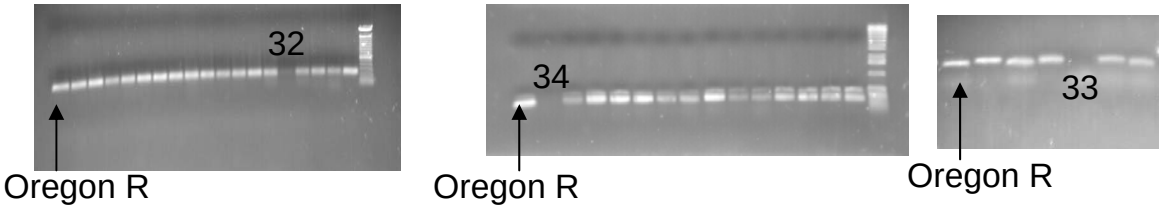
B



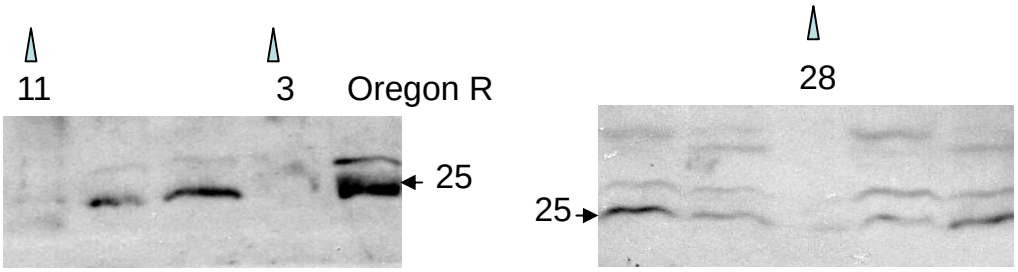
C

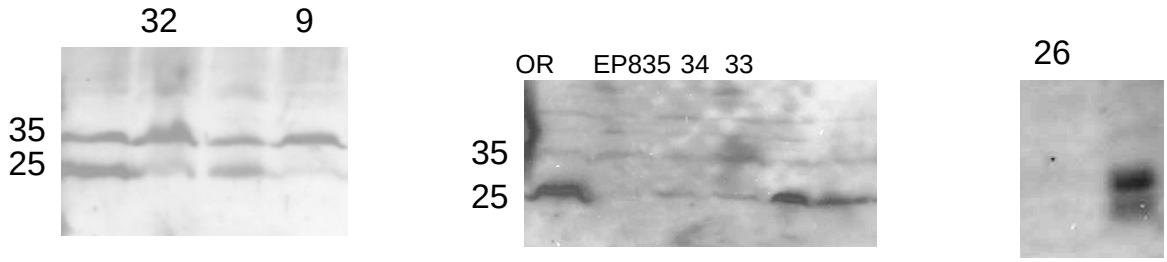


Primers: P29F-2 and P29R-2



D





Reference

Sabatier, L., Jouanguy, E., Dostert, C., Zachary, D., Dimarcq, J.-L., Bulet, P. and Imler, J.-L., 2003. Pherokine-2 and -3: Two *Drosophila* molecules related to pheromone/odor-binding proteins induced by viral and bacterial infections. Eur J Biochem 270(16), 3398-3407.