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Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster*

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Abstract

Mutation of members of the Halloween gene family results in embryonic lethality. We have shown that two of these genes code for enzymes responsible for specific steps in the synthesis of ecdysone, a polyhydroxylated sterol that is the precursor of the major molting hormone of all arthropods, 20-hydroxyecdysone. These two mitochondrial P450 enzymes, coded for by *disembodied* (*dib*) (CYP302A1) and *shadow* (*sad*) (CYP315A1), are the C22 and C2 hydroxylases, respectively, as shown by transfection of the gene into S2 cells and subsequent biochemical analysis. These are the last two enzymes in the ecdysone biosynthetic pathway. A third enzyme, necessary for the critical conversion of ecdysone to 20-hydroxyecdysone, the 20-monooxygenase, is encoded by *shade* (*shd*) (CYP314A1). All three enzymes are mitochondrial although *shade* has motifs suggesting both mitochondrial and microsomal locations. By tagging these enzymes, their subcellular location has been confirmed by confocal microscopy. *Shade* is present in several tissues as expected while *disembodied* and *shadow* are restricted to the ring gland. The paradigm used should allow us to define the enzymes mediating the entire ecdysteroid biosynthetic pathway.

Keywords: Ecdysone; Insect molting; Halloween genes; Prothoracicotropic hormone (PTTH); Fruit fly

1. Introduction

1.1. Flies: history and advantages as an experimental organism

There has been a relationship between humans and flies for at least 5000 years as seen from the depiction of a fly next to a reclining gazelle in a cylinder from Mesopotamia (summarized by Greenberg and Kunich, 2002). During the course of civilization, as human settlements grew and wars ensued, fly populations increased exponentially. The first written reference to blowflies is in a 3600-year-old collection of cuneiform writings using clay tablets from the Hammarabi era. Ten of the recorded 396 names in this animal inventory are flies. The term "fly" is derived from the old English *fleoge*, the middle English *flie*, the Sweedish *fluga*, the Norwegian *flue*, and the German *fliege* (Greenberg and Kunich, 2002). In Babylonian times (about 1700 BC), the fly was revered, and some of the writings suggest that the gods can transform into flies. Among the Phoenicians, a god known as "Lord of Flies" appeared as a fly to ward off fly plagues or send his winged legions as punishment. Thousands of years later this god reappeared as Beelzebub, the first lieutenant of Satan, in the witchcraft of New England (Greenberg and Kunich, 2002). In Greek mythology, Zeus scattered flies and saved the life of Hercules who was almost destroyed by flies. This is part of the myth attributed to the potency of flies by the ancients (and now by *Drosophila* geneticists). The Egyptians wore carved amulets of flies to ward off evil, but the fly also symbolized impudence, persistence, and courage, and a necklace of gold flies was awarded to the bravest of soldiers. In the Egyptian "Book of the Dead" it was noted "that shiny, metallic looking, calliphorid flies found inside houses represent spirits of dead individuals who once lived there. People did not kill these flies for fear of harming an ancestor," and in some areas of rural Egypt this belief is maintained (Greenberg and Kunich, 2002). The ancients therefore believed that flies are both a scourge and are sacred. Now fly maggots are used for wound cleansing and for forensics, the main subject of the excellent book by Greenberg and Kunich (2002).

These superstitions and historic observations are of course not the reasons that the fruit fly, *Drosophila melanogaster*, has become such an important experimental organism in

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classical and molecular genetics. Flies and other insects grow by a series of molts in which a new and larger exoskeleton (cuticle) is synthesized by epidermal cells and the old cuticle is digested away and shed (ecdysis). Basically, the same process occurs in the metamorphosis of the larva (maggot) to the pupa and then to the adult fly. The hormone responsible for eliciting the manifold physiological and biochemical processes in insects, including longevity (Simon et al., 2003), other arthropods and perhaps even some nematodes, has been termed ecdysone, the word derived from ecdysis. Actually, the major molting hormone of insects is 20-hydroxyecdysone although ecdysone appears to have roles of its own in addition to being the immediate precursor of 20-hydroxyecdysone (Gilbert et al., 2002). These polyhydroxylated sterols are derived from cholesterol, a compound that insects cannot synthesize from acetate (Gilbert, 1967). They either obtain it directly from their food, dealkylate certain plant sterols or use a homolog of 20-hydroxyecdysone. Although we have known for some 50 years that ecdysone is a steroid, many researchers (including this writer) have attempted, but failed, to elucidate the biosynthetic scheme for the conversion of cholesterol to ecdysone using classical biochemical and chemical paradigms. It is only recently that striking progress has occurred by using a molecular genetic approach with *Drosophila* as the experimental organism and using the knowledge we have gained from the elucidation of the *Drosophila* genome (fly database). Subsequently, I summarize the work of my lab in collaboration with the laboratories of Michael O'Connor at the University of Minnesota and Chantal Dauphin-Villemant at the Université P. and M. Curie in Paris.

There are obvious advantages to working with a genetic organism such as *D. melanogaster*. This fruit fly has been an experimental organism in the field of genetics for about 100 years and in the last several decades has been a model organism in molecular genetics, and particularly in the fields of developmental biology, neurobiology and recently as a model organism for the study of endocrinological phenomena. Its small size and very short life cycle as well as the presence of polytene chromosomes (giant chromosomes resulting from multiple cycles of DNA replication without cell division) in certain tissues such as the salivary glands, makes the fruit fly highly advantageous for studies in life sciences. In the past it has been used very successfully to understand extremely complex processes by dividing these processes into discrete steps (Rubin, 1988). The polytene chromosomes have been extremely useful in cytogenetics to map genes to specific loci, and these data have been very critical for the studies described herein. As Rubin (1988) points out, under the light microscope one can see bands that correspond to less than 10 kb of the DNA with the average of these bands being about 25 kb. Therefore, cytogenetic analysis in *Drosophila* has a resolving power many orders of magnitude greater than for other animals. Indeed, it was the classic work of Clever and Karlson (1960) who showed that steroid hormones act at the level of the nucleus by observing the puffing pattern (sites of mRNA transcript accumulations) of polytene chromosomes of another dipteran. This indeed was the first demonstration that the genome was the site of action of steroid hormones. Just recently it has been shown that the enzyme involved in DNA repair is also crucial for puff formation (Tulin and Spradling, 2003). In *Drosophila*, one can also introduce genes into the genome by using P transposable elements as vectors, a method established well before the much more laborious techniques developed for mammalian systems. Finally, one can grow thousands of fruit flies very cheaply and the recent elucidation of the sequence of the *Drosophila* genome has facilitated studies in many basic areas of life sciences as I will attempt to demonstrate here.

One might question the use of an insect model for an understanding of steroid hormone action in mammalian systems. It is obvious from the literature that steroid hormones in some cases control processes that are amazingly similar in flies and humans, and in fact the *Drosophila* genome is approximately 70% identical to the human genome. In many cases, these highly homologous genes modulate or control the same or similar physiological processes e.g. eye development (Ranganathan, 2003), but in some they have completely different functions. Perhaps, as in the latter instance, it is not so much the molecule that has changed during evolution but rather the roles played by the same class of molecules (Schneiderman and Gilbert, 1964).

A recent paper by Arbeitman et al. (2002) reported that about one-third of all the *Drosophila* genes undergo changes in expression patterns when the entire time course of development of whole insects was studied. Subsets of these genes were used to analyze tissue-specific gene expression programs and these investigators were able to compare transcriptional programs that form the basis for changes in the life cycle of the insect as well as those that appeared to be gender specific. Thus, they showed with this large scale gene expression data, that one can identify genes expressed in very specific tissues or organs or those involved with very specific physiological or biochemical processes. At least 80 genes that have some relevance to human cancers have homologues in *Drosophila* and there are even some model genes in *Drosophila* for anemias and Alzheimers disease. Indeed, of the approximately 290 human disease genes studied, there were homologous counterparts in about 180 of the *Drosophila* genes (Kling, 2002). With the availability of the *Drosophila* fly database, it has become much easier to study its genetics and biochemistry. As I will show subsequently, if the gene's location on a specific chromosome is known, one can explore the fly database, clone that particular gene, transfect it into a cell line and then determine its function by biochemical analysis.

1.2. Insect steroid metabolism

The major sterol ingested by plant-eating (phytophagous) insects is sitosterol while meat-eating insects obtain

cholesterol directly. It is of course cholesterol that is the ultimate precursor for steroid hormone biosynthesis including ecdysone. The plant-eating insects must dealkylate the sitosterol to cholesterol (Fig. 5), which in addition to its function as a hormone precursor is an important constituent of cell membranes. This inability to synthesize the basic ringed structure of sterols is a major difference between the arthropods and vertebrates and one can only speculate on the evolutionary significance (advantage) of this difference. The fact that insects appeared on this planet about a 1/2 billion years before the first mammal, and have persisted very successfully for this length of time, suggests that the basic mechanism for steroid hormone biosynthesis required exogenous cholesterol and it would be of interest to understand why the synthesis of cholesterol in higher organisms from simple precursors has provided a selective advantage during evolution.

Of interest along these lines is the relationship between 7-dehydrocholesterol and cholesterol. In the mammal 7-dehydrocholesterol is ultimately derived from lanosterol and is the precursor of vitamin D as well as cholesterol itself, the enzyme mediating that reaction being the 7-dehydrocholesterol reductase. In the insect, cholesterol is the precursor of 7-dehydrocholesterol which is the ultimate precursor of ecdysone, and therefore 20-hydroxyecdysone. In humans, a deficiency of 7-dehydrocholesterol results in the phenotype of the Smith-Lemli-Opitz syndrome (SOLS), a classical, autosomal recessive multiple malformation syndrome described almost 40 years ago (see Kelley and Herman, 2001)). The phenotype of this syndrome is diverse, but can lead to death and is presumably the result of the inability to synthesize cholesterol. Most patients are treated with 50–200 mg/kg per day of cholesterol, either in the food or they are given purified cholesterol. In *Drosophila* as well, there is a mutation involving 7-dehydrocholesterol and presumably due to the malfunctioning or non-functioning of the same or similar enzyme. In this case, the insect cannot convert cholesterol to 7-dehydrocholesterol and therefore is unable to make the required amount of molting hormone for development, molting and oogenesis. This *woc* (without children) mutant is a larval lethal (Wismar et al., 2000) but the animals can be rescued to adulthood by the inclusion of 7-dehydrocholesterol, but not cholesterol, in the diet, and the *woc* gene codes for a transcription factor (Warren et al., 2001). The mutant is one of a class of "low ecdysone mutants" that have been found in *Drosophila*, several of which are the topic of this contribution.

2. Prothoracicotropic hormone

Since the rigidity of insects is due to their exoskeleton (cuticle), they can only grow up to a point, at which time further growth is inhibited by this cuticle. They must therefore periodically remove the old cuticle and replace it by a larger version so that growth can continue. The secretion of the new cuticle by epidermal cells, the enzymatic digestion of most of the existing cuticle and ultimately the shedding of this cuticular remnant is what is known as the molting process. This molting process and many other developmental events in the insect are controlled or triggered by the insect molting hormone 20-hydroxyecdysone. The insect brain is an endocrine gland, and indeed work on this endocrine gland 80 years ago was the first to show that the nervous system is a neuroendocrine system in addition to possessing neurological function (see Gilbert et al., 2002). There is an amazing analogy between the regulation of ecdysteroid synthesis in special glands of the insect, the prothoracic glands (the ring gland in *Drosophila* and other flies is a composite gland containing prothoracic gland cells), that depend upon cues from the brain in a manner analogous to the ACTH-adrenal cortex axis in higher organisms. The cue from the brain takes the form of a neuropeptide, prothoracicotropic hormone (PTTH).

PTTH is produced by two large lateral neurosecretory cells in each hemisphere of the insect brain, and in moths such as the model insect *Manduca sexta*, PTTH is stored in neurohemal organs attached to the brain (corpora allata, see Fig. 1). PTTH release into the insect hemolymph (blood) is controlled by photoperiod, circadian factors, as well as the nutritional state of the animal. Once released into the hemolymph, this neuropeptide stimulates the prothoracic glands to synthesize an ecdysteroid which is ultimately converted to 20-hydroxyecdysone. PTTH itself is synthesized as a preprohomone and released as a shorter, glycosylated, homodimeric molecule of between 25 and 30 kDa having a single intermonomeric cysteine–cysteine bond and three intramonomeric cysteine–cysteine bonds (see Gilbert et al., 2002). The amino acid sequences of five lepidopteran PTTHs are known, four being deduced from the sequence of the recombinant form, and show considerable divergence. They are however a family of related proteins but are essentially species specific in their action.

My laboratory has been working on the PTTH transductory cascade in the prothoracic glands of *M. sexta* for several decades, having developed an in vitro assay system for PTTH. To summarize, the interaction of PTTH with a yet uncharacterized receptor at the cell membrane surface of prothoracic gland cells causes an influx of extracellular calcium followed by an increase in cAMP, suggesting that cAMP synthesis requires Ca^{2+}-calmodulin-dependent adenylyl cyclase activity (Fig. 2). This is followed by activation of PKA in the prothoracic gland and a number of rapid protein phosphorylations dependent on PKA, MAPKs, and perhaps some uncharacterized kinases. The multiple phosphorylation of ribosomal S6 is mediated by a 70 kDa S6 kinase. In other systems S6 phosphorylation appears responsible for increased rates of translation initiation, especially of mRNAs possessing a polypyrimidine tract at their 5′ transcriptional start site, and it likely plays a similar role in the prothoracic glands. Our most recent results suggest that PTTH also activates a MAPK pathway resulting in a rapid increase in ERK

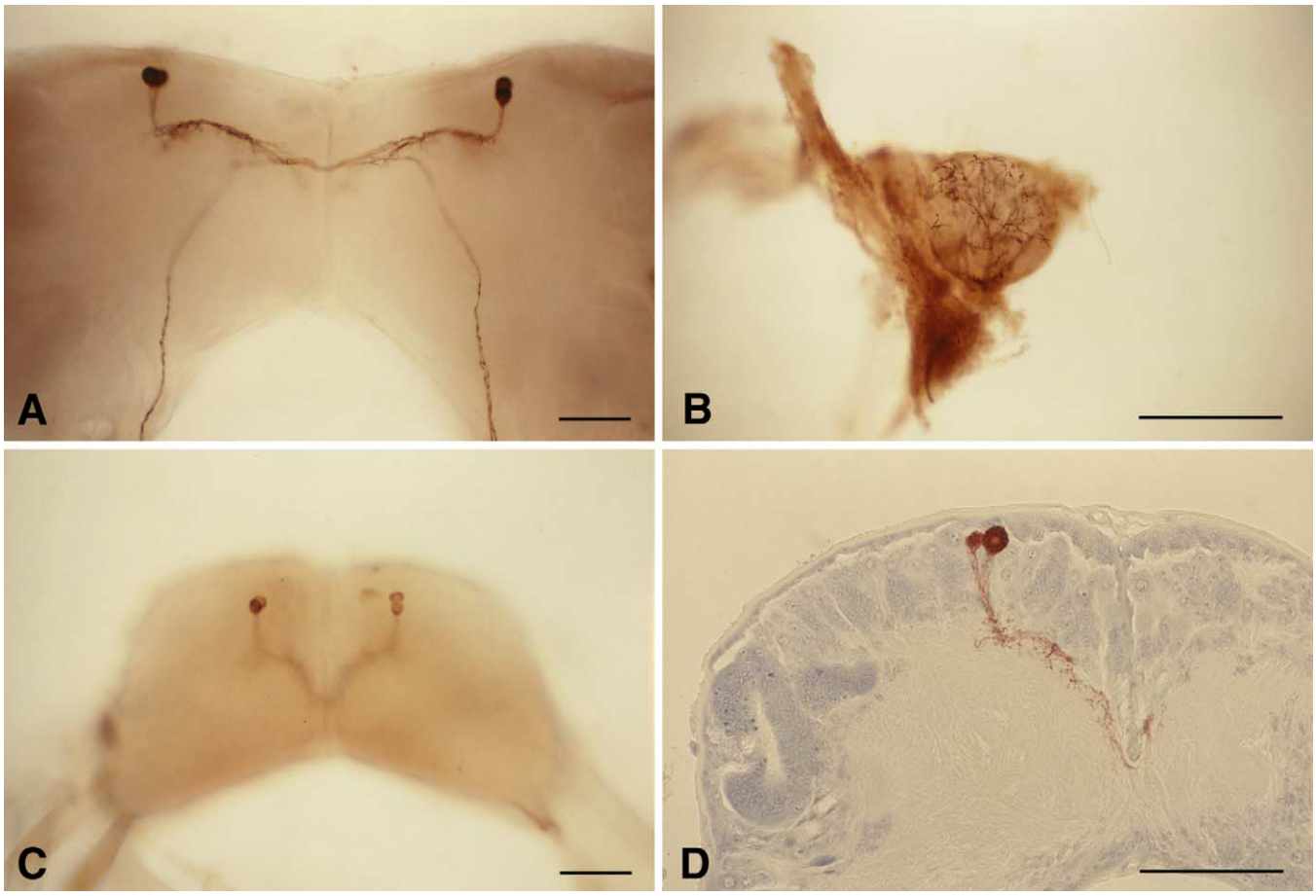

Fig. 1. Immunocytochemical detection of recombinant PTTH in the brain and corpus allatum. (A) Whole mount of the pupal *Manduca* brain showing staining for PTTH in cell bodies and axons of prothoracicotropes. (B) Whole mount of arborized axons in the pupal corpus allatum (PTTH neurohemal organ). (C) Whole mount of cell bodies and axons of prothoracicotropes in the larval brain. (D) Section of cell bodies and dendrites (arborized axons) of prothoracicotropes located at the periphery of the neuropile. Scale bars = 100 μm. (Gilbert et al., 2000).

phosphorylation. The composite data generated by our laboratory (Rybczynski et al., 2001; Rybczynski and Gilbert, 2003) suggest that ecdysteroidogenesis in the prothoracic gland requires the presence of a small basal population of phosphorylated (active) ERK molecules. This suggests that PTTH stimulates not only translation, but also transcription i.e. the stimulation of prothoracic gland MAPK results in effects at the nuclear level. Although the pathway depicted in Fig. 2 summarizes our knowledge of PTTH action in the *Manduca* prothoracic gland, recent studies in our laboratory indicate that the situation is much more complicated with cross-talk between a variety of transductory pathways. For our present purposes, the most relevant question is how the final step in the transductory pathway i.e. the multiple phosphorylation of S6 results in enhanced ecdysteroid biosynthesis by the cells of the prothoracic gland. We could not answer this because until recently we did not have a good sense of the biosynthetic pathway between cholesterol and ecdysone, and therefore could not identify the rate-limiting step. The remainder of this contribution concerns our attempts to identify several genes encoding specific P450 hydroxylases mediating steps in the biosynthesis of the molting hormone.

3. The Halloween genes: ecdysone and 20-hydroxyecdysone biosynthesis

A family of genes in *Drosophila* were identified and mapped, which when mutated resulted in embryonic lethality (Jürgens et al., 1984; Nüsslein-Volhard et al., 1984; Wieschaus et al., 1984). It appeared that at least one member of this Halloween gene family, *disembodied*(*dib*), was a low ecdysone mutant and it was suggested that the gene product was a P450 enzyme (Chávez et al., 2000). This initial study localized *dib* to a single transcription unit defined by a 1.7 kb cDNA isolated from an embryonic *Drosophila* cDNA library. This clone was shown to rescue the mutant phenotype following its insertion into the mutant germ line. The deduced gene product *dib* is a new member of the insect cytochrome P450 superfamily, designated as CYP302a1. In situ analysis revealed the restriction of expression of *dib* to the prothoracic gland cells of the *Drosophila* ring gland.

In addition to *dib*, other Halloween genes that disrupt embryonic development were identified, namely *shadow (sad)*, *shade (shd)*, *spook (spk)*, *and phantom (phm)*. These four other genes were also shown to code for P450 enzymes and

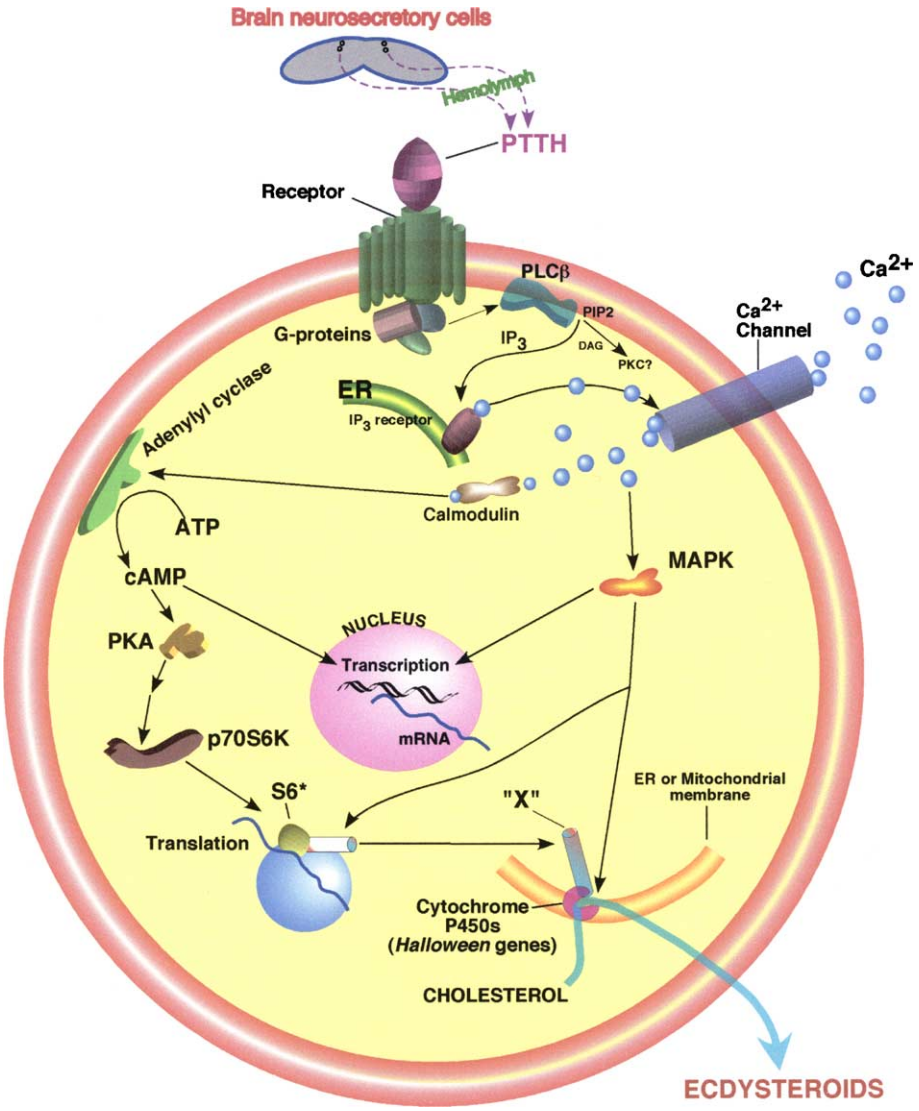

Fig. 2. Transductory cascade of PTTH interaction with a prothoracic gland cell. S6*, multiple phosphorylated form. (Graphics by Robert Rybczynski).

we have now been able to demonstrate the function of *dib*, *sad* (CYP315a1), and *shd* (CYP314a1). Before detailing our paradigm for the functional analysis of these genes, a succinct general summary of the procedure used is required. The three genes were isolated having the crucial knowledge of the location of the genes on the fly's polytene chromosomes from the cytogenetic research in the 1980s cited above. For *dib*, the difficult, laborious method of classical molecular cloning was utilized. For *sad* and *shd* we compared the known genetic map position obtained with the predicted chromosomal locations of the CYP enzymes by using the fly database derived from the *Drosophila* gene project and confirmed it by amplifying the entire cDNA coding sequence by PCR using a novel amplification procedure. Sequences were obtained and point mutations were identified within the putative coding region (Warren et al., 2002).

In order to characterize any complex P450 monooxygenase (hydroxylase), one must demonstrate specific enzymatic activity utilizing known substrates. For the work on the Halloween genes, the cDNA corresponding to the coding region of each gene was ligated into an expression vector and transfected into a *Drosophila* S2 cell line. We utilized similar promoter constructs constitutively expressing the GFP protein as the control transfected cell line. Three days post-transfection, the S2 cells expressing the gene of interest were incubated with radiolabeled intermediates, the radiolabeled products were analyzed, identified by RP-HPLC, TLC and in critical cases, by mass spectrometry, and enzyme kinetics were established.

3.1. Disembodied

The phenotype of the mutant homozygous embryos appears to be normal up to mid-embryonic development (Warren et al., 2002). The embryos subsequently display abnormal developmental characteristics including:

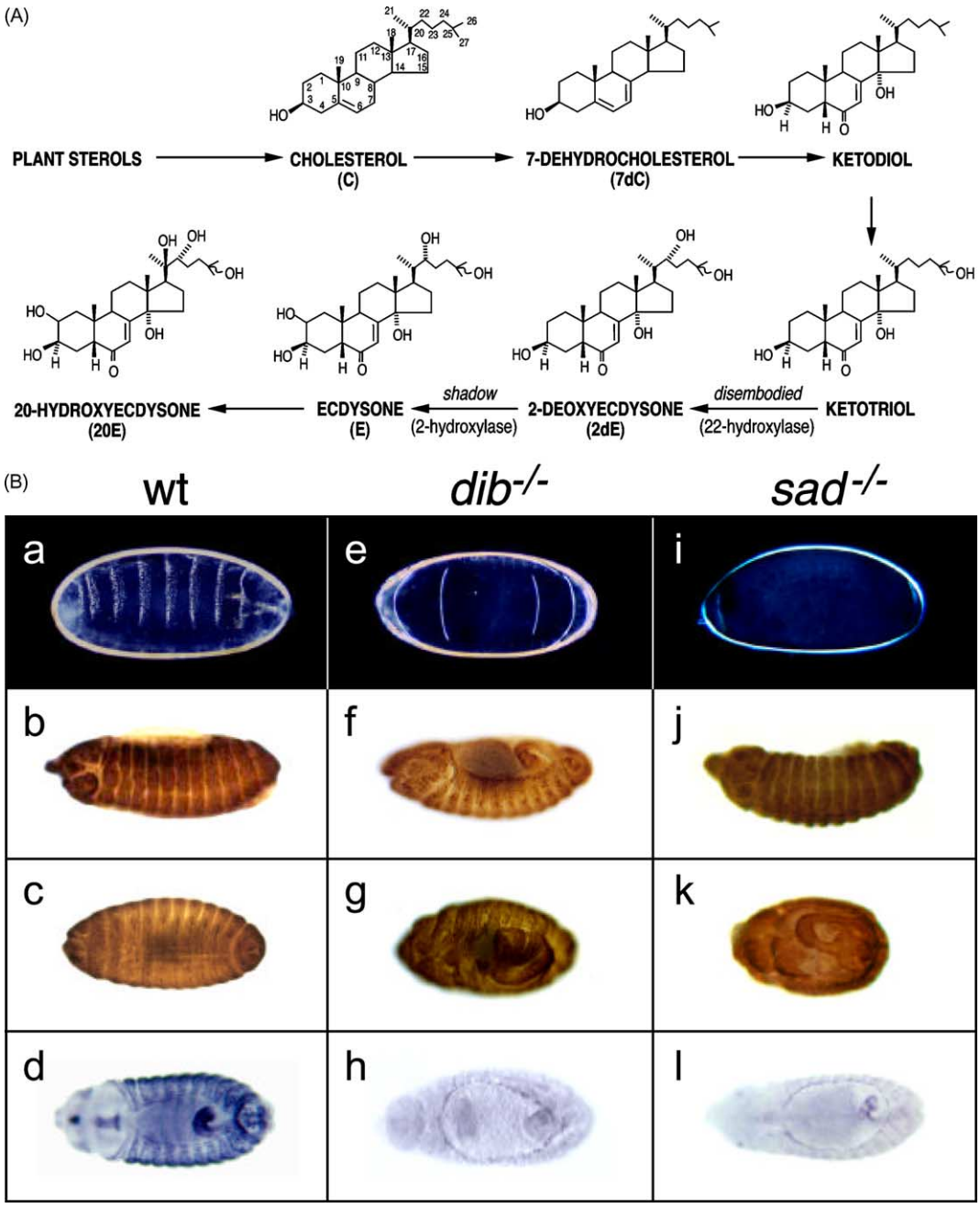

Fig. 3. (A): Abbreviated scheme of ecdysteroidogenesis. (B): Phenotypes of *sad* and *dib* mutant embryos. (a–d) Wild type: (a) cuticle; (b) spectrin staining, stage 14, lateral view; (c) spectrin staining, stage 16, dorsal view; (d) IMP-E1 expression, stage 14, dorsal view. (e–h) dib^{F8}/dib^{P3} embryos: (e) cuticle; (f) spectrin staining, stage 14, lateral view; (g) spectrin staining, stages 15–16, dorsal view; (h) IMP-E1 expression, stage 14, dorsal view. (i–l) sad^{Z804}/sad^{Z309} embryos: (i) cuticle; (j) spectrin staining, stage 14, lateral view; (k) spectrin staining, stage 15–16, dorsal view; (l) IMP-E1 expression, stage 14, dorsal view. IMP-E1 is an ecdysone inducible gene (Warren et al., 2002).

undifferentiated cuticle, a failure of head involution and dorsal closure, a compact appearance, and abnormal looping of the hindgut (Fig. 3). They then die well before the end of embryogenesis. In situ analysis of *dib* revealed its expression in the prothoracic gland cells of the wild type embryonic ring gland, and in the epidermis and epidermal stripes of early embryos in which the ring gland had not yet developed. These observations suggested a role for *dib* in ecdysteroidogenesis, and if that was correct, that the early embryonic epidermis has the ability to synthesize ecdysone. These data along with the original observations of a low ecdysteroid titer in the mutant embryos (Chávez et al., 2000 and confirmed by our laboratory) set the stage for a functional analysis of the *dib* gene.

For studies on *dib*, the substrate utilized was high specific activity [^{3}H]ketotriol (2,22-dideoxyecdysone; Figs. 3

and 5 for structure), a compound which has been hypothesized to be a precursor of ecdysone on the basis of indirect evidence. After incubation of the transfected cells with the [^{3}H]ketotriol, the resulting material was analyzed by RIA, RP-HPLC as well as normal phase TLC, and found to be 2-deoxyecdysone (82.8% conversion in 8 h). These data and the demonstration that when *Manduca* prothoracic gland cell homogenates were incubated with labeled 2-deoxyecdysone the product was [^{3}H] ecdysone, suggested very strongly that *dib* is the ecdysteroid C22-hydroxylase, that the ketotriol is its substrate, and that only the hydroxylation at C2 remained in order to obtain ecdysone (Fig. 3). We then turned our attention to *sad* as the putative gene encoding the C2-hydroxylase.

3.2. Shadow

The phenotype of *sad* (Warren et al., 2002) embryonic mutants is basically the same as that of the *dib* mutants (Fig. 3). The ecdysteroid titer of the homozygous mutant embryos was below the resolving power of the RIA, i.e. less than 2% of the wild type, and in situ analysis was very similar to that for the *dib* mutant embryos i.e. restriction of the expression of *sad* to the prothoracic gland cells of the ring gland and to the epidermis in the early embryo before development of the ring gland. It was also only the prothoracic gland cells of the larval ring gland that expressed *sad*. As in the case of *dib*, all the evidence pointed to *sad* being ultimately involved in ecdysone biosynthesis.

S2 cells transfected with *sad* were then incubated with 2-deoxyecdysone (the product of *dib* enzyme activity) and the incubation medium analyzed as with *dib*. The chromatographic and mass spectrometric data revealed that the sole metabolite of these incubations was ecdysone, indicating that *sad* is the 2-hydroxylase (Fig. 4). This was confirmed by incubation of the transfected cells with labeled ketotriol and demonstrating that the major metabolite was 22-deoxyecdysone. Thus, the final two steps in ecdysone biosynthesis utilized *dib* to mediate the conversion of the ketotriol to 2-deoxyecdysone and *sad* to mediate the conversion of the latter to ecdysone (Figs. 3 and 5). The final proof for this conclusion was the transfection of S2 cells with both *dib* and *sad*, and demonstrating that when incubated with the labeled ketotriol, the major product was ecdysone with lesser amounts of 2-deoxyecdysone and 22-deoxyecdysone. In all of these studies there was no conversion to these physiological products when the S2 cells were transfected with GFP (control) and incubated identically with the substrate of interest. It is of interest that both *dib* and *sad* contain a conserved N-terminal amphipathic region required for mitochondrial import and by using epitope-tagged *dib* and *sad* transiently transfected cells, we were able to show colocalization with a mitochondrial marker by confocal microscopy.

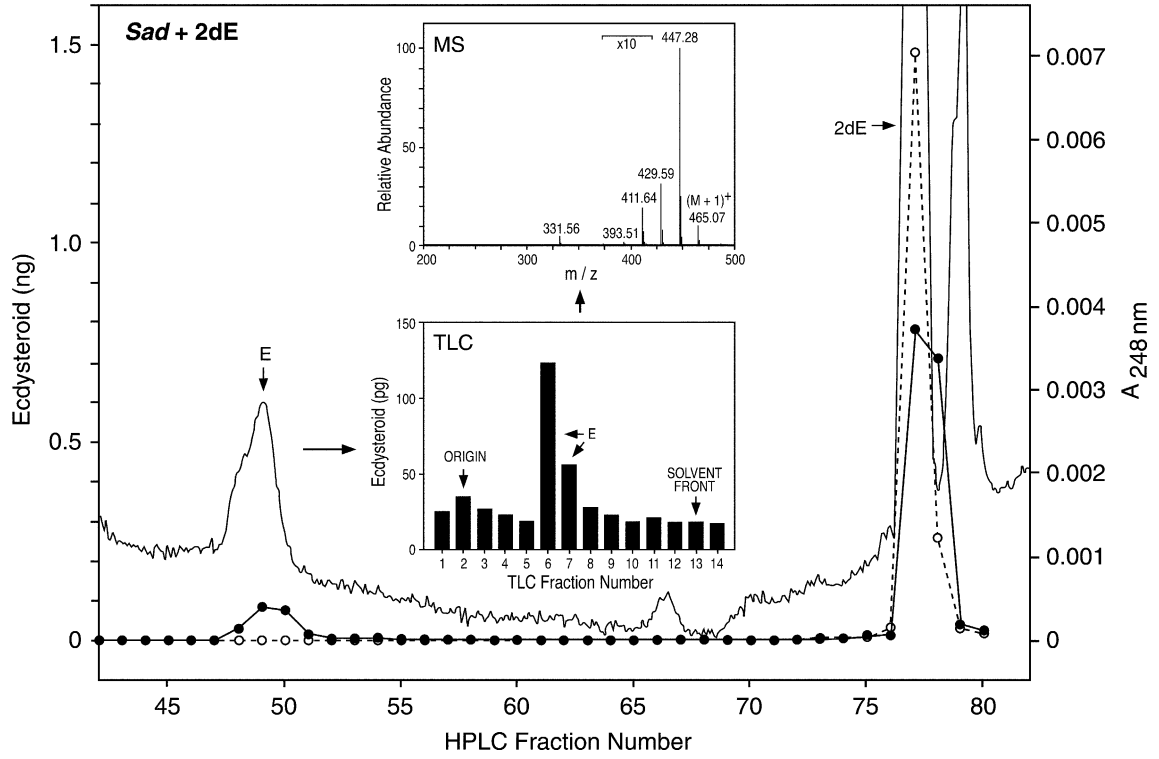

Fig. 4. RP-HPLC/TLC/RIA/MS analysis after *sad*- or GFP-transfected S2 cell incubation (8 h) with the 2-deoxyecdysone (2dE) substrate. Ecdysteroid immunoreactivity was quantified by RIA after incubations with *sad* (filled circles) or GFP (open circles). UV absorption was measured at 248 nm (solid line). (Inset, TLC) TLC/RIA (chloroform/ethanol) of RP-HPLC-purified ecdysone (E) product (Inset, MS) RP-HPLC/ESI-MS of the TLC-purified E product. (Warren et al., 2002).

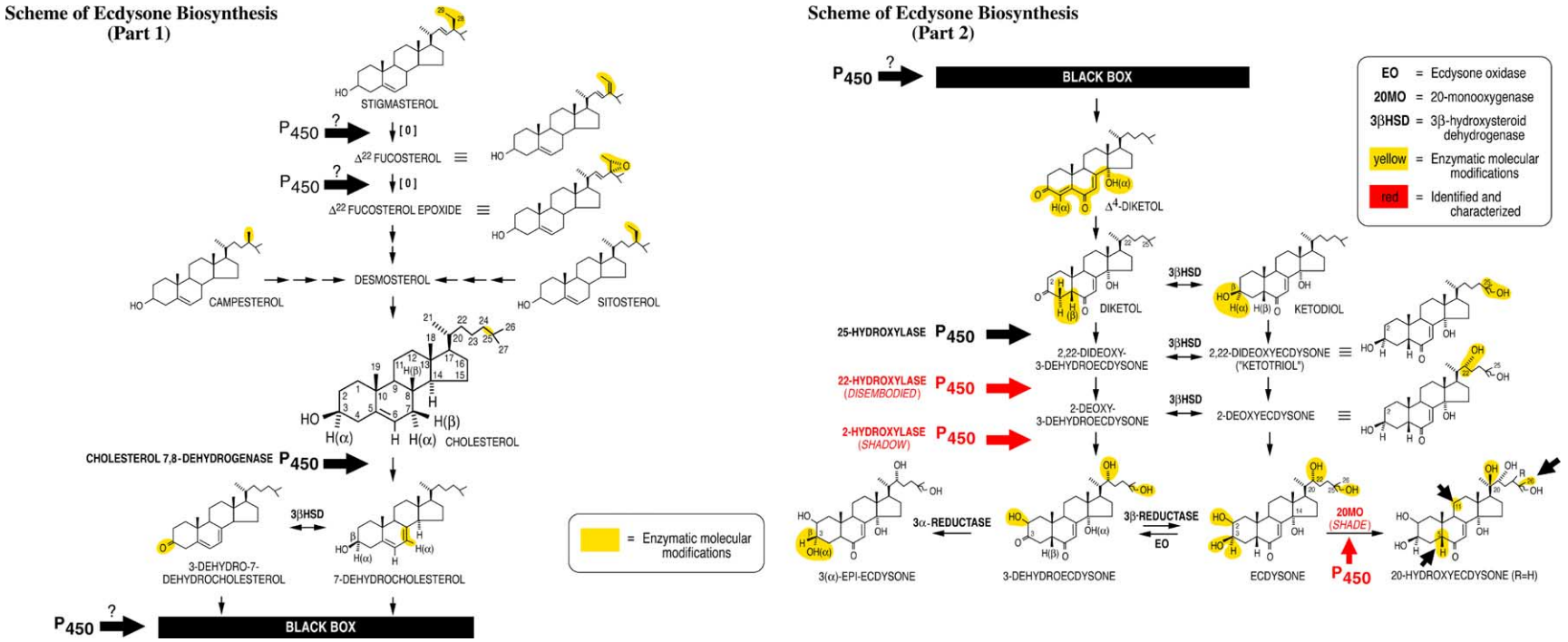

Fig. 5. (Parts 1 and 2). The biosynthesis of 20-hydroxyecdysone from plant sterols. Question marks denote possible involvement of P450 enzymes. Note specifically where the Halloween gene products act (red). 3-Dehydroecdysone is synthesized in the prothoracic glands of many insects (e.g. *Manduca sexta)* and converted to ecdysone in the hemolymph (left column of part 2). For *Drosophila*, ecdysone is synthesized in the prothoracic gland cells of the ring gland (right column of part 2).

We are currently using the above described paradigms to elucidate earlier steps in the biosynthetic pathway (e.g. C25 hydroxylation), but were also most interested in the final step in molting hormone biosynthesis i.e. the conversion of ecdysone to 20-hydroxyecdysone. That leads to the third Halloween gene whose function we have elucidated, *shade* (Petryk et al., 2003).

3.3. Shade

The final reaction in molting hormone biosynthesis, and thus of critical importance to the insect, is the 20-monooxygenation of ecdysone yielding 20-hydroxyecdysone. Although there have been many publications on crude 20-monooxygenase activity in various insect tissues and in subcellular fractions of particular tissues, the enzyme has never been purified or cloned despite several gallant efforts. The major insects that have been studied are the tobacco hornworm, *Manduca* (Bollenbacher et al., 1977) and the locust, *Locusta* (Feyereisen and Durst, 1978). For the most part 20-monooxygenase activity has been associated with the mitochondrial or microsomal fractions of tissues such as the fat body (the insect liver), Malphigian tubules (excretory organ), midgut, and several others, but not in the prothoracic glands, nerve cord, or muscle (Bollenbacher et al., 1976). For *Drosophila*, most of the research has been conducted by Mitchell and Smith (1986, 1988) who have demonstrated changing activity during both embryonic and post-embryonic life. Insofar as a role for the ecdysone 20-hydroxylase in embryogenesis, the data are scarce. The most cogent data on embryos except for our studies on embryonic lethals in *Drosophila*, come from research on the commercial silkworm, *Bombyx mori*, an insect that undergoes embryonic diapause (arrested development) under some environmental conditions. For embryonic development to be completed, 20-hydroxyecdysone is required continuously (Makka et al., 2002). Indeed, it has been suggested that the conversion of ecdysone to 20-hydroxyecdysone is rate-limiting in this developmental system (Sonobe et al., 1999).

Our data on the Halloween gene mutants led us to *shade* and using the same experimental paradigm used for *sad* we determined it to be CYP314a1 (Petryk et al., 2003). The mutant proved to be an embryonic lethal with morphological defects similar to those observed for *dib* and *sad*. This convinced us that it too was a gene implicated in ecdysteroid biosynthesis.

In situ hybridization studies showed that *shd* was not expressed during very early embryogenesis, unlike *dib* and *sad*, but later (5 h), expression was noted in the epidermis as with *sad*. With late third stage larvae, *shd* was expressed in the gut, Malpighian tubules and fat body (unlike the expression patterns of *dib* and *sad*), but not in the brain, ring gland or ventral ganglion (nerve cord). This tissue distribution of activity was identical to that found for ecdysone 20-monooxygenase activity in a variety of other insects including flies as noted above. This was our first direct evidence that *shd* codes for the 20-monooxygenase (hydroxylase), and was supported by the observations that the expression of *shd* lags that of *dib* and *sad* as might be expected if the product of those genes was the substrate for Shd i.e. ecdysone to 20-hydroxyecdysone and if E also had endocrine roles of its own as past work suggests. In addition, the *shd* mutant embryos appeared to have normal amounts of ecdysone but no 20-hydroxyecdysone.

Using the paradigm discussed previously, *shd* was transfected into S2 cells and the cells homogenized into media containing high specific activity [^{3}H] ecdysone as well as unlabeled ecdysone, incubated for 8 h and analyzed as described previously. The data were unequivocal, showing the conversion of ecdysone to 20-hydroxyecdysone (identified finally by mass spectrometry) while the GFP transfected cells (control) showed no such ability to mediate this hydroxylation step. These data, therefore, demonstrated that Shd is the ecdysone 20-monooxygenase. This was confirmed biologically by showing that wild type embryos were capable of mediating the hydroxylation of ecdysone to 20-hydroxyecdysone but similar stage *shd* mutant embryos were not so endowed and were therefore “low 20-hydroxyecdysone” mutants. Finally, genetic studies revealed rescue to the adult when the *shd* gene was inserted into the mutant.

As noted previously, the *dib* and *sad* enzymes are localized in the mitochondria of transfected S2 cells, as shown by confocal microscopic analysis of epitope-tagged versions of these proteins and using markers specific for mitochondrial and endoplasmic reticulum localization. The fact that Dib and Sad had the charged residues and other conserved motifs characteristic of mitochondrial localization signals at the N-terminus confirmed their localization in the mitochondria. Shd on the other hand has a hydrophobic signal type sequence typical of localization in the endoplasmic reticulum as well as a charged segment that typifies mitochondrial localization. Further, confocal analysis revealed mitochondrial localization. This “hybrid” P450 then may reside in either organelle depending on the organism, tissue, developmental state etc. perhaps as a result of differences in post-translational processing. The existence of 20-monooxygenase activity in insect mitochondrial and microsomal preparations is well established, and the present data may explain some of those past observations.

4. Summary

An examination of the fly data base for the cytochrome P450 gene superfamily in *Drosophila* by Tijet et al. (2001) revealed the presence of 83 functional genes coding for P450 enzymes, only eight of which appear to be mitochondrial based on their deduced protein sequences. Indeed, by 2001 only one *Drosophila* P450 had been characterized as to function. Our data from the functional analysis of three genes whose products are involved in the synthesis of the insect molting hormone has quadrupled that number. Further,

we have demonstrated that three of the eight mitochondrial P450s of the fruit fly are involved in steroid hormone synthesis. Fig. 5 summarizes our knowledge of ecdysteroidogenesis and denotes the reactions mediated by the products of the Halloween genes. Using the experimental paradigm described here we are confident that all the genes involved in this biosynthetic pathway will be identified in the next several years.

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