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Pesticide Biochemistry and Physiology 84 (2006) 236–242
PESTICIDE
Biochemistry & Physiology
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Deletion of *Cyp6d4* does not alter toxicity of insecticides to *Drosophila melanogaster*

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Received 29 April 2005; accepted 26 July 2005
Available online 26 October 2005

Abstract

Cytochrome P450-dependent monooxygenases are important in the activation and detoxification of numerous insecticides. In this study, a *Drosophila melanogaster Cyp6d4* null mutant was used to determine the role of this P450 in insecticide metabolism. This null mutant was generated by imprecise excision of a mobile *P* element located upstream to the P450 gene *Cyp6d4*. Comparative analysis between the non-functional mutant and relevant control strains shows that *Cyp6d4* does not appear to be involved in the metabolism of chlorfenapyr, cypermethrin, diazinon, imidacloprid, malathion, oxamyl, parathion, or pyrethrum extract, even though these insecticides are known to be activated or detoxified by P450-monooxygenases. No obvious abnormalities in development were seen in the *Cyp6d4* null mutant, indicating that *Cyp6d4* is not critical for the metabolism of vital endogenous substrates.

Keywords: Cytochrome P450 monooxygenases; Insecticide metabolism; *P* element; *Cyp6d4* null mutant; Insecta

1. Introduction

The cytochrome P450-dependent monooxygenases (monooxygenases) are a vital biochemical system because they metabolize xenobiotics such as pesticides, drugs and plant toxins, and because they regulate the titers of endogenous compounds such as hormones, fatty acids and steroids. Cytochrome P450 (P450) is a hemoprotein, which acts as the terminal oxidase in monooxygenase systems. There are multiple P450s in eukaryotic species, which vary dramatically in overall amino acid sequence between species. Based on available genome sequences [1] there are 90 P450 genes (including seven apparent pseudogenes) in *Drosophila melanogaster* [2] and 111 (including five pseudogenes) in *Anopheles gambiae* [3]. Monooxygenases are remarkable in that they can oxidize diverse substrates and are capable of catalyzing a bewildering array of reactions [4–6]. The breadth

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doi:10.1016/j.pestbp.2005.07.008

of substrates metabolized by monooxygenases is a function of both the large number of isoforms present and the broad substrate specificity of some isoforms. For example, CYP1A1 can metabolize more than 20 substrates. In contrast, CYP7A1 has only one known substrate [6]. Certain P450s have overlapping substrate specificity (e.g., CYP2C subfamily in humans) [6] so that a single compound may be metabolized by multiple P450s. In addition, some P450s can produce multiple metabolites. These features are further complicated by the fact that the change of a single amino acid in a P450 can alter its substrate specificity [7].

Drosophila melanogaster P450 genes are named *Cyp* (for cytochrome P450), followed by a number, a letter and a number indicating the family, subfamily, and isoform, respectively [8]. This nomenclature is based largely on overall amino acid sequence similarity, where P450 families are more than 40% identical [8]. In *D. melanogaster*, there are 23 P450s (and two pseudogenes) in family 6 [9]. Previous work has indicated that P450s belonging to the CYP6 family are capable of metabolizing several insecticides such as cypermethrin (CYP6B8, CYP6D1); [10,11], deltamethrin (CYP6D1)[12], diazinon (Cyp6a2, CYP6B8, CYP6D1); [10,13,14], aldrin (CYP6A1, Cyp6a2, Cyp6a8, CYP6B8); [10,14–16], and heptachlor (CYP6A1, Cyp6a2)[14,15].

Insect P450s are important toxicologically because they are involved in the detoxification (i.e., limit the toxicity) and bioactivation (i.e., produce more toxic metabolites) of insecticides. However, the large number of P450s present in any given species of insect makes it a formidable task to identify the isoform or combination of P450s responsible for the metabolism of a specific insecticide. The generation of null mutant lines of insects has potential as a powerful method for demonstrating the functional role of specific P450 genes.

Cyp6d4 is on chromosome 3R [2], and it is expressed in eggs, larvae, pupae, and both male and female adults [17]. Here, we have created a *Cyp6d4* null mutant to investigate its in vivo role in insecticide metabolism. This mutant was created by imprecise excision of a mobile *P* element inserted approximately 20 bp upstream of the *Cyp6d4* start codon. Animals homozygous for this mutation were tested, together with appropriate controls, for their susceptibility to eight insecticides that are known to be metabolized by insect P450 monooxygenases.

2. Materials and methods

2.1. Drosophila strains

A strain (HA-1829) containing a single *P* element inserted within the C*yp6d4* gene was isolated during a large-scale gene disruption project conducted in the Department of Genetics, University of Cambridge, UK (http://131.111.146.35/~pseq/index.html). This *P* element is non-autonomous and is marked with the recessive eye color gene *mini-white*. HA-1829 is homozygous viable and fertile. The *P* element is inserted ca. 20 bp upstream of the first ATG (start codon) of C*yp6d4*. The w^{1118} strain was obtained from the *Drosophila* stock center (Bloomington, IN). All flies were raised at 25 °C on standard cornmeal–agar media under a 12 h light/dark regimen.

2.2. Genetic screen for excisions in HA-1829

Excisions of HA-1829 were produced by introducing genetically a source of *P* element transposase then screening for loss of the eye color marker *mini-white* present on HA-1829, using standard techniques [18]. Male flies in which the *P* element had excised (i.e., had lost this marker) were individually crossed to *w*; *TM3*, *Ser*/*Sb* virgin females and used to set up balanced lines. The *w*; *TM3*, *Ser*/*Sb* line carries the third chromosome balancer, TM3, marked with the wing mutation *Serrate* (*Ser*), and a third chromosome bearing the dominant marker *Stubble* (*Sb*). It was obtained from the Bloomington *Drosophila* stock center (Bloomington, IN). Flies homozygous for each excision were then screened by PCR to identify lines in which precise and imprecise excision had occurred.

2.3. PCR screen for excisions of HA-1829

DNA preparations were either a crude extract made from single flies [19] or were prepared from 10 flies using the protocol developed by J. Rehm,

Berkeley *Drosophila* Genome project (www.fruitfly.org). PCR reactions were carried out using the forward primer "HAS2" (5′-GATTCCA TTTCAATTTCGAACC-3′) and reverse primer "HAA4" (5′-CATCCAAGCCAAAGATAGTC G-3′). These primers amplify a ca. 1.2 kb product from DNA of wildtype flies. PCR reactions (20 ul) were carried out using 0.4 U GoTaq (Promega; 2.75 mM $MgCl_2$) and 1 ul of DNA (equivalent to the DNA in approximately 1/5 to 1/10 of a fly). The following PCR program was used with a Hybaid Omn-E thermal cycler: 94 °C, 3 min, for 1 cycle; (94 °C, 45 s; 55 °C, 1.5 min; 72 °C, 4.5 min) for 40 cycles; 72 °C, 5 min, for 1 cycle. PCR products were separated by agarose gel electrophoresis and visualized using ethidium bromide staining and UV fluorescence. When needed, PCR products were purified using Wizard DNA purification system (Promega) following the manufacturer's instructions, and sequenced by the Cornell University BioResource Center.

Presence or absence of the *Cyp6d4* transcript was evaluated by RT-PCR. The QuickPrep *micro* mRNA purification kit (Amersham Biosciences, Piscataway, NJ) was used according to the manufacturer's instructions with 10 adult male *Drosophila*. The first-strand cDNA was synthesized using reverse transcriptase SuperScript III (Invitrogen, Carlsbad CA) following the manufacturer's instructions. The first-strand cDNA synthesis reaction was used to amplify *Cyp6d4* (forward primer 5′-tattggccgtaacgctattga-3′, reverse primer 5′-agtcgaggctataatgtcaatg-3′) using PCR ReddyMix Master Mix (ABgene, Rochester, NY). Thermal cycler conditions were: 95 °C for 3 min, for one cycle; (95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min) for 35 cycles; and 72 °C for 10 min, for one cycle. PCR products were detected by running on a 1% agarose gel with 1× TAE buffer.

2.4. Chemicals

Eight insecticides that can be metabolized by insect P450s were used in this study. Chlorfenapyr (99.7% purity) was from American Cyanamid (Princeton, NJ). Cypermethrin was from Roussel Uclaf (Paris, France). Diazinon (87%) was from Ciba-Geigy Corporation (Greensboro, NC). Imidacloprid (97.4%) was from Miles (Kansas City, MO). Malathion (99.2%), oxamyl (99%), and parathion (99.3%) were from Chem Service (West Chester, PA). Pyrethrum extract (51.3%) was from McLaughlin Gormley King Corporation (Minneapolis, MN). Four of these insecticides are activated and detoxified by P450 monooxygenases: chlorfenapyr [20], malathion, parathion, and diazinon [21]. Four of the insecticides are detoxified by P450 monooxygenases: cypermethrin, pyrethrum extract [22], oxamyl [23], and imidacloprid [24].

2.5. Bioassay

Bioassays were carried out by residual contact application. Test insecticides were dissolved in acetone solution and 0.5 ml of the dilution was applied evenly to the inside of a scintillation vial (Wheaton Scientific, Millville, NJ) of 38.6 cm^2. The applied dilutions were allowed to evaporate under a fume hood for at least 30 min before flies were placed inside. A piece of cotton lightly dipped in 20% sugar water and covered with white nylon tulle was placed in the opening of the scintillation vial. Treated vials containing flies were laid on their side and held at 23 °C with a photoperiod of 12 h:12 h (L:D). At least three replicates, each consisting of 20 flies (males and females mixed) per concentration and at least three concentrations (giving greater than 0% and less than 100% kill) of each insecticide were tested. Mortality was assessed 24 h after treatment. Flies were considered dead if they were ataxic. Bioassay data were pooled and analyzed by standard probit analysis [25], as adapted to personal computer use [26] using Abbott's correction [27] for control mortality. LC_{50} values were judged as significantly different if the 95% confidence intervals did not overlap. Chlorfenapyr data could not be analyzed by probit analysis (points did not fit a line) and LC_{50} values were approximated using logarithmic regression.

3. Results and discussion

A total of 305 HA-1829 excision lines were screened by PCR using primers HAS2 and HAA4 which flank the *P*-element insertion site. A single line (called here HAexc#25) was isolated which

produced a PCR product that was noticeably shorter than that of wildtype DNA, suggesting that a portion of the intervening genomic DNA had been deleted. Direct sequencing of the resulting PCR product (Fig. 1) showed that this line carried a 200 bp deletion that includes the predicted TATA box, the first 16 nucleotides (including the start codon) of the *Cyp6d4* coding region, and thus most likely inactivates the C*yp6d4* gene. In addition, several apparently precise excisions were isolated, one of which (called here HAexc#133) was determined to be a precise excision by direct sequencing (Fig. 1). We used RT-PCR to confirm that the excision present in HAexc#25 had inactivated the *Cyp6d4* gene. Fig. 2 shows that no Cyp6d4 was detectable in this strain. By contrast, both excision #133 and the wildtype control produced readily detectable levels of transcript. HAexc#25 and HAexc#133 are homozygous viable and fertile. HAexc#133 carries a wildtype allele of *Cyp6d4* (Fig. 1), has a genetic background most similar (i.e., isogenic) to that of HAexc#25, and was used as one of the control strains for the bioassays. The other control carried the *white* mutant allele w^{1118}, but is otherwise wildtype.

Cyp6d4 (FlyBase ID FBgn0039006) is a cytochrome P450 found on chromosome 3R in *D. melanogaster* [2]. It is expressed throughout the life cycle of *D. melanogaster* and in both adult males and females [17,28]. Certain P450s are important in the metabolism of endogenous compounds, such as ecdysone, the molting hormone [29]. Mutations in these genes lead to severe disruption in development [30]. No gross abnormalities in development were seen in HAexc#25, indicating *Cyp6d4* is not critical for the metabolism of vital endogenous substrates.

The role of *Cyp6d4* in the metabolism of insecticides was evaluated by comparing the toxicity of insecticides to the w^{1118} (non-manipulated) and HAexc#133 (precise excision) strains relative to the HAexc#25 (*Cyp6d4* null allele) strain. HAexc#25 did not differ in susceptibility from both the w^{1118} and HAexc#133 strains for any insecticide (Table 1). LC_{50} values for imidacloprid, malathion, parathion, and pyrethrum

gattccatttcaatttcgaaccctggtccgagaagcaacagccccaggatcttcatagtg
ccggcataagcaggggatccacttcggtgcgagtaatgtccaaggcaaatcatcaagccc
acaacgtaaccctacatattgataaaggaatctagttagaaacaagattcaattttgatt
aggaaggatttaatattaaatattataaaaaacatatacttctaaaccggttccttctat
tttaaaatagagttgctatgctaaagttagttaactaaacagcagcccatcataaatcaa
caataagtcaacgaattctaagattataaatcaacttttaagtattttgtatataataat
tgttagcttataacataagagctttgttttgcatttcaccattaatataaagctgtcatc
aagactatcaatgta[gctttgttcatttagaattcttcgctgtaaagaattaagagaac
gttactgccaaagagaacaaataatcgacgaatttcagcgcatataagtggaggctctcc
agtttgatagtcagttatatattgactttcgcagagaaagggtggatttaaaaaaaagaa
aaggtgcactacttcgact<u>ATG</u>TTTTCGCTGATTT]***TATTGGCCGTAACGCTATTGA***CTT
TGGCGTGGTTCTATCTGAAGCGCCACTATGAGTACTGGGAGCGACGCGGATTTCCATTTG
AAAAACACTCCGGGATTCCATTCGGTTGCTTGGACAGTGTGTGGCGGCAGGAGAAGAGCA
TGGGCTTGGCCATCTACGATGTGTATGTGAAGAGTAAAGAGCGCGTCTTGGGCATTTATT
TGCTCTTCCGTCCGGCTGTTTTGATCAGAGACGCCGATCTGGCTCGCCGTGTTCTGGCCC
AGGATTTCGCCAGTTTCCACGATCGCGGCGTTTACGTTGATGAGGAACGGGATCCCCTGT
CGGCCAATATCTTCTCGCTTCGCGGTCAGAGCTGGCGATCGATGAGGCACATGTTGTCGC
CATGTTTCACATCCGGAAAGTTGAAGAGCATGTTCAGCACATCCGAGGATATTGGTGACA
AGATGGTGGCCCATCTGCAAAAGGAGCTGCCGGAGGAGGGCTTCAAGGAGGTGGACATAA
AGAAAGTGATGCAAAACTATGC*CATTGACATTATAGCCT*<u>*CGACTA*</u>TCTTTGGCTTGGATG

Fig. 1. Partial sequence of *Cyp6d4* from the w^{1118}, HAexc #133, and HAexc #25 strains. The 5′ UTR is in lower case and the coding region is in capitals. The start codon is underlined and the positions of the HAS2 and HAA4 primers are double underlined. The position of the primers used to detect Cyp6d4 transcript in Fig. 2 are shown in *italics*. The DNA deleted in HAexc#25 [in brackets] is indicated by gray text.

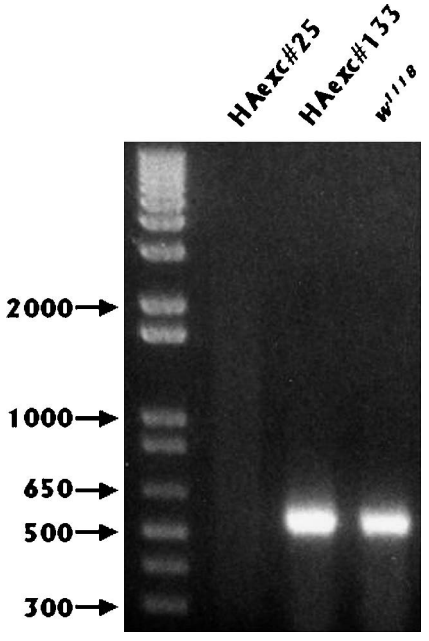

Fig. 2. RT-PCR from two strains having *Cyp6d4* (w^{1118} and HAexc #133) and a strain in which *Cyp6d4* had been deleted (HAexc #25).

extract were not significantly different between strains. For cypermethrin, the HAexc#133 strain was 10-fold more sensitive than the w^{1118} strain, while the HAexc#25 had an LC_{50} between these two strains. For diazinon, HAexc#133 was 1.6-fold less susceptible than the other two strains. The w^{1118} strain was 2.1-fold more susceptible to oxamyl compared to the other two strains. A similar pattern was observed for chlorfenapyr, however, probit analysis could not be used to obtain reliable LC_{50} values since there was a plateau in the mortality curves (Fig. 3). Therefore, we compared strains using 31 replicates (20 adults per replicate) at a concentration of 52 ng/cm^2. The mean mortalities were 48.0, 62.2, and 69.3 % for the HAexc#133, HAexc#25 and w^{1118} strains, respectively. These values were all significantly different from each other ($P < 0.05$, Student's two-tailed *t* test [31]). However, given that the LC_{50} value for the HAexc#25 strain was intermediate to the other two strains, the presence or absence of *Cyp6d4* is not a factor that determines susceptibility to chlorfenapyr. Thus, our results indicate that, at least in wildtype *D. melanogaster*, *Cyp6d4*

Table 1
Toxicity of eight insecticides to strains of *D. melanogaster* which have (w^{1118} and HAexc#133) or lack (HAexc#25) *Cyp6d4*

Insecticide	w^{1118}			HAexc#133				HAexc#25			
	LC_{50}[a] (95% CI)	*n*	Slope (SE)	LC_{50}[a] (95% CI)	*n*	Slope (SE)	RR[b]	LC_{50}[a] (95% CI)	*n*	Slope (SE)	RR[b]
Cypermethrin	3.01 (2.55–3.55)	480	2.0 (0.3)	0.26 (0.15–0.47)	440	1.4 (0.4)	0.1	0.84 (0.66–1.06)	480	1.9 (0.2)	0.3
Diazinon	3.24 (2.98–3.37)	400	7.2 (0.8)	5.44 (9.84–5.96)	560	6.4 (1.2)	1.7	3.50 (3.11–4.02)	900	4.0 (0.7)	1.1
Imidacloprid	4440 (2370–8400)	400	0.6 (0.1)	6700 (1580–31,000)	400	0.6 (0.1)	1.5	4790 (2760–8390)	400	0.6 (0.1)	1.1
Malathion	9.84 (6.61–14.3)	400	9.0 (6.2)	18.5 (14.9–23.2)	480	9.9 (5.0)	1.9	12.8 (6.48–25.4)	560	8.5 (0.9)	1.3
Oxamyl	24.1 (19.2–30.3)	890	2.5 (0.3)	50.4 (46.1–55.3)	840	3.2 (0.2)	2.1	50.6 (46.1–55.8)	840	3.0 (0.2)	2.1
Parathion	6.74 (5.70–8.03)	400	7.2 (1.9)	8.29 (5.70–12.2)	400	6.7 (3.8)	1.2	5.96 (5.57–6.48)	400	6.0 (0.6)	0.9
Pyrethrum extract	1290 (1140–1450)	560	2.8 (0.2)	1180 (1050–1310)	390	3.8 (0.4)	0.9	1200 (1040–1350)	360	3.2 (0.4)	0.9
Chlorfenapyr[c]	23	1110	0.8	35	1200	0.8	1.5	37	1140	0.8	1.6

[a] LC_{50} in units of ng/cm^2.
[b] Resistance ratio (RR) = LC_{50} resistant strain/LC_{50} susceptible strain.
[c] Estimated from best fit logrithmic regression lines in Fig. 3.

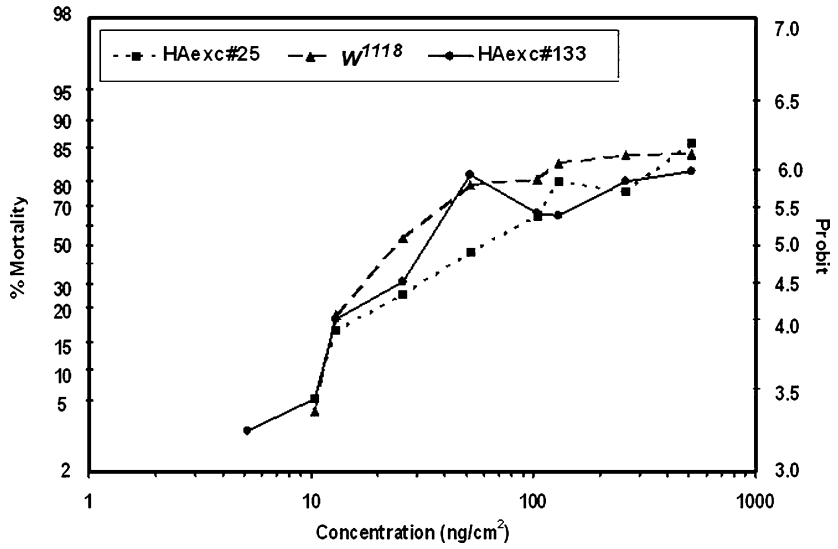

Fig. 3. Mortality vs. concentration of chlorfenapyr for two strains having *Cyp6d4* (w^{1118} and HAexc #133) and a strain in which *Cyp6d4* had been deleted (HAexc #25).

does not contribute significantly to metabolism of any of the eight insecticides previously recognized as activated and/or detoxified by P450s.

Despite the genetic similarities between all three strains, and the fact that HAexc#133 and HAexc#25 were isogenic, we observed differences in LC_{50} values of up to 10-fold between strains, and up to 3.2-fold between near-isogenic strains. Thus, differences in susceptibility, even between near-isogenic strains, may not always be due to the gene(s) being manipulated (*Cyp6d4* deletion in this study).

Our results suggest that *Cyp6d4* is not critically important for viability. *Cyp6d4* null mutants develop normally and do not differ in their ability to metabolize eight structurally different insecticides. Thus, either Cyp6d4 is not involved in the metabolism of these insecticides or other P450s are able to compensate for the lack of Cyp6d4. Our finding that *Cyp6d4* is not required for normal development is consistent with the idea that many genes in *D. melanogaster* (more than 2/3) are not vital [32]. While studies that delete individual P450s will continue to be of importance, identification of P450s involved in metabolism of insecticides, or those involved in metabolism of endogenous compounds, may progress faster by the simultaneous deletion of multiple P450s (for those that exist in clusters). For instance, this could be accomplished using homologous recombination, as was recently used to successfully delete several *Hsp70* (heat shock) genes from *D. melanogaster* [33].

Acknowledgments

We thank C. Leichter for technical assistance. This work was supported by the Daljit S. and Elaine Sarkaria Professorship. SAB and JE were funded by USDA NRICGP grant # 2000-02829.

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