A Cytochrome P450 Conserved in Insects Is Involved in Cuticle Formation

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Abstract

The sequencing of numerous insect genomes has revealed dynamic changes in the number and identity of cytochrome P450 genes in different insects. In the evolutionary sense, the rapid birth and death of many P450 genes is observed, with only a small number of P450 genes showing orthology between insects with sequenced genomes. It is likely that these conserved P450s function in conserved pathways. In this study, we demonstrate the P450 gene, Cyp301a1, present in all insect genomes sequenced to date, affects the formation of the adult cuticle in Drosophila melanogaster. A Cyp301a1 piggyBac insertion mutant and RNAi of Cyp301a1 both show a similar cuticle malformation phenotype, which can be reduced by 20-hydroxyecdysone, suggesting that Cyp301a1 is an important gene involved in the formation of the adult cuticle and may be involved in ecdysone regulation in this tissue.

Introduction

Cytochrome P450s are an evolutionarily ancient gene family found in virtually all organisms [1-5]. P450s were originally characterised for their roles in the detoxification of xenobiotics, but further studies have shown that some P450s possess catalytic characterised for their roles in the metabolism of many essential endogenous molecules [6-8]. In vertebrates, it has been suggested that evolutionarily conserved P450s function in endogenous pathways while those that are poorly conserved between species arise as evolutionary responses to xenobiotic challenges [9].

In insects, the number of cytochrome P450s in sequenced genomes ranges from 37 in the body louse, Pediculus humanus [10], to 160 in the dengue mosquito, Aedes aegypti [11]. Throughout evolution, selection results in tailoring an organism's genome. Thus genes involved in essential developmental processes are likely to be retained whereas those involved in specific detoxification responses, depending on an organism's environment, may not be under the same functional constraints. Some P450s are highly conserved and considered stable, with single ortholog found between species for those genes with proven biosynthetic and/or housekeeping roles [9]. Of the P450s conserved between D. melanogaster and A. aegypti [11], Cyp302a1, Cyp306a1, Cyp307a1, Cyp307a2, Cyp314a1, Cyp315a1 and Cyp10a1, are involved in the biosynthesis, activation and inactivation of the essential growth hormone 20-hydroxyecdysone [12-19], a conserved pathway found in all insects. Cyp48l1, which is involved in lipid metabolism [20] and Cyp49l1, which is expressed in the brain and central nervous system [21] are also highly conserved in distant insect genomes.

In this study, we investigate Cyp301a1, another P450 found in all sequenced insect genomes to date. A previous study has shown that Cyp301a1 is expressed in both the embryonic and larval hindgut as well as the embryonic epidermis of D. melanogaster [22]. Here we show that Cyp301a1 is likely to possess an important function in the developing epidermis. RNAi knockdown of Cyp301a1, and a Cyp301a1 piggyBac element insertion mutant both result in adults with a cuticle defect. Histological analyses suggest a retention of larval epidermal cells down the central portion of the abdomen. As Cyp301a1 is a conserved P450 in insects, further investigations into Cyp301a1 function may reveal critical biochemical insights into the formation of the adult cuticle.

Materials and Methods

Phylogenetic analysis of Cyp301a1

The D. melanogaster Cyp301A1 predicted protein sequence was used as a query against representative databases from selected Diptera, Lepidoptera, Coleoptera, Hymenoptera and Phthiraptera species using a BLASTp search (NCBI; http://flybase.bio.indiana.edu/blast). Corresponding orthologs to both Cyp301a1 and Cyp301B1 were identified and annotated using Artemis [23]. The sequences were aligned using ClustalX [24] and a maximum likelihood tree (bootstrapping = 1000) was compiled using MEGA 5.05 [25]. D. melanogaster Cyp49A1 was used as an outgroup.

Drosophila stocks and vectors

The D. melanogaster stocks y; cn, bw; sp [stock number 2057], y1 w1 ; P[tubP-GAL4]LL7/TM3, Sb1 [stock number 5138], the piggyBac transposase stock w1118; CyO, P{Tub-P-Bac\T}2/wgSp-1 [110], CyO, P{Tub-P-BCAT\T}2/wgSp-1 [26].
obtained from the Drosophila Genomics Resource Center (DGRC), Indiana. All stocks were maintained on glucose, semolina and yeast medium at 25°C. Drosophila transformation vectors were obtained from the Drosophila Genomics Resource Center (DGRC), Indiana.

**Synthesis of cDNA, real-time PCR and in situ hybridisation**

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies). RNA samples were treated with RNase-free DNase (Promega). cDNA was synthesised from 2 μg of each RNA sample in a 20 μl reaction using Superscript III Reverse Transcriptase (Invitrogen Life Technologies) and oligo(dT)20 primer following the supplier’s instructions. Quantitative PCR (QPCR) was performed as previously described using RpL11 as a housekeeping gene [26]. Primers for QPCR of Cyp301a1 were Cyp301a1-RT-F (5’-ACCGCGAATACACTCCACTT-3’) and Cyp301a1-RT-R (5’-TGATGATCGTCTCATGTTATT-3’). For in situ hybridisation, PCR was performed on cDNA using primers spanning the complete Cyp301a1 open reading frame (ORF) (Cyp301a1-ORF-F AGTAAACATCTGCTGCTGAGAGT-3’ and Cyp301a1-ORF-R CTTAACCCTCTGATCCTTAAAGCCGAG) and the PCR product was cloned into pGEM-T Easy (Promega). DIG-labeled RNA probe synthesis and in situ hybridisation were performed as previously described [22], [27]. In situ hybridisations were performed on the; in bw sp strain. Primers for Cyp4g1 and Cyp6g1 were previously reported [22], [26]. For adult integument RNA preparations, the integument was carefully removed from the inner abdominal layer and cDNA was synthesised using previously described methods.

**RNAi knockdown experiments**

The UAS-Cyp301a1-RNAi lines were constructed using the pWIZ vector [28]. BLASTn searches were performed on the ORF of Cyp301a1 to determine regions of similarity to other genes in the D. melanogaster genome. A region was chosen that contained less than 17 base pair similarity to any other gene (to avoid off-targets) and that spanned between 300–500 base pairs. The sequence GGCCTCTAGA (which contains an XhoI cleavage site) was added to the end of both primers. Primers used were 5’-AAAGCTTCTATGGAGATATCCTT-3’ and 5’-GTAAGCTTCTATGTTACCATGTT-3’. The fragment was amplified by PCR from cDNA using Expand High Fidelity PCR system and cloned into pGEM-T Easy to confirm the correct sequence. The fragment was digested with XhoI before being cloned into pWIZ via the ActII site. The construct was sequenced to determine the orientation of the inserted fragment before the second fragment was cloned into the XhoI site. PCR and restriction digest confirmed that the second fragment to be cloned in the opposite orientation to form a hairpin. Transformation into D. melanogaster w1118 strain was performed using standard techniques.

**Histological analyses**

Adult female flies were taken within 24 hours of eclosion, partially dissected and fixed in formalin for at least eight hours at room temperature before being embedded in paraffin. Sections were performed using the cut-4060 microtome (Microme) at 15 μm. Transverse sections were taken through the disrupted portion of the cuticle in Cyp301a1pWIZ strain and corresponding regions in controls. Sections were mounted on poly-lysine slides and either stained with Hematoxylin and Eosin or Calcofluor White (Sigma). Tissues were visualised using a Zeiss AxioImager Z1 microscope and a Leica SP5 confocal.

**Excision of piggyback element from Cyp301a1**

Cyp301a1pWIZ females were crossed to males from the piggyBac transposase strain w1118; Cyo, P(Tub-PBac)(T2/3)::sp. F1 males (genotype w1118; Cyp301a1pWIZ/Cyo, P(Tub-PBac)(T2/3)::sp) were then crossed to a double balancer strain (w1118; Sb/TM6, Sb/ TM3,Df(Tb) to isolate transposition events and identified by progeny with white eyes. These flies were then made homozygous for the second chromosome (where Cyp301a1 is located) and analysed by PCR.

**20-hydroxyecdysone exposure assays**

Third instar larvae of the Cyp301a1pWIZ strain were exposed to 1 mg/ml 20-hydroxyecdysone (20E) (Enzo Life Sciences) in instant fly media formula 4–24 (Carolina Biological Supply Company) as described previously [29]. Ten replicates of 10 larvae per vial were exposed to 20E, and the abdomen phenotype of all emerging flies was scored. Controls were reared under the same conditions but in the absence of 20E.

**Results**

**Cyp301a1 is evolutionarily conserved within insect species**

To identify orthologs of D. melanogaster Cyp301a1, the predicted protein sequence was used to query the genomes of twelve Drosophila species as well as other related insect species. A single Cyp301a1 ortholog was found in each genome of the 12 Drosophila species with available sequence [30], as well as in representative genomes from sequenced insect orders [31–33]. Given the rapid evolution of most members of the P450 family, this conservation indicates the gene product has an important function in all insects. The inferred Cyp301 protein sequences were aligned and a phylogenetic analysis was performed. The tree shows an ancient duplication of the Cyp301 gene family prior to the divergence of the Phthiraptera species forming the Cyp301a1 and Cyp301b1 genes and then a subsequent loss of the Cyp301b1 copy in Diptera (Figure 1A). Interestingly, Cyp301a1 in all insect species analysed has a Tyr (Y) instead of the highly conserved Phe (P) present in the second position of the heme-binding domain (PFxxGxxxCxG) (Figure 1B). A Tyr in this position is not observed for any other D. melanogaster P450 [1]. Cyp301B1 orthologs do not possess this change in the heme-binding domain, suggesting some functional divergence between the Cyp301 genes following their duplication.

**Temporal and spatial expression of Cyp301a1 during D. melanogaster development**

Cyp301a1 was detected by reverse transcription (RT)-PCR at all life stages, from embryo to adult (Figure 2A). Cyp301a1 expression is high during second instar larval stage (from L4 to 8), but decreases during the late third instar larval stage (L96-L120) and is essentially absent in wandering (W) larval stages (Figure 2A). Cyp301a1 expression then increases shortly during pupal formation (WP) and then again during late pupal development prior to eclosion (Figure 2A).

To examine the spatial expression of Cyp301a1, in situ hybridisation was performed on various stages during D. melanogaster development. Cyp301a1 expression in stage 1–2 embryos, the embryonic hindgut (stage 12–17) and epidermis (stage 17), as well as the larval hindgut has previously been
characterised [22]. These expression patterns have been confirmed, and additional *Cyp301a1* expression has been detected in the pupal and adult hindgut (Figure 2B–F). *Cyp301a1* expression has also been reported in the larval trachea and larval carcass [34]. In addition, we detected *Cyp301a1* expression by RT-PCR using cDNA synthesised from dissected adult integument (Figure 2G). To confirm the validity of this expression we also tested the expression of *Cyp4g1* (known to be expressed in the oenocytes residing in the epidermis [22]), *Cyp6g1* (a gene primarily expressed in the midgut, Malphigian tubules and fat body [26]) and the *Rpl11* housekeeping gene (Figure 2G). As expected, *Rpl11* and *Cyp4g1* were expressed highly in both male and female integument samples whereas *Cyp6g1* could not be detected from either cDNA sample (Figure 2G).
A piggyBac insertion in Cyp301a1 results in malformation of the adult cuticle

A transgenic D. melanogaster line, Cyp301a1^PBac{WH}Cy-p301a1^f02301, contains a piggyBac element inserted within the Cyp301a1 predicted open reading frame [35]. Sequencing of the Cyp301a1^f02301 allele using primers specific to the piggyBac element confirmed the presence of the WH-piggyBac element insertion, predicted to change the final five amino acids of the Cyp301A1 protein (Figure 3A, B). These changes are predicted to extend the C-terminal end of the protein by three amino acids and to increase the hydrophobicity of the protein, possibly altering the protein structure (Figure 3B). It is unknown if this results in a complete loss of function of the Cyp301A1 protein. Analysis of Cyp301a1^f02301 flies revealed a cuticle phenotype in both males and females, ranging from a slight malformation of the cuticle between the tergites to a complete loss of the tanned cuticle layer down the dorsal midline of the abdomen. The banding on the abdomen appeared severed, causing a misalignment of symmetry between the tergites. The range of phenotypes observed was classified into three categories: those that were wildtype (no visible disruption to the cuticle), slight (slight tearing of the cuticle down the mid-line) and severe (complete cuticle disruption) (Figure 3C). The Cyp301a1^f02301 flies also showed reduced survival with only 80%
of larval forming pupae and of these, only 90% eclose to adults. Of the emerging progeny reared at 25°C, 63(±5)% showed no phenotype, 24(±4)% of progeny emerged with a slight cuticle phenotype and 10(±1.5)% of progeny emerged with a severe cuticle disruption (Figure 3D). We measured the Cyp301a1 mRNA levels by QPCR in Cyp301a1f02301 adult flies (collected at eclosion) and found no significant difference compared to the background w1118 strain (Figure 3E), although we did detect higher Cyp301a1 expression in males than females. This suggests that the insertion is likely to affect Cyp301a1 protein stability or structure, rather than transcription.

To determine whether the cuticle phenotype seen in the Cyp301a1f02301 line was due to a disruption of the Cyp301a1 gene, the piggyBac element was excised (Figure 3A). Two independent excised lines (Cyp301a1-ex1aR202301 and Cyp301a1-ex2aR202301) were obtained, which were screened by PCR and sequenced to confirm the presence of a functionally wild-type Cyp301a1 gene (Figure 3B).

Unlike other eukaryotic class II transposons, piggyBac excisions are precise and do not leave a footprint [36]. Phenotypic analysis of the Cyp301a1-exR202301 flies raised at 25°C showed a significant decrease in appearance of the cuticle malformation phenotype and increase in the wild type phenotype compared to the Cyp301a1f02301 line (Figure 3D).

RNAi of Cyp301a1 results in similar cuticle phenotypes as Cyp301a1f02301 lines

To further confirm that Cyp301a1 was causing the malformed cuticle phenotype, we constructed three independent RNAi lines targeted against Cyp301a1. When Cyp301a1 was silenced using the ubiquitous tubulin-GAL4 driver y1 w2 P(tubP-GAL4)LL7/TM3, Sb1 [37] and progeny were raised at 25°C, all Cyp301a1 RNAi progeny showed a similar range of cuticle phenotypes as seen in the Cyp301a1f02301 flies (Figure 3A). QPCR on RNA isolated from adult males at eclosion showed that Cyp301a1 expression was significantly decreased (70–90%) in the Cyp301a1 RNAi progeny compared to controls (Figure 4C). Akin to the previous phenotypic classifications used, Cyp301a1 adult progeny were classed in order of cuticle phenotype severity (Figure 4A). At 25°C, 61(±7)% of flies emerged with no phenotype, 17(±3)% of RNAi progeny emerged with a slight cuticle malformation and 22(±7)% of RNAi progeny emerged with a severe cuticle phenotype from all three lines (Figure 4B). The cuticle phenotype was equally prevalent in males and females (Figure 4A). These RNAi results corroborate those reported for the Cyp301a1f02301 insertion strain, suggesting that a loss of Cyp301a1 is likely to be responsible for the cuticle malformation.

Cyp301a1f02301 flies show a cellular defect in adult cuticle formation

To further understand the cellular pathology observed in the Cyp301a1f02301 flies, transverse sections of the abdominal cuticle at eclosion were stained with hematoxylin and eosin. Although there is a lack of cells in the central region, there appears to be an intact cuticle layer (albeit thinner and nonpigmented), which spans the two sides of the tergites in Cyp301a1f02301 flies (Figure 5B,B'). Calcofluor staining shows that chitin is not secreted in these areas, although the remaining cuticle appears correctly pigmented, similar to control sections (Figure 5C). Compared to control flies, where transverse sections clearly show an even distribution of abdominal sensory bristles lining the cuticle layer (Figure 5A,A'), the Cyp301a1f02301 region shows a cellular defect with a severe phenotype.
in Cyp301a1<sup>RNAi</sup> flies there appears to be a lack of abdominal sensory bristles in regions surrounding the central cavity, where this cuticle is improperly formed (Figure 5B,B’). The lack of sensory bristles in this central region may suggest a failure of proper histoblast migration, given that abdominal histoblasts are also responsible for correct formation of sensory bristles along the abdomen.

**Discussion**

Insect cytochrome P450s are a large gene family, with roles in development and xenobiotic detoxification [1], [42]. Despite the large numbers of P450s found in insects, the number of orthologous P450s conserved between insects is small. In general, it has been observed that conserved P450s have developmental roles, such as the P450s involved in 20E biosynthesis in *D. melanogaster* and *Manduca sexta*, [43–45]. The conservation of Cyp301a1 in insects indicates that Cyp301a1 is likely to play an important role. Phylogenetic analyses show that Cyp301a1 possesses a restricted pattern of evolution, typical for many stable P450s [9]. A single Cyp301a1 ortholog is present in 12 *Drosophila* species with sequenced genomes [30]. Interestingly the Tyr to Phe change, conserved in the heme-binding domain of CYP301A1 orthologs is not found in CYP301B1 sequences, the nearest ortholog. Although sequence changes in the conserved heme-binding domain of P450s are rare, they are characteristic for P450s that act as atypical monooxygenases, for example CYP74A, a plant allene oxide synthase, and CYP5A1, the vertebrate thromboxane synthase enzyme [1], [46].

Cyp301a1 is expressed in a number of tissues throughout *D. melanogaster* development. It is expressed in the larval, pupal and adult hindgut, the epidermis of stage 17 embryos and in the larval trachea and carcass [34]. Disruption of Cyp301a1 function, as demonstrated by both Cyp301a1 <sup>piggybac</sup> and RNAi knockdown experiments, produced adult flies with a distinct morphological disruption to the cuticle. Histological analyses revealed an improper fusion of tergites along the abdomen, which may be caused by an abnormal proliferation of the adult cuticle. This phenotype is significantly reduced in the Cyp301a1 <sup>RNAi</sup> control.
groups, and the \textit{Cyp301a1}\textsuperscript{ex02301} strains, suggesting that the cuticle malformation is specifically due to a disruption in \textit{Cyp301a1} function.

The cuticle malformation phenotype, although replicable in both genotypes, showed incomplete penetrance with approximately 60% of flies affected in each case. This incomplete penetrance could be due to variability in the loss of \textit{Cyp301a1}. \textbf{RNAi} of \textit{Cyp301a1} results in a decrease of \textit{Cyp301a1} mRNA levels of 70–90% as measured by QPCR, and it is not known if the \textit{Cyp301a1} piggyBac insertion completely abolishes CYP301A1 function. A more extreme phenotype may result if a \textit{Cyp301a1} null allele is created. Alternatively, the incomplete penetrance could result from a redundancy in \textit{Cyp301a1} function. Several other P450s, such as \textit{Cyp49a1} and \textit{Cyp18a1} are expressed in the integument during \textit{Drosophila} development [19], [22]. Although \textit{Cyp301a1} is highly conserved and thus likely to possess an essential function, additional P450s may be able to partially compensate for

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\caption{Histological analysis of \textit{Cyp301a1}\textsuperscript{f02301} flies showing a severe cuticle phenotype. (A-B) Hematoxylin and eosin staining of sections. (A and enlarged in A') Transverse section through the abdomen of a \textit{Cyp301a1} control sample shows a normal, pigmented cuticle layer lining the abdomen (arrow). (B and enlarged in B') Transverse section through the abdomen of a \textit{Cyp301a1}\textsuperscript{f02301} sample shows a central unpigmented layer, in the region where the cuticle is disrupted (arrowhead). The surrounding cuticle looks wild type (arrows). (C) Calcofluor staining (blue; overlaid with brightfield in C') of transverse sections from \textit{Cyp301a1} control samples showing staining across the cuticle. (D) Calcofluor staining (blue overlaid with brightfield in D') of transverse sections from \textit{Cyp301a1}\textsuperscript{f02301} samples showing staining across the cuticle but is absent from the central regions where the cuticle is disrupted. doi:10.1371/journal.pone.0036544.g005}
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\caption{Rescue of \textit{Cyp301a1}\textsuperscript{f02301} flies using 20E. (A) Quantification of the proportion of flies emerging from the \textit{Cyp301a1}\textsuperscript{f02301} line with or without 20E treatment, according to the categories outlined in Figure 3A. **p<.01. (B) Phenotypes of \textit{Cyp301a1}\textsuperscript{f02301} flies at eclosion with or without 20E treatment. doi:10.1371/journal.pone.0036544.g006}
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the reduced function of Cyp301a1 in the Cyp301a1R2301 and Cyp301a1 RNAi flies.

A similar abdominal phenotype has been documented and is caused by a lack of larval epidermal cell (LEC) replacement, which obstructs the closure of the adult abdominal epithelium [29], [41]. Usually newly formed adult abdominal tergitae arise during metamorphosis as polyploid LECs are replaced by the descendants of the histoblasts imaginal cells, derived from small lateral nests in the larva. The histoblasts divide and migrate dorsally and ventrally over the abdomen until its whole surface is covered with cells [41]. During this process, the LECs undergo apoptosis; they constrict apically, are extruded from the epithelium and are subsequently phagocytosed [41]. Sustained expression of Activating transcription factor 3 (Afβ3) alters the adhesive properties of LECs, thus preventing their extrusion and replacement by the adult epidermis [29]. Removal of LECs is normally complete by 36 hours after pupal formation, at which time sheets of histoblasts reach the dorsal midline [49]. Afβ3 expression is high during early larval development and is downregulated during early pupal formation with a late peak of expression just prior to eclosion [19], similar to a Cyp301a1 profile. Retention of LECs caused by sustained afβ3 expression appears to be Jun-mediated and can be partially rescued with the addition of 20E [29]. Similarly, we also supplied ecdysone via this feeding method [29] to Cyp301a1R2301 flies, which seems to allow progression through development without inhibiting adult ecysis (as seen when 20E is administered via injection). Survival could be seen because the actual concentration that is entering larvae (assuming the larvae are only exposed when feeding, and not at later life stages) was not high enough to alter metamorphosis, and this concentration is not maintained during pupal development. Our experiments show that 20E exposure during third instar larval stage partially rescues the abdominal closure defects in Cyp301a1R2291 flies, possibly due to altering the early 20E peak. 20E is also involved in activating several genes in the chitin biosynthesis pathway, forming the polysaccharide layer in the cuticle [50]. However, given that intact regions of chitin are present in the Cyp301a1 RNAi and Cyp301a1R2301 flies, it is unlikely that CYP301A1 is involved in chitin synthesis.

Although we cannot conclude that CYP301A1 is directly involved in 20E regulation, it is not unprecedented for P450s to be involved in 20E regulation during development. Cyp302a1, Cyp306a1, Cyp307a1, Cyp307a2, Cyp314a1 and Cyp315a1 are all involved in the biosynthesis and activation of the essential growth hormone 20-hydroxyecdysone [12-18] while Cyp18a1 is involved in the inactivation of 20E [19]. Mutants in Cyp18a1 affect the timing and shaping the 20E peaks during metamorphosis [19]. As the inactivation of ecdysteroids possibly involves more than one enzyme, with ecdysteroid metabolic breakdown products located in the gut and other tissues [51], it is possible that Cyp301a1 is somehow involved in ecdysteroid metabolism, or perhaps some other aspect of 20E signalling or regulation during adult cuticle formation.

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Author Contributions

Conceived and designed the experiments: TS HC PJD. Performed the experiments: TS SB. Analyzed the data: TS PJD HC. Contributed reagents/materials/analysis tools: PJD PB. Wrote the paper: TS HC PJD.

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