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CYP18A1, a key enzyme of *Drosophila* steroid hormone inactivation, is essential for metamorphosis

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ABSTRACT

Ecdysteroids are steroid hormones, which coordinate major developmental transitions in insects. Both the rises and falls in circulating levels of active hormones are important for coordinating molting and metamorphosis, making both ecdysteroid biosynthesis and inactivation of physiological relevance. We demonstrate that *Drosophila melanogaster Cyp18a1* encodes a cytochrome P450 enzyme (CYP) with 26-hydroxylase activity, a prominent step in ecdysteroid catabolism. A clear ortholog of *Cyp18a1* exists in most insects and crustaceans. When *Cyp18a1* is transfected in *Drosophila* S2 cells, extensive conversion of 20-hydroxyecdysone (20E) into 20-hydroxyecdysonoic acid is observed. This is a multi-step process, which involves the formation of 20,26-dihydroxyecdysone as an intermediate. In *Drosophila* larvae, *Cyp18a1* is expressed in many target tissues of 20E. We examined the consequences of *Cyp18a1* inactivation on *Drosophila* development. Null alleles generated by excision of a *P* element and RNAi knockdown of *Cyp18a1* both result in pupal lethality, possibly as a consequence of impaired ecdysteroid degradation. Our data suggest that the inactivation of 20E is essential for proper development and that CYP18A1 is a key enzyme in this process.

Introduction

Regulatory processes must be tightly timed in multicellular organisms to ensure appropriate cell growth and differentiation at each defined life-stage. In insects, ecdysteroids are the steroid hormones that coordinate major developmental transitions, and successive ecdysteroid pulses are necessary to induce larval molts and metamorphosis (Riddiford et al., 2003; Thummel, 2001). Both increases and decreases in the circulating levels of active hormones are important for the timing of developmental events (Apple and Fristrom, 1991; Thummel, 2001), making both biosynthesis and inactivation of ecdysteroids of physiological relevance. Despite this importance, the biosynthetic pathway leading to ecdysteroids is still not completely understood (Lafont et al., 2005; Warren et al., 2009) (Fig. 1). Moreover, only one gene encoding an ecdysteroid inactivating enzyme, ecdysone oxidase, has been so far identified in *Drosophila melanogaster* and no mutants have been characterized (Takeuchi et al., 2005).

Numerous ways to inactivate steroids have been identified in animals, consisting mainly of hydroxylation and conjugation reactions (Lafont et al., 2005; Rees, 1995; You, 2004). A widespread and prominent route of ecdysteroids inactivation is their irreversible conversion to 26-hydroxylated metabolites, and ultimately to the corresponding ecdysonoic acids (Lafont et al., 2005) (Fig. 1). In several insect orders, 26-hydroxylation of the active hormone 20-hydroxyecdysone (20E) has been reported (Lafont et al., 1983) with the formation of transient aldehyde intermediates (Chen et al., 1994; Kayser et al., 2002) (Fig. 1). Biochemical evidence suggests that 26-hydroxylation is catalyzed by a cytochrome P450 enzyme (CYP) (Kayser et al., 1997; Williams et al., 2000), but the identity of the gene encoding this enzyme has never been demonstrated (Bassett et al., 1997; Davies et al., 2006; Hurban and Thummel, 1993).

In the present study, we demonstrate that *D. melanogaster* CYP18A1 catalyzes the 26-hydroxylation of ecdysteroids and their further oxidation to 26-carboxylic acids. We show that, despite the apparent redundancy of hormone inactivation processes in insects, *Cyp18a1* loss-of-function mutants and *Cyp18a1* RNAi inactivation strains both result in an extended final larval instar and lethality during metamorphosis, whilst ectopic over-expression of *Cyp18a1* is

Abbreviations: CYP, cytochrome P450 enzyme; cpr, cytochrome P450 reductase; dib, disembodied; E, ecdysone; 20E, 20-hydroxyecdysone; 20, 20,26E, 20,26-dihydroxyecdysone; 20Eoic, 20-hydroxyecdysonoic acid; EIA, enzyme immunoassay; HPH, hours post hatching; HPL, hours post laying; ISH, in situ hybridization; JH, juvenile hormone; phm, phantom; PTTH, prothoracicotropic hormone; RP-HPLC, reverse phase high performance liquid chromatography; RT-PCR, reverse transcription-polymerase chain reaction; shd, shade.

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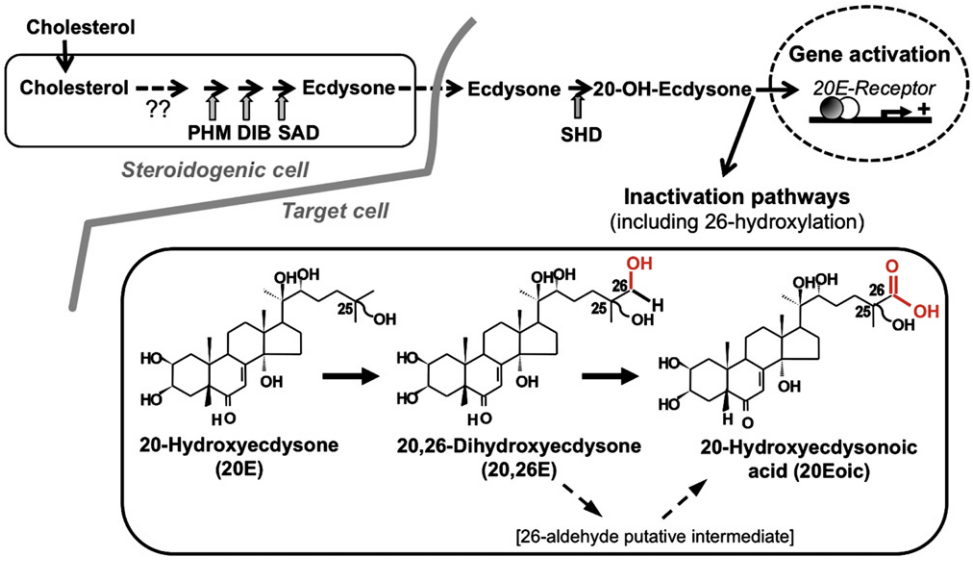

Fig. 1. Scheme of 20-hydroxyecdysone (20E) signaling pathway with emphasis on known steps of 20E biosynthesis and catabolism through 26-hydroxylation. The terminal steps of ecdysteroid biosynthesis have been identified and are catalyzed by the cytochrome P450 enzymes Phantom (PHM or CYP306A1), Disembodied (DIB or CYP302A1), Shadow (SAD or CYP315A1) and Shade (SHD or CYP314A1). After binding to the ecdysone receptor (EcR) and action at gene level, 20E is inactivated. 26-Hydroxylation and further oxidation to 20-hydroxyecdysonoic acid is a prominent inactivation pathway.

lethal. Our results therefore indicate that *Cyp18a1*, conserved in most arthropods, is essential for proper development of *D. melanogaster*.

Materials and methods

Drosophila feeding and staging

D. melanogaster strains were maintained at 25 °C on cornmeal agar medium. Control flies, unless specified, are w^{1118}. For experiments requiring staged larvae, animals were synchronized at the time of hatching by collecting the newly hatched larvae every hour and transferring to agarose plates coated with a paste of brewer's yeast. Larvae were synchronized again at the transition from the second to third larval instar at 30-minute intervals and transferred in groups of 30 into fresh tubes of cornmeal agar medium for further development.

Inactivation of Cyp18a1

Cyp18a1 knockdown flies were produced by generating flies with *Cyp18a1* RNAi transgenes. A 334-bp region specific to *Cyp18a1* was amplified from cDNA using *Cyp18a1*-RNAi-F and *Cyp18a1*-RNAi-R (Table S1) and cloned into pGEM-T Easy (Promega). This fragment was digested with XbaI, using restriction sites introduced in the primers, and sequentially cloned in both sense and antisense orientations downstream of the UAS sites in pWIZ (Lee and Carthew, 2003) using XbaI and SpeI. The resulting vector, marked with w^{+}, contained two copies of the *Cyp18a1* 334-bp fragment in an inverted repeat orientation separated by intron 2 of the *white* gene. Homozygous viable and fertile strains *Cyp18a1*RNAi-7a (transgene insert on chromosome 3) and *Cyp18a1*RNAi-14a (transgene insert on chromosome 2) were used in further analyses. Results obtained with strain *Cyp18a1*RNAi-7a are detailed in the results section. Similar results obtained with *Cyp18a1*RNAi-14a are shown in Supplementary Figs. S5A,B. The 6g1HR-GAL4 strain has been described previously (Chung et al., 2007). The 5′*phm*-GAL4 strain was established by generating transgenic flies carrying 674 bp upstream of the *phantom* gene fused to GAL4. The primers phm-F and phm-R (Table S1) were used to amplify the *phm* upstream region from w^{1118} gDNA. This was cloned into pGEM-T Easy (Promega) and, using NotI, subsequently cloned upstream of GAL4 in the pC3G4 vector. The construct was injected into the w^{1118} strain, and transgenic individuals recovered as w^{+} progeny. A single homozygous lethal insert on chromosome 2, maintained over a *CyO*, GFP balancer chromosome (5′*phm*-GAL4/*CyO*,GFP) was used. GAL4 expression is specific to the prothoracic cells of the ring gland in this strain (Fig. S5C). Other GAL4 drivers used were obtained from Bloomington Drosophila Stock Center, Indiana University. Two independent *Cyp18a1* null strains were generated by the imprecise excision of the modified *P* element (XP) located 601 bp upstream of *Cyp18a1* in the w^{1118} P(XP)*Cyp18a1*^{d07122} strain. The *P* element was mobilized using w^{*}; ry^{506} Sb^{1} *P*(*ry* + t7.2 = Delta2-3)99B/TM6B, Tb^{1}. Mobilization events creating deletions of *Cyp18a1* were detected by PCR using KO-F and KO-R primers (Table S1) spanning the *Cyp18a1* genomic region and confirmed by DNA sequencing. *Cyp18a1*^{null644} and *Cyp18a1*^{null748} are deletions of 644 bp and 748 bp respectively, including 43 bp and 147 bp of *Cyp18a1* coding sequence (from ATG). Both deletions remove the transcription and translation start sites of *Cyp18a1* (Fig. 6A and Fig. S6A). *Cyp18a1* null strains were maintained over a FM7i, *P*(w + mC = ActGFP)JMR3 (abbreviated FM7i-pAct-GFP) X-chromosome balancer. Results obtained with *Cyp18a1*^{null644} are detailed in the results section and similar results obtained with *Cyp18a1*^{null748} are shown as supplementary data (Figs. S6B,C). Survival of *Cyp18a1* null and knockdown individuals was determined by scoring for the presence/absence of GFP, or physical markers in adults. First instar larvae were reared in vials at 25 °C, with life-stage and mortality scored daily.

Cyp18a1 ectopic over-expression and rescue with 20-hydroxyecdysone

Ectopic over-expression of *Cyp18a1* was achieved using the GAL4/UAS system (Brand and Perrimon, 1993). The *Cyp18a1* ORF was PCR amplified using the primers 18a1-ORF-F and 18a1-ORF-R (Table S1) cloned into the pUAS vector (Brand and Perrimon, 1993) and transgenic flies generated in the w^{1118} strain. A single homozygous viable strain (UAS-*Cyp18a1*-6a) carrying an insert on chromosome III was used. All GAL4 driver strains were maintained as heterozygotes with GFP balancers (either *TM3*,GFP or *CyO*,GFP). GFP was used to distinguish control individuals from those driving *Cyp18a1* expression in crosses. Embryonic rescue experiments using 20E were conducted as described previously (Ono et al., 2006). 6–9 h old embryos were washed in (phosphate saline buffer with 0.1% Tween 20) and incubated with a 1 mL of 20E solution (100 μM) for 3 h before being placed on fly food media. UAS-*Cy18a1* survival to 1st instar of GFP and non-GFP was scored at 28 h post egg laying (HPL). Crossing experiments were conducted at 25 °C.