The Halloween genes code for cytochrome P450 enzymes mediating synthesis of the insect moulting hormone

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Abstract

The developmental events occurring during moulting and metamorphosis of insects are controlled by precisely timed changes in levels of ecdysteroids, the moulting hormones. The final four sequential hydroxylations of steroid precursors into the active ecdysteroid of insects, 20E (20-hydroxyecdysone), are mediated by four cytochrome P450 (P450) enzymes, encoded by genes in the Halloween family. Orthologues of the Drosophila Halloween genes phantom (phm; CYP306A1), disembodied (dib; CYP302A1), shadow (sad; CYP315A1) and shade (shd; CYP314A1) were obtained from the endocrinological model insect, the tobacco hornworm Manduca sexta. Expression of these genes was studied and compared with changes in the ecdysteroid titre that controls transition from the larval to pupal stage. phm, dib and sad, which encode P450s that mediate the final hydroxylations in the biosynthesis of ecdysone, were selectively expressed in the prothoracic gland, the primary source of ecdysone during larval and pupal development. Changes in their expression correlate with the haemolymph ecdysteroid titre during the fifth (final) larval instar. Shd, the 20-hydroxylase, which converts ecdysone into the more active 20E, is expressed in tissues peripheral to the prothoracic glands during the fifth instar. Transcript levels of shd in the fat body and midgut closely parallel the enzyme activity measured in vitro. The results indicate that these Halloween genes are transcriptionally regulated to support the high biosynthetic activity that produces the cyclic ecdysteroid pulses triggering moulting.

Introduction

Insect moulting hormones (ecdysteroids) control and coordinate the periodic moults of growing immature insects and the metamorphic differentiation into pupae and adult. The insect Halloween genes encode the terminal cytochrome P450 (P450) hydroxylases mediating the biosynthesis of ecdysteroids [1]. Mutations disrupting these specific P450 steroid hydroxylases in Drosophila result in morphogenetic abnormalities such as failure of head involution and cuticle formation, leading to embryonic death [2–6]. This emphasizes the physiological importance of the Halloween P450 enzymes, in contrast with many catalytically versatile P450 enzymes that metabolize xenobiotics, and which can be identified from their known cytological position and the catalytic competences were demonstrated by expression in Drosophila S2 cells [1]. It was found that Drosophila Phantom (Phm; CYP306A1), Disembodied (Dib; CYP302A1), Shadow (Sad; CYP315A1) and Shade (Shd; CYP314A1) catalyse the final four hydroxylations, yielding 20E (20-hydroxyecdysone) the principal insect moulting hormone [3–6].

phm, dib and sad are expressed in the prothoracic gland cells of the ring gland, the principal source of ecdysteroids during larval development, where they mediate the last three steps in the formation of ecdysone from dietary cholesterol [4,5]. In many lepidopteran insects, ecdysone is, however, not the primary product of the prothoracic glands [8]. Instead, 3-dehydroecdysone is produced and secreted, by what is believed to be an essentially similar biosynthetic pathway [8–10], and this prohormone is immediately converted into ecdysone in the haemolymph by a reductase [11]. Ecdysone is then converted into 20E in peripheral tissues such as the fat body, midgut and Malpighian tubules by a hydroxylation at C-20 catalysed by Shd, the E20MO (ecdysone 20-mono-oxygenase) [3,12]. These Halloween genes are expressed also in the ovaries, consistent with the importance of 20E for normal oogenesis [13].

Key words: cytochrome P450, ecdysone, Halloween gene, 20-hydroxyecdysone, moulting, prothoracic gland.

Abbreviations used: 7dC, 7-dehydrocholesterol; Dib, Disembodied; 20E, 20-hydroxyecdysone; E20MO, ecdysone 20-mono-oxygenase; ketodiol, 2,22,25-trideoxyecdysone; P450, cytochrome P450; Phm, Phantom; Sad, Shadow; Shd, Shade; Spa, Spook; Spok, Spookier.

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Here, we provide a short review of recent advances in unravelling the roles of the steroidogenic insect P450s with emphasis on their developmental regulation in the tobacco hornworm, *Manduca sexta*, during the final (fifth) larval instar.

**Ecdysteroid biosynthesis**

There has been major progress in this field over the last 5 years with the molecular characterization of terminal enzymatic steps [1]. Despite this great achievement, resulting from the utilization of molecular genetics, details of earlier steps in the biosynthetic pathway remain to be elucidated [1].

**Early steps: sterol precursors to the ketodiol (2,22,25-trideoxyecdysone)**

Insects, which are sterile heterotrophs, obtain mainly cholesterol or phytosterols depending on their dietary habits. Phytophagous insects primarily ingest phytosterols that are first dealkylated to cholesterol, the immediate sterol precursor of ecdysteroids. In the prothoracic gland cells, cholesterol is first converted into 7dC (7-dehydrocholesterol) by the action of the 7,8-dehydrogenase. Both dealkylation and the cholesterol to 7dC conversion are believed to involve P450-catalysed reactions by yet unidentified enzymes [11]. However, a recent study indicates that the 7,8-dehydrogenation may be carried out by Neverland, a Rieske domain protein [19].

The product of the 7,8-dehydrogenation, 7dC, is subjected to a unique and mysterious transformation to 5β-[H]-3β, 14α-dihydroxy-cholesta-7-ene-6-one (ketodiol), the first recognizable ecdysteroid-like molecule (extensively reviewed in [1,11,14]). No intermediates have been characterized between 7dC and the ketodiol and even the subcellular site of this biochemical transformation remains conjectural. The nature of this so-called ‘Black Box’ reaction has eluded molecular and even biochemical characterization despite a great deal of investigation. Various biochemical scenarios have been proposed for this transformation that may well include P450-catalysed reactions by yet unidentified enzymes [11]. So far, the P450 enzymes involved in ecdysteroid biosynthesis (i.e. the Halloween P450s) have all been identified from ‘low ecdysteroid’ mutants of *Drosophila*. The only ‘orphan’ P450 enzyme in the Halloween family of low ecdysteroid mutants is Spook (Spo; CYP307A1). The experimental paradigm that determined the function of the other Halloween P450s has failed to assign a specific enzymatic role to Spo [15,16]. The rescue of homozygous spo mutants provided an artificial pulse of ecdysteroid intermediates, which indicates that Spo probably acts upstream of Phm [i.e. upstream of the ketodiol (2,22,25-trideoxyecdysone)]. However, 7dC does not rescue spo mutants as it does with the woc [17], dpnca [18] and neverland mutants [19], indicating that Spo is not the 7,8-dehydrogenase. Several functions have been proposed for Spo including the possibility that Spo is either involved in the Black Box reaction or synthesizes a novel signal molecule required for ecdysteroid biosynthesis [15,16].

**Ecdysteroidogenic Halloween P450s: *M. sexta***

Because all insects utilize the same active ecdysteroid, namely 20E, synthesized from cholesterol through the same series of sterol modifications, the Halloween P450 genes may have been structurally and functionally conserved through some 400 million years of insect evolution. This is substantiated by the orthologous relationship of *Drosophila*, *Manduca* and silkmoth (*Bombyx mori*) Halloween genes shown in Figure 1. The conserved primary structure of these genes allowed for the identification of the *Manduca* orthologues using RT (reverse transcriptase)–PCR [12,16,20]. Unequivocal evidence that these genes are the *Manduca* orthologues of *Drosophila* Phm, Dib, Sad and Shd came from functional characterization by expression of the genes in *Drosophila* S2 cell lines. When expressed in S2 cells with specific ecdysteroid substrates, *Manduca* Phm is the C25-hydroxylase, Dib the C22-hydroxylase, Sad the C2-hydroxylase and Shd the C20-hydroxylase (Figure 2) [12,20]. While Phm is a microsomal P450, Dib and Sad are mitochondrial. Thus ecdysteroid intermediates are shuttled between intracellular compartments during biosynthesis. In *Manduca*, E20MO activity is observed in both mitochondrial and microsomal fractions [21] and it is possible the Shd may reside in either of these locations [3,12] as demonstrated for some mammalian P450s [22,23].

**Expression of the *Manduca* Halloween genes**

Throughout embryonic, larval and pupal development of insects, changes in the ecdysteroid titre promote transition from one developmental stage to the next [11]. We carried out an extensive expression analysis of the *Manduca* Halloween genes to investigate developmentally related changes in their expression [12,16,20] in this established model insect for endocrinological studies.

Consistent with previous studies showing that the *Drosophila* phm, dib and sad genes are predominantly expressed in the prothoracic gland cells [4,5], and the classical dogma that these glands are the principal source of ecdysone [11], the *Manduca* orthologues of these genes are selectively expressed in the prothoracic glands during the fifth larval instar [20].

The haemolymph ecdysteroid titre is for a large part directly a result of the production by the prothoracic glands [11], thus depending on the activity of the steroidogenic enzymes including Phm, Dib and Sad. To examine whether changes in the production of ecdysteroids by the prothoracic glands are associated with transcriptional regulation of the Halloween genes, we carried out detailed analyses of their expression during the fifth instar and during the beginning of pupal–adult development [20]. The final larval instar of *Manduca* is characterized by two pulses of ecdysteroids; the first small peak around days 3–4 (commitment peak) programs the animal to undergo a metamorphic moult in response to the second and large peak (moulting peak) at day 7 that elicits mouling to the pupa [24]. Figure 2 shows that increases in the prothoracic glands’ expression of phm, dib and
sad were temporally coincident with the two haemolymph ecdysteroid pulses during the fifth larval instar.

During the beginning of pupal–adult development, the prothoracic glands are believed to support the rise in the haemolymph ecdysteroid titre consisting mainly of ecdysone [25]. Six days after pupation, these glands begin to undergo programmed cell death [26]. Although the expression of phm, dib and sad does not closely parallel the ecdysteroid titre during the beginning of pupal–adult development, increases are observed in this period (Figure 2). This agrees with previous reports indicating a role of the prothoracic glands in the production of ecdysone necessary for normal pupal–adult development.

The expression pattern of sad, catalysing the final step in the formation of ecdysone, is reminiscent of the 2-hydroxylase, dib, catalysing the preceding step (Figure 2). It is intriguing that spo and phm exhibit very similar patterns of expression given evidence that Spo may act as an integral protein in the biosynthetic pathway upstream of Phm [15,16].

In Drosophila, spo is not expressed in the ring gland, a composite organ housing the prothoracic gland cells, during larval development but only in the embryo prior to the formation of this gland [15,16]. This indicates that Spo is not required for ecdysteroid production during larval development, even though ecdysteroid production by the prothoracic glands must take place for moulting to occur at these stages. The fact that Manduca spo was expressed in the prothoracic glands during larval development perplexed but encouraged us to re-examine the Drosophila genome. It turned out that Cyp307a2p, believed to be a truncated pseudogene, encoded a complete P450 enzyme. Expression analysis revealed that this closely related parologue of spo, dubbed spookier (spok; Cyp307a2), is expressed in the embryonic and larval ring gland [16]. Thus it seems that Drosophila (dipteran), in contrast with Manduca and Bombyx (lepidopterans), has two proteins, Spo and Spok, that provide Spo-like activity at different life stages [16]. Distinct spatiotemporal expression of duplicated steroidogenic P450s, like spo and spok, is also known with paralogues of the aromatase (CYP19) in teleosts [27].

Overall, our expression data indicate that transcriptional up-regulation of phm, dib and sad may occur to support the zenith of ecdysteroid production by the prothoracic glands during the fifth larval instar of Manduca. If Spo is directly involved in the biosynthesis of ecdysone, regulation of its expression probably serves the same purpose, that is, to support the prothoracic glands’ changing requirements for synthesizing ecdysteroids.

The E20MO is responsible for the conversion of ecdysone into 20E and thus is of critical importance to normal insect development. Biochemical characterization of the E20MO as being a typical P450 enzyme was done more than 20 years ago [28] and Smith et al. [29] described the in vitro activity of this enzyme in the fat body and midgut of Manduca. In these tissues, the activity fluctuates dramatically during the fifth instar. Analysis of Manduca shd mRNA levels reveals the preponderance of expression in the fat body, midgut...
Data showing the developmental expression of the *Manduca* Halloween genes in the prothoracic glands (i.e., *spo*, *phm*, *dib* and *sad*) are from Rewitz et al. [20] and Ono et al. [16]. Data for *shd* expression are from Rewitz et al. [12] and for the haemolymph ecdysteroid titre is from Grieneisen et al. [32] and Warren and Gilbert [25]. Note that biochemical evidence indicates that the conversion of cholesterol into 7dC is catalysed by an as yet undescribed microsomal P450 enzyme [32], although recent evidence indicates that perhaps the Rieske-domain protein Neverland may be involved [19]. Multiple arrows between 7dC and the ketodiol indicate an uncharacterized series of oxidations called the ‘Black Box’ reaction that may involve P450 enzymes [11]. *Spo* may be involved in this reaction although its function is currently unknown [16]. E, ecdysis.

Future prospects
While characterization of the Halloween gene family of P450s has brought conclusive molecular characterization of the terminal hydroxylations in the biosynthetic scheme, attempts to identify enzymes acting prior to the formation of the ketodiol (the substrate of Phm) have been unsuccessful.
Identification and characterization of the enzymes involved in these early steps are the final frontiers in the elucidation of the insect enzymes involved in ecdysoidogenesis.

The apparent transcriptional control of the Halloween genes, including the prominent and rapid changes of sbd expression, merits further investigation as knowledge of regulation of these genes is important for understanding modulation of ecdysoidogenesis. In *Drosophila*, it was recently demonstrated that βFTZ-F1 (β fushi tarazu factor 1), a homologue of SF1 (steroidogenic factor 1) controlling the expression of vertebrate steroidogenic P450s, is involved in regulation of Phm and Dib [31]. This indicates fundamental conservation of the transcriptional machinery controlling vertebrate and insect steroidogenic P450s.

Uncovering the complete biosynthetic pathway of ecdysteroids and the mechanisms regulating the ecdysteroid titre are important for our understanding of moulding and metamorphosis, which are fundamental processes in the lives of arthropods that have contributed to the indisputable success of this group of animals. Such knowledge may also provide the basis for the development of target-specific agents for pest control.

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