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Juvenile hormone signaling during reproduction and development of the linden bug, *Pyrrhocoris apterus*

Vlastimil Smykal [a,b,1], Adam Bajgar [a,b,1], Jan Provaznik [a,b], Silvie Fexova [a,b], Marcela Buricova [a,b], Keiko Takaki [a], Magdalena Hodkova [a], Marek Jindra [a,c,*], David Dolezel [a,b,*]

[a] Biology Center, Academy of Sciences of the Czech Republic, 37005 Ceske Budejovice, Czech Republic
[b] Department of Molecular Biology, Faculty of Sciences, University of South Bohemia, 37005 Ceske Budejovice, Czech Republic
[c] Animal, Food and Health Sciences Division, Commonwealth Scientific and Industrial Research Organization, North Ryde, NSW 2113, Australia

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ABSTRACT

Juvenile hormone (JH), a sesquiterpenoid produced by the insect corpus allatum gland (CA), prevents metamorphosis in larvae and stimulates vitellogenesis in adult females. Whether the same JH signaling pathway regulates both processes is presently unknown. Here, we employ the robust JH response during reproduction and development of the linden bug, *Pyrrhocoris apterus*, to compare the function of key JH-signaling genes encoding the JH receptor, Methoprene-tolerant (Met), its binding partner Taiman (Tai), and a JH-inducible protein, Krüppel-homolog 1 (Kr-h1). RNA interference (RNAi) with Met or Tai, but not Kr-h1, blocked ovarian development and suppressed vitellogenin gene expression in the fat body of females raised under reproduction-inducing conditions. Loss of Met and Tai matched the effects of CA ablation or the natural absence of JH during reproductive diapause. Stimulation of vitellogenesis by treatment of diapausing females with a JH mimic methoprene also required both Met and Tai in the fat body, whereas *Kr-h1* RNAi had no effect. Therefore, the Met-Tai complex likely functions as a JH receptor during vitellogenesis. In contrast to Met and Kr-h1 that are both required for JH to prevent precocious metamorphosis in *P. apterus* larvae, removal of Tai disrupted larval ecdysis without causing premature adult development. Our results show that while Met operates during metamorphosis in larvae and reproduction in adult females, its partner Tai is only required for the latter. The diverse functions of JH thus likely rely on a common receptor whose actions are modulated by distinct components.

1. Introduction

The sesquiterpenoid juvenile hormone (JH) controls multiple events in insect's life, from development to reproduction, seasonal diapause, and various polyphenisms (Nijhout, 1994). How JH exerts all of its functions is unclear as our knowledge of the mode of JH action remains limited. JH was discovered and named for its capacity to maintain the juvenile state of insect larvae until they have attained an appropriate stage (Wigglesworth, 1934). Only during the final larval instar, a temporal drop in JH secretion from the corpora allata (CA) glands permits metamorphosis to the adult form (Hiruma and Kaneko, 2013; Jindra et al., 2013). In adult insects, JH reappears to fulfill its other major function during reproduction, particularly in oogenesis (Raikhel et al., 2005). Since JH occurs even in wingless insects that do not undergo metamorphosis, stimulating reproduction is thought to be the evolutionarily older of the JH roles (Sehnal et al., 1996).

The molecular basis of JH signaling has been partially unveiled owing to studies in several insect models [see (Jindra et al., 2013) for a review]. A candidate JH receptor gene, *Methoprene-tolerant* (*Met*), was originally uncovered through resistance to the JH mimic methoprene in *Drosophila melanogaster* mutants (Wilson and Fabian, 1986). Met was identified as a novel member of the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) family of transcription factors (Ashok et al., 1998). RNA interference (RNAi)-mediated knockdown of a *Met* ortholog in the flour beetle, *Tribolium castaneum*, revealed that Met was required for JH to prevent precocious metamorphosis (Konopova and Jindra, 2007). The anti-metamorphic JH pathway was soon extended to the zinc-finger

* Corresponding authors. Biology Center, Academy of Sciences of the Czech Republic, 37005 Ceske Budejovice, Czech Republic.
E-mail addresses: jindra@entu.cas.cz (M. Jindra), david.dolezel@entu.cas.cz (D. Dolezel).
[1] These authors contributed equally to this work.

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transcription factor Krüppel-homolog 1 (Kr-h1) that acts immediately downstream of JH and Met to block metamorphosis in holometabolous (Minakuchi et al., 2009, 2008) as well as in hemimetabolous (Konopova et al., 2011; Lozano and Bellés, 2011) insects. Met was shown to bind JH with a nanomolar affinity (Charles et al., 2011; Miura et al., 2005), and its ligand-binding domain was defined through specific amino acid mutations (Charles et al., 2011). In response to JH, Met forms complexes with at least two other bHLH-PAS family proteins: Taiman (Tai), also called FISC or steroid receptor coactivator (SRC) (Charles et al., 2011; Li et al., 2011; Zhang et al., 2011), and the circadian clock protein Cycle (Cyc) (Shin et al., 2012).

Besides regulating metamorphosis, the JH receptor Met has been implicated in oogenesis. *Drosophila Met* mutants showed delayed onset of vitellogenesis and oviposition (Wilson and Fabian, 1986), and a milder reduction of fecundity was also found in flies lacking *germ cell-expressed* (*gce*), a gene paralogous to *Met* (Abdou et al., 2011). RNAi knockdown of *Met* in females of *Tribolium* nearly stalled egg production and prevented expression of a vitellogenin gene (Parthasarathy et al., 2010). JH and Met were shown to regulate *Tribolium* vitellogenesis indirectly, via activating insulin signaling (Sheng et al., 2011). In the *Aedes aegypti* mosquito, where JH is required for female reproductive maturation prior to blood meal that then stimulates vitellogenesis (Raikhel et al., 2005), *Met* RNAi retarded ovarian development (Zou et al., 2013) and lowered egg production (Li et al., 2011).

Tai is currently the best-known protein partner of Met, but its function in JH-regulated processes is much less evident than it is for Met itself. A role for Tai/SRC in metamorphosis has been postulated in recent models (Hiruma and Kaneko, 2013; Riddiford, 2012) but not yet experimentally demonstrated. A role for Tai in oogenesis is suggested by reduced number of eggs laid by *A. aegypti* females deficient for the Tai ortholog FISC (Li et al., 2011). One intriguing possibility is that Met might engage in interactions with multiple protein partners in order to exert the distinct functions of JH.

We have chosen to address the roles of Met and Tai in reproduction and in the maintenance of the larval state using the linden bug, *Pyrrhocoris apterus* (Hemiptera). Besides efficient systemic RNAi, this species offers a robust model of JH-dependent reproduction that can be manipulated by the photoperiod. Under short day length, the bug females have inactive CA and remain in a state of reproductive diapause, whereas extended photoperiod promotes JH synthesis and oogenesis. The animals can be repeatedly switched between diapause and reproduction using the photoperiod alone, without changing temperature or access to food, and oogenesis can be triggered in short-day diapausing females upon JH mimic treatment. In this experimental setup, we have shown recently that diapause to reproduction reprogramming of gene expression in the gut requires JH signaling from the CA that is mediated by Met and two circadian clock genes, *cyc* and *Clock* (*Clk*) but not Tai (Bajgar et al., 2013a, 2013b).

Our present data indicate that in *P. apterus*, JH-dependent development and reproduction both rely on the same JH receptor, Met. However, other components of the JH signaling pathway may have specific functions, as Tai is required for JH to stimulate vitellogenesis but not to maintain the juvenile program in penultimate-instar larvae.

2. Methods and materials

2.1. Insect rearing

P. apterus bugs (short-winged form) were maintained at 25 °C on dry linden seeds and were supplemented with water. The cultures were kept at either of two photoperiod regimes: long-day (LD; 18 h light, 6 h dark) that permits reproduction, or short-day (SD; 12 h light, 12 h dark) that induces adult reproductive diapause. Adult females of specific age after adult ecdysis or newly ecdysed penultimate (fourth instar) larvae were selected for experiments.

2.2. cDNA cloning

Cloning of *P. apterus* cDNAs encoding Met and Kr-h1 (Konopova et al., 2011), Tai, Cyc, and Clk (Bajgar et al., 2013b) was described in the cited references. Sequences encoding Vitellogenin (Vg1 and Vg2) and Hexamerin proteins were retrieved by using BLAST from *P. apterus* cDNA subjected to 454 pyrosequencing (GATC Biotech), PCR-amplified, and verified by Sanger sequencing. Sequences are available from the GenBank under the following accession numbers (*Vg1*, KF583751; *Vg2*, KF583752; *Hex*, KF583750).

2.3. RNA interference (RNAi), methoprene treatment, and organ culture

Double-stranded RNA (dsRNA) was prepared using the T3 and T7 RNA polymerases with the MEGA script kit (Ambion) from plasmids containing the appropriate gene fragments and injected into *P. apterus* adult females or larvae as described previously (Bajgar et al., 2013b; Konopova et al., 2011). Adult females received 4 μl of dsRNA at a concentration of 4 μg/μl in Ringer's solution; fourth-instar larvae were injected with 1 μl containing 3 μg of dsRNA. Control animals were injected with heterologous dsRNAs derived from bacterial *β-galactosidase* (adults) and *malE* (larvae) genes, *egfp* (larvae) or with the Ringer's solution alone.

For JH mimic treatments, diapausing SD adult females were anesthetized under CO_2 and treated on the dorsal side with 5 μl of 0.3 mM methoprene (VUOS Pardubice, Czech Republic) dissolved in acetone; controls were treated with acetone only. When RNAi and JH mimic treatments were to be combined, methoprene application followed dsRNA injection by four days. Animals were then sacrificed and subjected to mRNA expression analysis four days after methoprene administration. For experiments on isolated fat body, the tissues were dissected from adult females four days after dsRNA injection. Part of fat body with the Ringer's solution (approximately 50 μl) from a single female was placed in a small culture dish with 100 μl of Grace's medium and exposed to 10 μM methoprene or solvent (acetone) alone for 24 h before mRNA expression was analyzed.

2.4. mRNA quantification

Analyzed tissues (fat body, epidermis) were dissected in RNAse-free Ringer's solution. Total RNA was isolated with the Trizol reagent (Invitrogen). After Turbo DNAse (Ambion) treatment, 1 μg of total RNA was used for cDNA synthesis using the SuperScript III reverse transcriptase (Invitrogen). Relative transcript levels were measured by quantitative PCR using the iQ SYBR Green Supermix kit and the C1000 Thermal Cycler (both Bio-Rad). All measured transcripts were normalized to relative levels of the ribosomal protein (*rp49*) mRNA as described previously (Dolezel et al., 2007). Sequences of primers used for quantification of the specific mRNAs are listed in Table 1.

2.5. Ablation of the corpus allatum

Females selected from the colony within 24 h after adult ecdysis were deprived of linden seeds. One day later, females were anesthetized by submergence in water for 15 min and the corpus allatum was removed through the neck membrane incision under