Methoprene-tolerant 1 regulates gene transcription to maintain insect larval status

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Abstract

Insect molting and metamorphosis are regulated by two hormones: 20-hydroxyecdysone (20E) and juvenile hormone (JH). The hormone 20E regulates gene transcription via the nuclear receptor EcR to promote metamorphosis, whereas JH regulates gene transcription via its intracellular receptor methoprene-tolerant (Met) to prevent larval–pupal transition. However, the function and mechanism of Met in various insect developments are not well understood. We propose that Met1 plays a key role in maintaining larval status not only by promoting JH-responsive gene transcription but also by repressing 20E-responsive gene transcription in the Lepidopteran insect *Helicoverpa armigera*. Met1 protein is increased during feeding stage and decreased during molting and metamorphic stages. Met1 is upregulated by JH III and a low concentration of 20E independently, but is downregulated by a high concentration of 20E. Knockdown of *Met1* in larvae causes precocious pupation, decrease in JH pathway gene expression, and increase in 20E pathway gene expression. Met1 interacts with heat shock protein 90 and binds to JH response element to regulate *Krüppel homolog 1* transcription in JH III induction. Met1 interacts with ultraspiracle protein 1 (USP1) to repress 20E transcription complex EcRB1/USP1 formation and binding to ecdysone response element. These data indicate that JH via Met1 regulates JH pathway gene expression and represses 20E pathway gene expression to maintain the larval status.

Key Words

- methoprene-tolerant 1
- juvenile hormone
- gene transcription
- 20-hydroxyecdysone

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Introduction

Insect growth, molting, and metamorphosis are coordinately regulated by two hormones, namely juvenile hormone (JH) and 20-hydroxyecdysone (20E): 20E promotes molting and metamorphosis and JH prevents larval–adult transition until insects have attained an appropriate stage (Riddiford *et al.* 2003). The hormone 20E binds to its nuclear receptor EcR and forms a transcription complex with the heterodimeric partner ultraspiracle protein (USP) in *Drosophila* (Yao *et al.* 1992). The EcR/USP transcription complex binds to ecdysone response

element (EcRE) to promote gene transcription in the 20E pathway in *Drosophila* (Riddihough & Pelham 1987), such as transcription factor Broad (Br) that initiates metamorphosis in *Manduca sexta* (Zhou & Riddiford 2001) and transcription factor hormone receptor 3 (HR3) to promote molting and metamorphosis in *M. sexta* (Lan *et al.* 1999).

Methoprene-tolerant (*Met*) gene is cloned in a *Drosophila melanogaster* mutant that showed resistance to toxic doses of JH or JH analog (Ashok *et al.* 1998). Met protein

has a typical basic helix–loop–helix Per-ARNT-Sim (bHLH-PAS) domain. As Met binds to JH III with a high affinity through its C-terminal PAS domain (Charles *et al.* 2011) and regulates gene transcription in the JH pathway in *Tribolium*, Met is considered as the JH intranuclear receptor (Jindra *et al.* 2013). In a recent paper, De Loof *et al.* have advanced arguments in favor of the view that the still missing membrane receptor of JH is probably nothing else than the Ca^{2+} -homeostasis system of cells. Indeed, at least some isoforms of Ca^{2+} -ATPase have a binding site for farnesol-like sesquiterpenoids, the family of compounds to which JH belongs. These authors think that a low cytoplasmic Ca^{2+} concentration is causal to the status quo effect of JH (De Loof *et al.* 2014).

Met forms homodimers with Met or germ cell expressed (Gce) without hormone regulation in *Drosophila* (Godlewski *et al.* 2006). JH regulates the formation of the complex of Met with the steroid co-activator p160/SRC in *Tribolium castaneum* (Zhang *et al.* 2011, Jindra *et al.* 2013), which is known as FISC in *Aedes aegypti* (Li *et al.* 2011), Taiman in *D. melanogaster* (Charles *et al.* 2011), and NcoA/Src1/p160 in mammals (Beischlag *et al.* 2002). In *A. aegypti*, Met interacts with Cycle (CYC) (Shin *et al.* 2012) and $\beta\text{Ftz-F1}$ (Zhu *et al.* 2003) by the induction of JH. Met2 interacts with SRC and binds to JH response element (kJHRE) in the Krüppel homolog 1 gene (*Krh1*) to promote *Krh1* transcription to repress metamorphosis in *Bombyx mori* (Kayukawa *et al.* 2012). However, Met is also involved in the 20E pathway. Met RNAi prevents tissue remodeling, which results in lethality and disruption of the 20E-triggered transcriptional cascade reaction in *B. mori* (Guo *et al.* 2012). In *Drosophila*, the overexpression of Met in fat body results in precocious and enhanced programmed cell death and cell dissociation (Liu *et al.* 2009). Met can also interact with EcR and USP in insect cells in an ecdysteroid or in a JH-regulation-independent manner, suggesting that the interaction among Met, EcR, and USP mediates the crosstalk between these two important hormones (Bitra & Palli 2009). Therefore, the basic function of Met in various insects is not really clear.

Helicoverpa armigera (Noctuidae, Lepidoptera) is a severe agricultural pest throughout the world. We reveal that Met1 has an important function in maintaining the larval status in this insect. Knockdown of *Met1* causes precocious pupation by repressing the JH pathway gene expression and promoting 20E pathway gene expression. The mechanism used by Met1 in controlling 20E and JH pathways is studied in this work.

Materials and methods

Experimental animal

H. armigera larvae were raised on the artificial diet, which was made from powder of wheat germs and soybeans at 27 °C with 60–70% relative humidity. The illumination condition consisted of 14 h light:10 h darkness cycles in an insectarium (Zhao *et al.* 1998).

H. armigera epidermal cell line culture

The *H. armigera* epidermal cell line (HaEpi) was established in our laboratory (Shao *et al.* 2008). Cells were cultured as a loosely attached monolayer and maintained at 27 °C in tissue culture flasks. The tissue culture flasks had an area of 25 cm² with 4 ml of antibiotic-free Grace's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cells were subcultured weekly to a near confluent monolayer.

Bioinformatic analyses of Met1

The translation and prediction of Met1 were analyzed by ExPASy Proteomics Server (<http://www.expasy.ch/tools/>). The nucleotide sequence of Met1 has been submitted to GenBank with GenBank Number: KJ184572. Protein domain prediction was performed using SMART (<http://smart.embl-heidelberg.de/>). The phylogenetic tree was obtained using the neighbor-joining method in MEGA 3.1 (<http://www.megasoftware.net/>).

Recombinant expression and polyclonal antibody preparation

The fragment of Met1 81 aa–277 aa was expressed in *Escherichia coli* Rosetta host cells by the vector pGEX-4T-1, as inclusion body with the primers (sequence-Met1-F1–BamHI: TACTCAGGATCCCGGAGCCAATACAACCTCT; Met1-R1–XhoI: TACTCACTCGAGTCAATTCCCACTG–GATGA). Protein was purified by SDS–PAGE. The antiserum was prepared by the method described previously (Dong *et al.* 2013). The specificity of the antiserum was determined by western blot analysis.

Quantitative real-time RT-PCR

Total RNA of larvae was extracted using Unizol reagent (Biostar, Shanghai, China). The RNA was reverse transcribed into the first-strand cDNA as the template for quantitative real-time RT-PCR (qRT-PCR) with the primers

listed in [Supplementary Table S1](#), see section on [supplementary data](#) given at the end of this article. The 10 μ l mixture, which was used to perform qRT-PCR, comprised 5 μ l SsoFast EvaGreen Supermix (Bio-Rad), 1 μ l cDNA (1:50 diluted), 2 μ l of 1 μ mol/l forward primer, and 2 μ l of 1 μ mol/l reverse primer in 1 ml FBS-free Grace's medium for 12 h. β -actin was used for quality control. Data were analyzed using the following formula: $R = 2^{-(\Delta Ct \text{ sample} - \Delta Ct \text{ control})}$, where R is the relative expression level, ΔCt sample is the difference between the Ct of the gene and the average β -actin in the experimental sample, and ΔCt control the difference between the Ct of the gene and the average of β -actin in the control sample.

Hormone treatment on larvae

Stock solutions (20 mM) of 20E and JH III (Sigma) were prepared in dimethyl sulfoxide (DMSO). The 20E and JH III stock solute were diluted to 0.1 mg/ml with PBS (10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 140 mM NaCl, and 2.7 mM KCl, pH 7.4). The hormone 20E or JH III (0.1 mg/ml) was injected in the 6th instar 6 h larvae (500 ng/larva). The untreated larvae were injected with an equivalent amount of DMSO.

Western blot analysis

Tissues were homogenized in Tris-buffered saline (TBS, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl), and protein quantification was performed using a β -actin antibody. The proteins were separated in SDS-PAGE and then transferred onto a nitrocellulose membrane. After being blocked with 2% non-fat milk in TBS for 1 h at room temperature, the membrane was incubated with the antibody for 12 h at 4 °C. The membrane was washed with TBST (0.1% Tween 20 in TBS) for 15 min thrice. Subsequently, the blot was probed with the HRP-conjugated goat anti-rabbit IgG (1:10 000 in the blocking solution). To visualize the peroxidase activity, 4-chloro-1-naphthol was used as a HRP substrate.

RNAi in the HaEpi cell line and larvae

MEGA-script RNAi Kit (Ambion, Inc., Austin, TX, USA) was used to synthesize dsRNA with the T7 promoter-containing PCR primers (Met1-RNAi-F and Met1-RNAi-R sequence in [Supplementary Table S1](#)). HaEpi was cultured to 80% confluence at 27 °C in Grace's medium supplemented with 10% FBS. Approximately 1 μ g dsRNA and 125 μ l FBS-free Grace's medium with 8 μ l lipofectamine were mixed to incubate cells in 1 ml FBS-free Grace's medium for 12 h.

Subsequently, cells were cultured in Grace's medium supplemented with FBS for 12 h and incubated with 20E or JH III for 12 h. For the RNAi experiment in larvae, dsRNA was diluted in nuclease-free water to 100 ng/ μ l. Approximately 5 μ l of dsRNA were injected in a sixth instar 6 h larva. The control larvae were injected with 5 μ l *dsGFP* individually. Experiments were carried out in 30 larvae and repeated thrice for statistical analysis.

Hematoxylin-eosin staining

The dewaxed histological sections were stained with hematoxylin for 10 min at room temperature. The sections were washed consecutively with running water and Scott liquid for 1 min. The sections were then differentiated with 1% hydrochloric acid in alcohol for 20 s, washed with Scott liquid for 1 min, stained with 0.5% water-soluble eosin dye solution for 30 s, and washed with running water. After sealing, the sections were observed under an Olympus BX51 fluorescence microscope.

Co-immunoprecipitation

The open reading frame (ORF) of the genes was cloned into the pLEX-4-vector fused with red fluorescent protein (RFP) or green fluorescent protein (GFP) using the primers ([Supplementary Table S1](#)) respectively. USP1-RFP-His, EcRB1-His, Met1-GFP-His, and Met1-RFP were over-expressed in HaEpi cells via transfection with Cellfectin II Reagent (Invitrogen) according to the method described previously ([Hou et al. 2012](#)). After 48 h of culture, cells were incubated with 1 μ M 20E or JH III for 2 h. DMSO treatment was used as the control, and RFP-His was expressed as the control. The proteins were extracted from cells using the RIPA buffer, three wells of six well plate/800 μ l (0.1 M Tris-HCl buffer, pH 8.0 containing 0.15 M NaCl, and 1% NP-40) and harvested by centrifugation at 12 000 g for 20 min at 4 °C. Approximately 30 μ l to 40 μ l of supernatant were used as input proteins. The rest of the supernatant was added the antiserum against Met1 (1:100), incubated for 3 h with gentle shaking at 4 °C, and then incubated with 40 μ l Protein A resin for 3 h with gentle shaking at 4 °C. After centrifugation at 12 000 g for 10 min at 4 °C, the supernatant was discarded. After being washed with PBS, the resin was treated with the SDS-PAGE loading buffer and boiled for 10 min for western blot analysis with various antibodies. The molecular masses of the proteins are shown in [Supplementary Figure S3](#) (see section on supplementary data given at the end of this article).

Electrophoretic mobility shift assay

Various proteins were overexpressed in HaEpi cells. The nuclear proteins were extracted from HaEpi cells and diluted to 1 µg/µl. *T. castaneum* *Krh1*-JHRE-digoxin-labeled double-stranded probe contains E-box (sequences: 5'-GCGG-TGGGCTCCACGTGTCGAACG-3' and 3'-CGTTCGA-CACGTGGAGGCCACCGC-5'). The E-box 'CACGTG' is conserved in *Apis mellifera*, *Acyrtosiphon pisum*, *B. mori*, *D. melanogaster*, *N. vitripennis*, and *T. castaneum* (Kayukawa *et al.* 2012). *H. armigera* HR3-EcRE (Liu *et al.* 2014) digoxin-labeled double-stranded probe (sense 5'-gggtccgggtcaat-gaactgtgtga-3' and antisense 5'-tcacagcagttcattgacccgg-gacc-3') was synthesized by Shengong (Shanghai, China). The probes were incubated with nuclear proteins for 20 min at room temperature in a binding buffer (Beyotime, Shanghai, China). The detailed method was described in our previous paper (Liu *et al.* 2014). The 100 fmol probes were incubated with 5 µg nuclear proteins for 20 min at room temperature in 15 µl binding buffer (Beyotime, Shanghai, China). A fifty-fold amount of non-labeled-probe was used as specific competitor. For antibody mediated super shift assay, antiserum (1:2000) was incubated with the nuclear proteins in reaction mixture for 20 min, followed by incubation with a digoxin-labeled probe for another 20 min. The protein-DNA complex was separated from the free probe in 5.75% non-denaturing polyacrylamide gel.

Results

The full cDNA sequence of *Met1* is 2410 bp, with an ORF of 1581 bp, and encodes 526 amino acid proteins with a molecular mass of 59 kDa. Met1 has the typical characteristics of b-HLH-PAS family proteins with four structural domains of b-HLH, two PAS, and one PAC. Met1 of

H. armigera is similar to Met1 of *B. mori* (Supplementary Figure S1, see section on supplementary data given at the end of this article). HLH is a DNA-binding domain (Murre *et al.* 1989). PAS is used as a signal sensor domain (Taylor & Zhulin 1999). PAC is a motif C-terminal to PAS, which is proposed to contribute to the PAS domain fold by SMART analysis (<http://smart.embl-heidelberg.de/>).

Met1 expression level increased during feeding but decreased during metamorphosis

To study the function of Met1, we examined the expression profile during development from fifth instar to prepupae. Met1 showed a higher expression level at the fifth instar feeding stage (F) and the sixth instar feeding stage (F) compared with the fifth instar molting stage (M) and the metamorphic molting stage (MM) in the integument, midgut, and fat body (Fig. 1A and a). This expression pattern suggests that Met1 expression level is regulated during larval development.

Considering that JH titer is higher during feeding and 20E is higher during molting and metamorphosis in *M. sexta* (Riddiford *et al.* 2003), we injected sixth instar 6 h larvae with JH III and 20E to analyze the hormone regulation in Met1 expression. Results showed that 20E and JH III upregulated the expression of Met1 in the midgut independently. However, the higher concentration of 20E repressed Met1 expression (Fig. 1B and b). These results suggest that Met1 expression is coordinately regulated by JH and 20E.

Knockdown of *Met1* causes precocious pupation by changing the gene expression

Met1 was knocked down by injecting dsRNA of *Met1* into 6th instar 6 h larvae to examine the function of Met1 in

Figure 1

Western blot analysis showing Met1 expression profiles during larval development. The specificity of the antibody against *H. armigera* Met1 is shown in Supplementary Figure S2, see section on supplementary data given at the end of this article. (A) Profile of Met1 expression in the integument, midgut, and fat body. F, feeding; M, molting; MM, metamorphic molting. β-actin was used as the loading control. (a) Quantification of Met1 expression in (A) using the ImageJ software

(National Institutes of Health, <http://imagej.nih.gov/ij/download.html>) based on three independent experiments. (B) The 6th instar 6 h larvae were injected with JH III, 20E (500 ng/larva), or JH III (500 ng/larva) plus 20E (250, 500, 1000, and 2500 ng/larva) for 1, 3, 6, and 12 h, respectively, and the midgut proteins were examined. An equal volume of DMSO is the hormone solvent control. β-actin was used as the loading control. (b) Quantification analysis of hormone induction of Met1 in B using the ImageJ software.

the larval development. The knockdown of *Met1* caused precocious pupation (Fig. 2A and B). The pupation time from 6th instar 0 h to pupa in *dsMet1*-injected larvae was 92 h, which was 28 h more precocious than the 120 h

in *dsGFP*-injected control larvae (Fig. 2C). These results suggest that *Met1* is involved in maintaining the larval status.

JH III was injected in the 6th instar 6 h larval hemocoel to examine the involvement of *Met1* in JH III function of preventing metamorphosis. In the DMSO-injected control, the midgut performed normal remodeling by separating from the imaginal midgut 70 h after the first injection. By contrast, injection of JH III prevented midgut remodeling. *dsGFP* injection did not repress the JH III-prevented midgut remodeling. However, *dsMet1* injection repressed the JH III-prevented midgut remodeling (Fig. 2D). These results suggest that *Met1* is involved in the JH III function to prevent metamorphosis.

To address the mechanism of *Met1* knockdown causing precocious metamorphosis, we examined a set of gene expression after *Met1* knockdown in larvae, including the 20E pathway genes *EcRB1*, *USP1*, *HR3*, and *Br*, and JH pathway gene *Krh1* using qRT-PCR. *EcRB1*, *USP1*, *HR3*, and *Br* significantly increased in the midgut after *Met1* knockdown compared with the *dsGFP*-injected control. However, *Krh1* was downregulated after *Met1* knockdown (Fig. 2E). These results suggest that *Met1* knockdown caused precocious metamorphosis because of the change in gene expression, and *Met1* is a promoter of JH pathway gene expression and a repressor of 20E pathway gene expression.

Figure 2

Knockdown of *Met1* causes precocious pupation by altering the gene expression. (A) Phenotypes after knockdown of *Met1* (injection of *dsMet1* to 6th instar 6 h larvae, 500 ng/larva, twice in 48 h interval). (B) Western blot analysis showed the efficacy of *Met1* knockdown. (C) Statistical analysis of the pupation time of 50% larvae (P_{50}) after *Met1* knockdown (30 larvae, three replicates) by Student's *t*-test. (D) HE staining showed the midgut remodeling 70 h after first *dsMet1* injection. DMSO as the JH III solvent control; JH III injection; *dsGFP* and JH III injection (12 h after *dsGFP* injection); and *dsMet1* and JH III injection (12 h after *dsMet1* injection). A total of 500 ng of dsRNA or JH III per larva. Lu, midgut lumen; LM, larval midgut; IM, imaginal midgut. Bar is 50 μ m. (E) Gene expression in the midgut after *Met1* knockdown in larvae (500 ng *dsMet1*/larva, extracted RNA 48 h after first injection). Asterisks indicate significant differences between the groups ($P < 0.05$) by Student's *t*-test based on three independent experiments. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0019>.

Knockdown of *Met1* in HaEpi cells represses gene expression in JH pathway and promotes gene expression in 20E pathway

To confirm the conclusion regarding the *Met1* knockdown in larvae, the function of *Met1* in JH and 20E pathways was examined in HaEpi cells by RNAi. JH III increased expression of *Met1*, *USP1*, *Br*, and *Krh1* transcripts. However, after knockdown of *Met1*, JH III could not increase expression of these gene transcripts (Fig. 3A). By contrast, 20E increased expression of *Met1*, *EcRB1*, *USP1*, *HR3*, *Br*, and *Krh1* transcripts in cells. However, after knockdown of *Met1*, 20E induced expression of *EcRB1*, *USP1*, *HR3*, and *Br* to a greater extent (Fig. 3B). These results confirm that JH via *Met1* promotes *USP1*, *Br*, and *Krh1* expression, and *Met1* inhibits 20E-induced *EcRB1*, *USP1*, *HR3*, and *Br* expression.

Overexpression of *Met1* in HaEpi cells promotes JH III-induced gene expression and represses 20E-induced gene expression

To corroborate the data in *Met1* knockdown in HaEpi cells, we induced overexpression of *Met1* in HaEpi cells by fusing

Figure 3

qRT-PCR analyses showing gene expression in HaEpi cells after *Met1* knockdown. (A) Decreased gene expression in JH induction. (B) Increased gene expression in 20E induction (1 μg/ml of dsMet1, 1 μM JH or 20E for 12 h). Asterisks indicate the significant differences between the groups ($P < 0.05$) by Student's *t*-test based on three independent experiments.

it at the front of RFP in the pEx-RFP-His vector. The overexpression of RFP-His alone is used as the nonspecific protein control. The red fluorescent signal was detected in the entire cell in the RFP-expressing cells. However, the red fluorescent signal was detected in the nucleus in *Met1*-RFP-expressing cells (Fig. 4A). The expression of *Met1*-RFP-His was confirmed by western blot analysis (Fig. 4B). The overexpressed *Met1*-RFP-His increased the JH III-induced expression levels of *USP1*, *Br*, and *Krh1* compared with the RFP-His control (Fig. 4C). By contrast, the overexpressed *Met1*-RFP-His repressed 20E-induced *EcRB1*, *USP1*, *HR3*, and *Br* expression (Fig. 4D). This result confirms that *Met1* promotes the JH pathway gene expression and represses 20E pathway gene expression.

Met1 interacts with Hsp90 and binds to *Krh1*-JHRE

Met binds to the E-box 'CACGTG' in the core region of kJHRE (GGCCTCCACGTG) and the E-box is conserved in six species (*A. mellifera*, *A. pisum*, *B. mori*, *D. melanogaster*,

N. vitripennis, and *T. castaneum*) (Kayukawa *et al.* 2012). To confirm that JH III regulates *Krh1* transcription via *Met*, we overexpressed *Met1*-RFP in HaEpi cells and performed an electrophoretic mobility shift assay (EMSA) experiment using kJHRE (GGCCTCCACGTG) in the *Krh1* promoter of *Tribolium* as a probe. In the RFP-expressing cells, the nuclear extracts from normal cells, or DMSO- or 20E-treated cells could not change the mobility of kJHRE (Fig. 5A and lane 1, 2, and 3). However, the nuclear extracts from JH III-treated RFP-expressing cells changed the mobility of kJHRE (Fig. 5A and lane 4), suggesting that JH III-induced nuclear proteins can bind to kJHRE. In the *Met1*-RFP-expressing cells, the nuclear extracts from DMSO- or 20E-treated cells could not change the mobility of kJHRE (Fig. 5A and lanes 5 and 6). However, the nuclear extracts from JH III-treated cells changed the mobility of kJHRE and increased the amount of mobilized kJHRE compared with RFP-expressing control cells (Fig. 5A and lane 7 compared with lane 4). When the 50-fold excess of unlabeled kJHRE was added, the amount of the mobilized kJHRE decreased (Fig. 5A and lane 8 compared with lane 7), suggesting that *Met1*-RFP binds to kJHRE. After the addition of the antibodies against either *Hsp90* or *Met1*, the mobilized kJHRE band disappeared (Fig. 5A and lane 9 and 10). The data were statistically analyzed (Fig. 5B). These results confirm that *Met1*-RFP binds to kJHRE, and *Hsp90* is necessary for this binding.

Given that *Met1* can interact with *Hsp90* to regulate the gene expression in the JH pathway in *H. armigera* (Liu *et al.* 2013), Co-IP was conducted to confirm the interaction between *Met1* and *Hsp90* in JH induction. *Hsp90* was co-precipitated by an anti-*Met1* antibody from the JH III-treated cells, but not from the 20E-induced cells (Fig. 5C and D). These findings suggest that *Met1* interacts with *Hsp90* and binds to kJHRE to promote *Krh1* transcription in the JH pathway.

Met1 represses *EcRB1*-*USP1* interaction

Considering that the overexpression of *Met1* repressed 20E-induced gene transcription, we performed an EMSA experiment by examining the binding of *EcRB1* to EcRE in *H. armigera* *HR3* (5'-GGGGTCAATGAAGT-3' and 3'-CAGTTCATTGACCCC-5') (Liu *et al.* 2014) to investigate the mechanism. In the RFP-expressing control cells, when the nuclear extracts from DMSO- or JH III-induced RFP-expressing cells were incubated with Dig-EcRE, no mobilized Dig-EcRE was detected (Fig. 6A and lanes 2 and 4). However, Dig-labeled EcRE was mobilized by the nuclear extracts from 20E-treated cells (Fig. 6A and lane 3

Figure 4

The overexpression of Met1 in HaEpi cells promotes JH-induced gene expression and represses 20E-induced gene expression. (A) Overexpression of Met1-RFP-His in HaEpi cells. RFP (red); Met1-RFP (red); DAPI indicates the cell nucleus (blue); and merge is the overlapped red and blue. Bar: 20 μm. (B) Western blot analysis showing the expression of RFP (35 kDa) and Met1-RFP-His (94 kDa) in HaEpi cells, as detected using the

anti-His antibody. (C and D). JH and 20E (1 μM for 12 h) induction of the gene expression in Met1-RFP-His overexpressing cells by qRT-PCR analysis respectively. Asterisks indicate significant differences between the groups ($P < 0.05$) by Student's *t*-test based on three independent experiments. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0019>.

compared with lane 2), and the non-Dig-labeled EcRE competitor could compete to decrease the amount of mobilized Dig-EcRE (Fig. 6A and lane 5 compared with lane 3), suggesting 20E-induced nuclear extracts binding to the Dig-EcRE probe. The overexpressed Met1-RFP decreased the 20E-induced amount of mobilized Dig-EcRE (Fig. 6A and lane 7 compared with lane 3). The non-Dig-labeled EcRE competitor decreased the amount of mobilized Dig-EcRE (Fig. 6A and lane 8 compared with lane 7). The antibody against Met1 increased the amount of mobilized Dig-EcRE (Fig. 6A and lane 9 compared with lane 7). JH III repressed the binding of nuclear extracts to the Dig-EcRE probe (Fig. 6A and lane 10). The EcRB1-His was overexpressed in cells to confirm 20E-induced binding of EcRB1 to EcRE. The amount of mobilized Dig-EcRE by 20E-induced nuclear extracts (Fig. 6C and lane 3) was decreased using a anti-His antibody that recognizes EcRB1-His (Fig. 6C and lane 4), indicating that the nuclear protein in the nuclear extracts binding to EcRE was EcRB1. Statistical analysis showed the significant difference in the amount of the mobilized Dig-EcRE in various treatments (Fig. 6B and D). These results suggest that Met1 represses the EcRB1 binding to EcRE.

To address the mechanism of overexpressed Met1 repressing the EcRB1 binding to EcRE, we induced

overexpression of EcRB1-His, USP1-RFP, and Met1-GFP in HaEpi cells simultaneously. Three proteins were equally expressed in cells, as shown in the input proteins (Fig. 6E, Input). From the co-immunoprecipitates (Co-IP) by anti-RFP that precipitated USP1-RFP as well as the interacting proteins, Met1-GFP and USP1-RFP, were detected either in the DMSO- or JH III-treated cells, whereas, EcRB1-His was detected together with Met1-GFP and USP1-RFP in 20E-treated cells. After addition of JH III to cells, the amount of 20E-induced EcRB1-His was reduced and that of Met1-GFP was increased (Fig. 6E, Co-IP). Statistical analysis revealed that the differences in the protein amounts in various treatments were significant (Fig. 6E and F). These results suggest that Met1 interacts with USP1-independent hormone regulation; however, 20E promotes the interaction between EcRB1 and USP1, and JH increases the interaction between USP1 and Met1.

Given that USP is phosphorylated by 20E induction and the phosphorylation of USP is essential for its function in gene transcription (Sun & Song 2006, Wang *et al.* 2012), the phosphorylation status of USP1 was examined by a 7.5% low-concentration SDS-PAGE gel to demonstrate the mechanism of EcRB1 and USP1 interaction in 20E induction. The overexpressed USP1-His in HaEpi cells partially increased the molecular mass 6 h after 20E

Figure 5

Met1 interacts with Hsp90 and binds to kJHRE. (A) EMSA analysis of Met1-Hsp90 binding to kJHRE. Lane 1, normal cells; lanes 2, 3, and 4, RFP-overexpressing cells treated with DMSO, 20E, or JH III (1 μ M for 6 h); lanes 5, 6, 7, 8, 9, and 10, Met1-RFP-overexpressing cells treated with DMSO, 20E, JH III (1 μ M for 6 h), competitor kJHRE, or antibodies respectively. kJHRE, as a non-labeled competitor, is in 50-fold excess of the labeled probe; and anti-Met1 and anti-Hsp90 are the rabbit polyclonal antibodies against *H. armigera*. (B) Statistical analysis of the data in (A). Asterisks indicate significant differences between the groups ($P < 0.05$)

by Student's *t*-test based on three independent experiments. (C) Co-IP experiments show that JH regulates Met1 and Hsp90 interaction. Input: protein expression levels of Hsp90 and Met1 in various treated cells. Co-IP: Met1 was immunoprecipitated with an anti-*Helicoverpa*-Met1 antibody, whereas co-precipitated Hsp90 was detected by an anti-*Helicoverpa*-Hsp 90 antibody. β -actin was used as the loading control in the experiments. (D) Statistical analysis of the data in Co-IP in (C). Asterisks indicate significant differences between the groups ($P < 0.05$) by Student's *t*-test based on three independent experiments.

treatment, compared with DMSO and JH III treatments. The increased molecular mass of USP1-His was decreased upon lambda protein phosphatase treatment, indicating that it was the phosphorylated form of USP1 (Fig. 6G). These results suggest that 20E regulates USP1 phosphorylation to interact with EcRB1, and JH III maintains USP1 nonphosphorylation to interact with Met1.

Discussion

Met is undoubtedly an important transcription factor with a crucial function in insect development. However, the function and mechanism of Met in regulating insect development are not well understood because of its complicated roles in either the JH pathway or the 20E pathway. In this research, we demonstrated that Met1 promotes JH-regulated *Krh1* transcription and represses 20E-induced *EcRB1*, *USP1*, *Br*, and *HR3* transcription to maintain the larval status. The expression level of Met1 is upregulated in larval life when JH is present, i.e. during the feeding stages. It is downregulated during molting and metamorphosis, thus in stages when the JH titer is lowered or even zero. In these stages, the ecdysteroid titer is increased. JH mediates Met1 binding

to kJHRE via interaction with Hsp90 to promote *Krh1* transcription. Met1 represses EcRB1/USP1 binding to EcRE for *EcRB1*, *USP1*, *Br*, and *HR3* transcription by interacting with USP1.

Met1 promotes *Krh1* transcription to maintain the larval status

Tribolium Met functions in JH response to mediate the antimetamorphic effect of JH (Konopova & Jindra 2007). Double mutation of *Met* and *gce* in *Drosophila* larvae totally blocks the *Krh1* expression (Abdou *et al.* 2011). *Krh1* is a JH early-inducible gene identified in *D. melanogaster* (Minakuchi *et al.* 2008). Knockdown of *Tribolium* *Krh1* causes precocious metamorphosis (Minakuchi *et al.* 2009). In our study on *H. armigera*, *Met1* knockdown causes precocious pupation and midgut remodeling. Correlated with the precocious pupation, *Met1* knockdown repressed the *Krh1* expression. These results are similar to the observations in *Drosophila* and *Tribolium*. Therefore, Met1 promotes the JH pathway in *H. armigera*. The function of Met1 at the level of the nucleus is the maintenance of the larval status.

Figure 6

Overexpressed Met1 represses EcRB1 binding to EcRE. (A) EMSA analysis of nuclear protein binding to EcRE. Lane 1, the nuclear extracts from normal cells; lanes 2, 3, 4, and 5, the nuclear extracts from the RFP-overexpressing control cells treated with DMSO, 20E, JH III (1 μ M for 6 h), and a competitor (non-labeled probe EcRE, 50-fold molar excess of Dig-labeled probe EcRE); and lanes 6, 7, 8, 9, and 10, the nuclear extracts from Met1-RFP-overexpressing cells treated with DMSO, 20E, competitor, anti-Met1, and JH III. (B) Statistical analysis of data in (A) using Student's *t*-test (asterisks indicate significant differences ($P < 0.05$) between the groups based on three independent experiments for all the following statistical analyses). (C) EMSA analysis of EcRB1 binding to EcRE in the induction of 20E. (D) Statistical analysis of data in B by Student's *t*-test. (E) Co-IP showing the protein interaction among Met1, EcRB1, and USP1 (12.5% SDS-PAGE gel). pLEX-4T1-EcRB1-His, pLEX-4T1-USP1-RFP, and pLEX-4T1-Met1-GFP

plasmids were co-transfected in cells. Input: anti-His detected EcRB1-His; anti-GFP detected Met1-GFP; anti-RFP detected USP1-RFP; and anti- β -actin detected β -actin in various treatments. Co-IP: USP1-RFP was immunoprecipitated with an anti-RFP monoclonal antibody. Co-precipitated EcRB1-His and Met1-GFP were detected by anti-His or anti-GFP antibodies, respectively, in cells treated with DMSO, 20E, JH III, and 20E plus JH III (1 μ M for 6 h). β -actin was used as the loading control in the experiments. (F) Statistical analysis of data in (E) by Student's *t*-test. (G) Western blot analysis showed the phosphorylation of USP1 in the induction of 20E (7.5% SDS-PAGE gel). USP1-His-overexpressing cells were treated with DMSO, 20E, or JH III (1 μ M for 6 h). The 20E-treated protein was incubated with Lambda protein phosphatase (λ -phosphatase) with β -actin as the loading control. (H) Statistical analysis of data in (G) by Student's *t*-test.

JH regulates PKC-phosphorylated Hsp90 and interaction with Met1 to participate in *Krh1* expression (Liu *et al.* 2013). In this study, we further revealed that the complex of Hsp90 and Met1 binds to kJHRE to promote *Krh1* transcription. Considering the critical function of *Krh1* in the suppression of insect metamorphosis (Kayukawa *et al.* 2012), we proposed that JH maintains the larval status by upregulating Met1 expression and then directing the *Krh1* expression.

The fact that JH acts not only through a nuclear receptor but through a membrane receptor(s) as well should not be overlooked. According to De Loof *et al.* (2014), a low cytoplasmic Ca^{2+} concentration is causal to maintaining the larval status of holometabolous insects. At least some of the proteins that constitute the Ca^{2+} homeostasis system have a binding site for sesquiterpenoids, JH being one of them. At a higher Ca^{2+} concentration, Ca^{2+} -induced programmed cell death, a typical aspect of metamorphosis, is

activated. JH may play a role through the membrane receptor and protein kinase C in the accessory gland of male fruit flies and the follicle of vampire bugs (Sevala & Davey 1993). However, the specific membrane receptor of JH needs to be elucidated.

Met1 represses the transcription of a set of 20E-responsive genes to prevent metamorphosis

Met1 represses a set of 20E-responsive gene transcription, including *EcRB1*, *USP1*, *HR3*, and *Br*. EcRB1 and USP1 form a transcription complex and bind to EcRE to initiate gene transcription (Jindra *et al.* 1996). HR3 is a reporter gene in the 20E pathway that is involved in molting and metamorphosis (Lam *et al.* 1999). Br is a metamorphosis initiator that promotes programmed cell death gene expression (Cakouros *et al.* 2002). Knockdown of *Met1* helps 20E to induce *EcRB1*, *USP1*, *HR3*, and *Br*

Figure 7

Chart to interpret the function and mechanism of Met1 in regulating larval development. (1) JH upregulates Met1 expression and interaction with phosphorylated Hsp90 and nonphosphorylated USP; Met1 binds to kJHRE to promote gene transcription to maintain the larval status. (2) The hormone 20E represses Met1 expression and regulates USP1 phosphorylation to interact with EcRB1; EcRB1/USP1 binds to EcRE to promote 20E-responsive gene transcription for metamorphosis. (3) Met1 interacts with USP1 to repress the interaction between EcRB1 and USP1 and its binding to EcRE. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0019>.

transcription, thus promoting precocious metamorphosis, suggesting that Met1 is a repressor of the 20E pathway.

By EMSA experimental analysis, the overexpressed Met1 can repress EcRB1 binding to EcRE. This might be due to the fact that Met1 interacts with USP1 that disturbs the formation of EcRB1/USP1 complex and its binding to EcRE. USP1 is the heterodimeric partner of EcRB1 in the formation of a transcription complex for gene transcription in the 20E pathway. JH also binds to USP1 and participates in JH-regulated gene transcription, suggesting its role in JH pathway (Jones & Sharp 1997). We found that 20E regulates USP1 partially phosphorylated to interact with EcRB1. JH maintains USP1 nonphosphorylation to repress its interaction with EcRB1 and increases its interaction with Met1. However, the interaction between Met1 and USP1 may not rely on JH regulation because the overexpressed Met1 is co-immunoprecipitated with nonphosphorylated USP1 in DMSO-treated cells also. The 20E- or JH hormone-independent interactions between Met and USP and between Met and EcRB1 has been reported in *D. melanogaster* (Bitra & Palli 2009) and *B. mori* (Guo *et al.* 2012) respectively. Therefore, Met1 might repress 20E pathway gene transcription by repressing the formation of EcRB1/USP1 transcription complex and its binding to EcRE.

Expression of Met1 is coordinately regulated by JH and 20E

In *Tribolium*, Met is continuously expressed throughout embryonic development (Konopova & Jindra 2007).

In *Bombyx*, the developmental profiles of Met1 and Met2 from day 2 of the 4th instar to day 2 of the prepupal stage, when the 20E titer is high, have been demonstrated by Guo *et al.* (2012). In *H. armigera*, Met1 is expressed during the entire larval stage from the 5th instar stage up to the prepupal stage. However, the expression levels increased during the feeding stage and decreased at molting and later metamorphic stages. The different expression profiles in various insects may be attributed to species specificity. JH III and 20E may independently upregulate Met1 expression. However, a higher concentration of 20E represses Met1 expression. Thus, Met1 is coordinately regulated by the two hormones.

Conclusion

This study deepens our understanding of how interactions among a number of key transcription factors contribute, at the level of the nucleus, to the maintenance of the larval status by JH. JH upregulates the expression of Met1 and interaction with phosphorylated Hsp90 and nonphosphorylated USP1, which binds to kJHRE to promote *Krh1* transcription for larval growth. The hormone 20E mediates the interaction between EcRB1 and phosphorylated USP1. Met1 interacts with USP1 to repress the formation of EcRB1/USP1 complex and its binding to EcRE and, therefore, to repress 20E-induced gene transcription and metamorphosis (Fig. 7).

The challenge for future experiments is to uncover which genes are activated by the JH- and 20E-dependent transcription factors. Perhaps, the transcription of genes coding for some of the proteins that make up the Ca^{2+} homeostasis system, which seems to play a crucial role in realizing some JH-dependent effects, is controlled this way.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-14-0019>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

W-L Z performed the major experiments, C-Y L cloned the gene, W L helped on EMSA, D W performed overexpression experiments, J-X W directed the study, and X-F Z designed the study and wrote the final version of the manuscript.

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