Hormonal regulation and developmental role of Krüppel homolog 1, a repressor of metamorphosis, in the silkworm Bombyx mori

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A R T I C L E   I N F O

Article info
Received 3 September 2013
Received in revised form 17 January 2014
Accepted 26 January 2014
Available online 4 February 2014

Keywords:
Krüppel homolog 1
Juvenile hormone
Ecdysteroid
Molt
Metamorphosis
Ecdysone-inducible gene

A B S T R A C T

Juvenile hormone (JH) has an ability to repress the precocious metamorphosis of insects during their larval development. Krüppel homolog 1 (Kr-h1) is an early JH-inducible gene that mediates this action of JH; however, the fine hormonal regulation of Kr-h1 and the molecular mechanism underlying its ant metamorphic effect are little understood. In this study, we attempted to elucidate the hormonal regulation and developmental role of Kr-h1. We found that the expression of Kr-h1 in the epidermis of penultimate-instar larvae of the silkworm Bombyx mori was induced by JH secreted by the corpora allata (CA), whereas the CA were not involved in the transient induction of Kr-h1 at the prepupal stage. Tissue culture experiments suggested that the transient peak of Kr-h1 at the prepupal stage is likely to be induced cooperatively by JH derived from gland(s) other than the CA and the prepupal surge of ecdysteroid, although involvement of unknown factor(s) could not be ruled out. To elucidate the developmental role of Kr-h1, we generated transgenic silkworms overexpressing Kr-h1. The transgenic silkworms grew normally until the spinning stage, but their development was arrested at the prepupal stage. The transgenic silkworms from which the CA were removed in the penultimate instar did not undergo precocious puation or larval–larval molt but fell into prepupal arrest. This result demonstrated that Kr-h1 is indeed involved in the repression of metamorphosis but that Kr-h1 alone is incapable of implementing normal larval molt. Moreover, the expression profiles and hormonal responses of early ecdysone-inducible genes (E74, E75, and Broad) in transgenic silkworms suggested that Kr-h1 is not involved in the JH-dependent modulation of these genes, which is associated with the control of metamorphosis.

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Introduction

Insect metamorphosis is coordinated by the actions of ecdysteroids and juvenile hormone (JH), which are secreted by the prothoracic gland and corpora allata (CA), respectively. In holometabolous insects, 20-hydroxyecdysone (20E), the active metabolite of ecdysteroids, induc es the larval–larval molt in the presence of JH, whereas in the absence of JH, it induces larval–pupal and pupal–adult molts (Riddiford, 1994). In many insects, the topical application of JH analogs during larval development inhibits normal metamorphosis, leading to supernumerary larval molt or prepupal arrest (Retnakaran et al., 1985; Kamimura and Kiuchi, 2002). Conversely, the deprivation of JH by the removal of CA (allactectomy) from the penultimate larvae induces precocious metamorphosis (Dominick and Truman, 1985).

Thus, the major function of JH is to prevent larvae from precociously turning into adults (status quo action) (Riddiford, 1994).

The molecular actions of 20E in larval–pupal metamorphosis have been elucidated in detail: 20E binds to a complex of the ecdysone receptor (EcR) and ultraspiracle (USP), both of which belong to the nuclear receptor superfamily. The liganded EcR/USP complex directly activates the transcription of early ecdysone-inducible genes such as E74, E75, and the Broad complex (Broad), all of which are transcriptional regulatory factors. The early ecdysone-inducible genes subsequently regulate large groups of late ecdysone-inducible genes, some of which are involved in the formation of pupal traits (Dubrovsky, 2005; Nakagawa and Henrich, 2009). JH has been reported to modify the expression of several isoforms of the early ecdysone-inducible genes in Manduca sexta (Stilwell et al., 2003; Keshan et al., 2006; Zhou et al., 1998), but the molecular mechanism underlying this modulation remains largely unknown.

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http://dx.doi.org/10.1016/j.ydbio.2014.01.022
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Minakuchi et al. (2008) revealed for the first time that the Krüppel homolog 1 (Kr-h1) gene, a C2H2 zinc-finger type transcription factor, is a JH-early-inducible gene and represses the metamorphic differentiation of adult abdominal epidermis in *Drosophila melanogaster*. This gene plays a key role in the repression of larval–pupal and nymphal–adult transitions in holometabolous and hemimetabolous insects, respectively (Minakuchi et al., 2009; Lozano and Belles, 2011; Konopova et al., 2011). Recent studies clarified the mechanism underlying JH-mediated induction of *Kr-h1* in insects: JH binds to its receptor, methoprene tolerant (Met) (Wilson and Fabian, 1986; Ashok et al., 1998; Miura et al., 2005; Charles et al., 2011); then, JH-ligated Met interacts with steroid receptor coactivator (SRC) (Li et al., 2011; Zhang et al., 2011; Kayukawa et al., 2012), and the JH/Met/SRC complex activates *Kr-h1* by interacting with JH response element (kJHRE) in the *Kr-h1* gene (Kayukawa et al., 2012, 2013). Although *Kr-h1* plays a crucial role in the repression of insect metamorphosis, only a few studies have attempted to elucidate its molecular mechanisms. In *D. melanogaster*, mutations in *Kr-h1* cause changes in the expression patterns of edcsyne-inducible genes such as E74A, E75B, E93, HR3, and FJFZ-F1 during metamorphosis (Pecas et al., 2000). Recently, *Kr-h1* was found to be required for the suppression of *Broad* in the fat body of young *D. melanogaster* larvae (Huang et al., 2011). These studies suggest that *Kr-h1* may repress metamorphosis via modification of the expression of early-edcsyne inducible genes. However, no further studies have been conducted to elucidate the molecular mechanisms of the repression of metamorphosis by *Kr-h1*.

In the present study, we investigated the hormonal regulation of *Kr-h1* across the postembryonic development of *B. mori*, and we analyzed the functions of *Kr-h1* in the larval–larval molt and larval–pupal transition by using transgenic silkworms overexpressing *Kr-h1*. Based on our findings, we reviewed the JH signaling cascade from the viewpoint of the mode of action of *Kr-h1* in the repression of metamorphosis.

### Material and methods

#### Experimental animals and dissection

*B. mori* (Kinsyu × Showa strain) larvae were reared on an artificial diet (Nosan Corp, Japan) at 25 °C under a 12-h light/dark photoperiod. The epidermis was dissected from the insects at various stages in phosphate-buffered saline (137 mM NaCl, 8 mM KCl, 2.7 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4). The integuments of larvae were cultured in phosphate, pH 7), each at a concentration of 0.2 mg/mL. To generate a transgenic vector, *BmKr-h1α* was coinjected with the helper plasmid into preblastoderm G1 embryos were screened under a fluorescence stereomicroscope (20E, Sigma-Aldrich), or both, for 3 h.

#### qPCR

Total RNA was extracted from the epidermis by using the RNeasy Plus mini kit (Qiagen) and was used to synthesize the cDNA with the PrimeScript RT reagent kit (Takara Bio). The primers designed to quantify the transcripts of *BmKr-h1* (all isoforms), *BmKr-h1α*, *BmKr-h1β*, *BmBRC* (all isoforms), *BmE74A*, *BmE74B*, *BmE74C*, *BmE75A*, *BmE75B*, *BmE75C*, *BmE75D*, and *BmRp49* are shown in Table S1. *BmRp49* was used as the internal reference. The reaction was carried out in a 10-μL reaction volume containing the template cDNA derived from 1 ng of total RNA, 5 μL of SYBR Premix Ex Taq (Takara Bio), and 0.2 μM of each primer by using the LightCycler 480 real-time thermal cycler (Roche). The PCR conditions were 95 °C for 5 min, followed by 55 cycles each at 95 °C for 5 s and 60 °C for 20 s. The relative molarities of the gene transcripts were obtained with crossing point analysis involving standard curves generated using plasmids containing a fragment of each gene. The expression levels of the target genes were normalized against that of *BmRp49*.

#### Results

##### Developmental expression profile

Developmental changes in the expression of *BmKr-h1α* and *BmKr-h1β*, two isoforms of *Kr-h1*, in the epidermis of *B. mori* were determined by qPCR (Fig. 1). Although the levels of *BmKr-h1α* were approximately tenfold higher than those of *BmKr-h1β* (see the inset of Fig. 1), the overall expression patterns of the two isoforms were very similar. The transcripts of *BmKr-h1α/β* were
continuously detected during the 3rd and 4th instar stages, with fluctuations (Fig. 1). The *BmKr-h1*α/β transcripts disappeared completely by day 1 of the 5th instar, reappeared when the larvae began spinning (day 6), peaked during the prepupal stage (days 7–8), and disappeared again at 1 d before pupation (day 9) (Fig. 1). In the pupal stage, the expression of *BmKr-h1*α/β remained undetectable for the first half of the stage, but gradually increased from day 6 toward the end of the stage, and the high level was maintained during the adult stage (Fig. 1). Little difference was observed between the expression levels of *BmKr-h1*α/β in females and males. The expression patterns of *BmKr-h1*α/β were closely correlated with the titer of ecdysteroids in the hemolymph of *B. mori*. Notably, the peaks of the transcripts on day 3 of the 4th-instar larvae and at the prepupal stage corresponded well with those of ecdysteroids (Fig. 1).

**Hormonal regulation of BmKr-h1 expression at the prepupal and adult stages**

In the epidermis of the 4th-instar larvae, the transcription of *BmKr-h1* is positively regulated by JH (Kayukawa et al., 2012). To examine whether *BmKr-h1* is regulated by JH also in other stages, we removed the CA from 4th-instar larvae on day 3. Because the allatectomized larvae had already committed to larval–larval molt, they normally molted to the 5th instar and became normal pupae and normal adults. The *BmKr-h1* transcripts in the allatectomized adults were drastically decreased irrespective of the sex (Fig. 2A). The transcript levels in the allatectomized adults treated with JHA remained high in both sexes (Fig. 2A), suggesting that the expression of *BmKr-h1* in the adult stage is also induced by JH secreted by the CA. In contrast, the *BmKr-h1* transcript level in the prepupal stage was barely affected by the allatectomy (Fig. 2B). Given the occurrence of a peak ecdysteroid titer at the prepupal stage (Fig. 1), this finding led us to consider the possibility that the expression of *BmKr-h1* in the allatectomized larvae was induced by the ecdysteroids (Fig. 1). We therefore examined the effect of 20E and JH on the cultured epidermis of day-5 5th-instar larvae, in which the expression of *Kr-h1* was undetectable (Fig. 1). Contrary to our expectations, 20E alone did not induce the expression of *Kr-h1* (Fig. 2C). However, JHA significantly induced the *Kr-h1* expression and 20E enhanced the induction by 2.3-fold (Fig. 2C).

**Phenotypes of B. mori overexpressing BmKr-h1α**

To investigate the function of *Kr-h1* in vivo, we generated transgenic silkworms overexpressing *BmKr-h1α* (A3-GAL4/+; UAS-BmKr-h1α/+; referred to as *BmKr-h1α*O/E silkworms for brevity). Because the expression level of *BmKr-h1β* was by far lower than that of *BmKr-h1α* throughout the development (Fig. 1) (Kayukawa et al., 2012), we focused on the function of *BmKr-h1α* in this study.

The level of the *BmKr-h1* transcripts in the epidermis of the 4th-instar *BmKr-h1αO/E* silkworms was approximately fourfold higher than that of the control (+/+; UAS-BmKr-h1α/+; ) (Fig. 3A and B, IV0). The allatectomy of the control 4th-instar larvae on day 0 resulted in the depletion of the *BmKr-h1* transcripts, and the level of transcripts was restored by the topical application of JHA (Fig. 3A). In contrast, allatectomy and JHA barely affected the
expression of BmKr-h1 in the BmKr-h1αO/E silkworm (Fig. 3B), indicating that the high level of BmKr-h1 expression is mostly attributable to the transgene. The BmKr-h1αO/E silkworms showed normal development until the 5th instar and started spinning at the same time as the control insects (day 5). However, they spun less silk than the control insects and produced an unusually thin-skinned cocoon (Fig. 4A and B). The growth of BmKr-h1αO/E silkworms was eventually arrested at the prepupal stage (prepupal arrest) (Table 1 and Fig. 4B). At this stage, the formation of a new pupal cuticle was observable in the dorsal abdomen under the apolysed old cuticle (Fig. 4B’ and B’ arrowheads). Although no difference was found between the body weights of BmKr-h1αO/E and control silkworms until the spinning stage, the BmKr-h1αO/E larvae thereafter tended to be heavier than the controls (Fig. S1). The silk glands in BmKr-h1αO/E silkworms on day 3 in the spinning stage were substantially larger than those of the control larvae (Fig. 4E), suggesting that the difference in the larval weight is mainly attributable to the remaining unspun silk protein. Thus, the overexpression of BmKr-h1α appeared to have no significant effect on the larval development but did affect the metamorphic processes.

To further analyze the function of BmKr-h1 as a repressor of metamorphosis, we allatectomized the 4th-instar larvae of BmKr-h1αO/E on day 0 and observed their phenotypes. The allatectomized control larvae (+/+ , UAS-BmKr-h1α/+ ) underwent precocious metamorphosis at the end of the 4th instar. They formed small thick cocoons and became miniature pupae (Table 1, Fig. 4C and C). The allatectomized BmKr-h1αO/E larvae formed thin cocoons and entered precocious prepupal arrest, without molting into the 5th instar (Table 1 and Fig. 4B, B’, D, and D’). When JHA was topically applied to the allatectomized BmKr-h1αO/E larvae, they molted into the normal 5th instar and eventually entered prepupal arrest, as observed in the intact BmKr-h1αO/E larvae (Table 2).

Overexpression of BmKr-h1α does not affect early ecdysone-inducible genes

To address the molecular actions of BmKr-h1 in the repression of the metamorphosis, we investigated the expression of early ecdysone-inducible genes in BmKr-h1αO/E silkworms. Although the expression level of BmKr-h1 in normal 5th-instar larvae decreased as they aged, that in the transgenic silkworms was high throughout the feeding stage, including the time critical for pupal commitment (Muramatsu et al., 2008) (Fig. 5A).

BmBRC is a transcription factor found in the epidermis and is crucial for pupal commitment (Muramatsu et al., 2008); however, the ectopic expression of BmKr-h1α did not induce any change in the expression of BmBRC, compared with that for the controls (Fig. 5B). The expression of other transcription factors examined in this study (i.e., BmE74A, BmE74B, BmE75A, BmE75B, BmE75C, and BmE75D) was also barely affected by the overexpression of BmKr-h1α (Fig. 5), indicating that BmKr-h1 does not modulate the expression of the early ecdysone-inducible genes in the epidermis during the larval period investigated in this study, which includes the time critical for larval–pupal commitment.

We further analyzed whether the ectopic expression of BmKr-h1 altered the responsiveness of the early ecdysone-inducible genes to JH and/or ecdysone by tissue culture experiments. When the epidermis of day-2 5th-instar larvae of normal and transgenic B. mori were cultured with the hormones for 24 h, the expression of BmBRC was induced by 20E regardless of the overexpression of BmKr-h1α (Fig. 6A and B). The induction of BmBRC by 20E was suppressed by JH I also, regardless of overexpression (Fig. 6). Moreover, there was no or little difference between the control and BmKr-h1αO/E silkworms with respect to the responsiveness of
other early genes (E74 isoforms and E75 isoforms) to JH and/or 20E (Fig. S2).

Discussion

Kr-h1 has been shown to be induced by JH and to mediate the ant metamorphic action of JH during the larval or nymphal stage in several insect species (Minakuchi et al., 2009; Lozano and Belles, 2011; Konopova et al., 2011). In a previous study, we reported that the expression of BmKr-h1 is also induced by JH in the penultimate-instar larvae of B. mori (Kayukawa et al., 2012). However, in the present study, we found that the expression of Kr-h1 is not always regulated in the same way across postembryonic development.

In the adult stage, Kr-h1 is induced by JH and has been supposed to contribute to the reproductive maturation in Aedes aegypti and T. castaneum (Zhu et al., 2010; Parthasarathy et al., 2010). Kr-h1 is also induced by JH in the adults of the male moth Agrotis ipsilon and is suggested to be involved in the maturation of the behavioral and central nervous responses to female sex pheromone (Duportets et al., 2012). The drastic decrease in BmKr-h1 transcript levels in the allatectomized adults and their restoration by JHA suggested that the expression of BmKr-h1 in the adult stage of B. mori is also induced by JH secreted by the CA. However, since these adults showed no abnormality in reproductive maturation and sexual behavior, the function of Kr-h1 in adult B. mori is yet to be clarified.

Allatectomy of B. mori larvae showed that the CA are not involved in the induction of BmKr-h1 at the prepupal stage. In D. melanogaster, the expression of Kr-h1 is reported to be upregulated by 20E, and Kr-h1 is involved in the ecdysone signaling pathway (Pecasse et al., 2000; Beckstead et al., 2005). Our tissue culture experiments have shown that 20E alone does not induce BmKr-h1 at the prepupal stage, but significantly synergizes the induction by JHA (Fig. 2C). The prepupal peak of Kr-h1 also occurs in T. castaneum (Minakuchi et al., 2009). This peak coincided with the peak of expression of JHAMT, a key enzyme for JH biosynthesis (Minakuchi et al., 2008), suggesting the involvement of JH in the prepupal induction of Kr-h1. Although the CA did not show any JH synthetic activity at the prepupal stage (Kinjoh et al., 2007), JH was indeed detected in the hemolymph of the larvae at this stage (Niimi and Sakurai, 1997; Furuta et al., 2013). Taken together, the transient peak of BmKr-h1 at the prepupal stage is likely to be induced cooperatively by JH originating from non-CA tissues and the prepupal surge of ecdysteroid. Nevertheless, we cannot rule out the possibility that the induction is due to unknown factor(s) other than JH and ecdysteroid.

In the tobacco hornworm, M. sexta, the CA of the late last instar larvae do not produce JH; instead, the CA secrete JH acid into the hemolymph, which is supposedly converted to JH by JHAMT present in the imaginal discs (Sparagana et al., 1985). Meanwhile, de novo biosynthesis of JH by non-CA tissues (accessory glands) has been found in male mosquitoes of A. aegypti (Borovsky et al., 1994). Since the expression of JHAMT was not detected in the CA but was clearly detected in the testes and ovaries of the prepupal silkworm (Shinoda and Itoyama, 2003), these tissues are likely candidates for JH synthesis during this stage. Whether these tissues produce JH de novo or by the conversion of JH acid secreted by the CA remains to be determined.

Intriguingly, allatectomy of the final instar larvae of other lepidopteran species such as Hyalophora cecropia and M. sexta caused partial precocious adult development during larval–pupal transition (Williams, 1961; Kiguchi and Riddiford, 1978), suggesting that JH originating from the CA is necessary for preventing premature development of imaginal structures at the prepupal stage (Riddiford, 2012). However, as mentioned above, allatectomized B. mori developed into normal pupae. We hypothesized that the role of Kr-h1 at the prepupal stage in holometabolous insects is to prevent precocious adult differentiation and that the allatectomized B. mori can pupate normally because the expression of BmKr-h1 at the prepupal stage is induced in a CA-independent manner characteristically in this species. In order to test this hypothesis, the prepupal expression of Kr-h1 in allatectomized larvae of H. cecropia or M. sexta should be examined in the future.

We investigated the functions of BmKr-h1 by using transgenic silkworms. Silkworms overexpressing BmKr-h1α enter prepupal arrest at the end of the 5th instar (Table 1 and Fig. 4). RNAi-mediated knockdown of Kr-h1 in several insects has shown that Kr-h1 plays a crucial role in the repression of insect metamorphosis (Minakuchi et al., 2009; Lozano and Belles, 2011; Konopova et al., 2011). However, RNAi cannot be used for functional analysis of the BmKr-h1 gene because RNAi does not work efficiently in B. mori. Using a gain-of-function approach, the present study directly proved that BmKr-h1 functions as a repressor of larval–pupal metamorphosis. Moreover, when the BmKr-h1αOE silkworms were allatectomized in the 4th instar, they did not undergo the next larval
molt and eventually entered prepupal arrest, while the topical application of JHA rescued this failure (Table 2). These results suggest that BmKr-h1 alone is not capable of completely repressing the larval–pupal transition; that is, an additional JH-mediated factor(s) is required to implement larval–larval molt normally.

Several early ecdysone-inducible genes, such as E74, E75, and Broad, are involved in larval–pupal transition [Dubrovsky, 2005; Nakagawa and Henrich, 2009], and their expression has been reported to be modulated by JH. Experiments using the epidermis of the final instar M. sexta larvae showed that E74B, E75D, and

**Table 1**
Effect of allatectomy on the phenotype of *B. mori* overexpressing BmKr-h1a.

<table>
<thead>
<tr>
<th>Operation*</th>
<th>N</th>
<th>4th</th>
<th>5th</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Molting arrest</td>
<td>Prepupal arrest</td>
<td>Pupation</td>
</tr>
<tr>
<td>+/+, UAS-BmKr-h1a/+</td>
<td>Sham</td>
<td>15</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CAX</td>
<td>19</td>
<td>–</td>
</tr>
<tr>
<td>A3-GAL4/+, UAS-BmKr-h1a/+</td>
<td>Sham</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CAX</td>
<td>14</td>
<td>14</td>
</tr>
</tbody>
</table>

Sham, sham operation; CAX, allatectomy.

* The corpora allata (CA) were removed from the larvae on day 0 of the 4th instar.
Table 2
Effect of allatectomy and JHA treatment on the phenotype of *B. mori* overexpressing BmKr-h1α.

<table>
<thead>
<tr>
<th>Operationa</th>
<th>Treatment</th>
<th>N</th>
<th>4th Molting arrest</th>
<th>Preupal arrest</th>
<th>Pupation</th>
<th>5th Molting or molting arrest</th>
<th>Preupal arrest</th>
<th>Pupation</th>
</tr>
</thead>
<tbody>
<tr>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>10</td>
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<tr>
<td></td>
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<td>–</td>
<td>9</td>
<td>–</td>
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<td>1</td>
</tr>
<tr>
<td></td>
<td>CAX Acetone</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>6</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>A3-GAL4/+, UAS-BmKr-h1α/+</td>
<td>Sham Acetone</td>
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<td>–</td>
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<td>10</td>
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<td>–</td>
<td>–</td>
<td>1</td>
</tr>
</tbody>
</table>

JHA, juvenile hormone analog (methoprene); Sham, sham operation; CAX, allatectomy.

a The CA was removed from the larvae on day 0 of 4th instar.

Fig. 5. Effect of the overexpression of BmKr-h1α on the developmental expression of the early ecdysone-inducible genes. The expression of *BmKr-h1* (A), *BmBRC* (B), *BmE74A* (C), *BmE74B* (D), *BmE75A* (E), *BmE75B* (F), *BmE75C* (G), and *BmE75D* (H) in the epidermis of days 3–7 5th-instar larvae were determined by q-PCR. Mean ± SD (4 replicates) have been provided.
induction was suppressed by JH (Stilwell et al., 2003; Keshan et al., 2006; Zhou et al., 1998). The current knowledge on the function of Broad (unspecified isoform) in B. mori epidermis of day-2 5th-instar larvae were quantified by qPCR after exposure to several combinations of 100 ng/mL 20E and 500 ng/mL JH I for 24 h in vitro. (A) Control larva (+/+, UAS-BmKr-h1α/+ ) and (B) BmKr-h1α-overexpressing larva (A3-GAL4/+, UAS-BmKr-h1α/+ ). Mean ± SD (4 replicates).

Fig. 7. JH signaling pathways involved in larval molt and metamorphosis in the epidermis of B. mori, inferred from the present study. The red arrows and characters represent the new findings of the present study.

Broad (unspecified isoform) were induced by 20E and that their induction was suppressed by JH (Stilwell et al., 2003; Keshan et al., 2006; Zhou et al., 1998). The current knowledge on the function of Kr-h1 in the control of metamorphosis is summarized as follows: (1) Kr-h1, which is induced by JH, represses the premature expression of Broad during the larval stage. (2) At the onset of metamorphosis, when JH is depleted, Broad is induced by a small surge of ecdysosterone in the absence of Kr-h1, which leads to larval–pupal transition (Riddiford, 2012; Hiruma and Kaneko, 2013). Indeed, the larval–pupal metamorphosis was interrupted by the overexpression of BmKr-h1α (Table 1 and Fig. 4). However, the formation of the pupal cuticle and the induction of Broad by 20E in the epidermis were not suppressed by the overexpression of BmKr-h1α although they were suppressed by JH until the occurrence of pupal commitment (Figs. 4 and 7) (Muramatsu et al., 2008). These findings indicate that (a) JH regulates metamorphosis through Met and Kr-h1, and (b) a separate JH signaling pathway, which does not involve Kr-h1, plays an essential role in the modulation of ecdysone response (Fig. 7).

In conclusion, the following two important findings on the JH signal pathways in B. mori were obtained in the present study (Fig. 7): first, ecdysosterone synergizes the induction of Kr-h1 by JH. Second, Kr-h1 regulates metamorphosis but not via modulating early ecdysone-inducible genes. Identification of the targets of Kr-h1 will contribute to better understanding of the molecular mechanism underlying the control of metamorphosis in insects.

Acknowledgments

We thank Drs. Chieka Minakuchi and Toshiki Namiki for technical assistance and Ms. Yumiko Satoh for rearing the silk-worms. This study was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), JSPS KAKENHI Grant Number 25850230, and the NIAS Strategic Research Fund.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.01.022.

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