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Edysone receptor isoforms play distinct roles in controlling molting and metamorphosis in the red flour beetle, *Tribolium castaneum*

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Abstract

Ecdysteroids regulate insect growth and development through a heterodimeric complex of nuclear receptors consisting of edysone receptor (EcR) and ultraspiracle (USP). In the red flour beetle, *Tribolium castaneum*, two isoforms each of EcR and USP have been identified. Quantitative real-time reverse-transcriptase PCR (qRT-PCR) analysis showed isoform-specific developmental expression of both EcR and USP in the epidermis and the midgut dissected from the final instar larvae and pupae. Injection of double-stranded RNA (dsRNA) prepared using the common or isoform-specific regions of EcR or USP as templates caused derailment of development. EcR common region (EcRC) or EcRA dsRNA caused more severe effects, and most of the treated larvae died prior to pupation. EcRB dsRNA caused less severe effects and most of the treated larvae became pupae but showed developmental defects. Only dsRNA prepared against USP common region but not against USPA or USPB isoform-specific region caused developmental defects during larval-pupal metamorphosis. Determination of mRNA levels of EcR isoforms and 20-hydroxyecdysone-response (20E) genes (broad, E75, E74, HR3 and FTZ-F1) by qRT-PCR in the larvae injected with EcRA, EcRB or EcRC dsRNA showed that EcRA initiates ecdysteroid action by regulation the expression of EcRB and 20E-response genes. These data suggest that the EcR but not USP isoforms play distinct roles during the larval-pupal metamorphosis and EcRA plays a dominant role in transduction of ecdysteroid response in *T. castaneum*.

Keywords

Ecdysteroid; Metamorphosis; Nuclear receptors; Gene regulation; Ultraspiracle

1. Introduction

Ecdysteroids and juvenile hormones play critical roles in the regulation of insect growth and development. The most active form of ecdysteroids, 20-hydroxyecdysone (20E), transduces its signal through a heterodimeric complex of two nuclear receptors, the edysone receptor (EcR) and the ultraspiracle (USP), a homolog of the vertebrate retinoid X receptor (RXR) (Yao et al., 1993). The high affinity binding of ecdysteroids to the EcR requires the presence of its heterodimeric partner, USP. Recent studies showed that the USP of *T. castaneum* adopts

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an apo structure and therefore is not able to bind to ligands (Iwema et al., 2007). The EcR and USP heterodimer binds to the hormone response elements present in the promoters of ecdysone-response genes and regulate their expression (King-Jones and Thummel, 2005; Palli et al., 2005).

In *Drosophila melanogaster*, the EcR gene encodes three isoforms, EcRA, EcRB1 and EcRB2 that contain unique amino termini but a common carboxy-terminal region including DNA-binding and ligand-binding domains (Talbot et al., 1993). These three isoforms showed distinct temporal and spatial expression patterns and exhibited functional differences during development (Talbot et al., 1993; Bender et al., 1997). Mutational analysis of EcR isoforms identified several different lethal phases, including developmental arrest during embryogenesis and failure of pupariation (Bender et al., 1997; Davis et al., 2005). Using inducible expression of double-stranded RNA (dsRNA) in *D. melanogaster*, EcR was demonstrated to be essential for larval molting and metamorphosis (Lam and Thummel, 2000). The larvae that had dsRNA synthesis induced by heat shock died as prepupae with defects in larval tissue cell death and adult leg formation (Lam and Thummel, 2000). EcR isoforms, especially EcRA and EcRB1, have been identified in several other insects (Palli et al., 2005 and references cited there in). In a hemimetabolous insect, *Blattella germanica*, RNAi experiments showed that EcRA is required for adult-specific developmental processes including wing development, prothoracic gland degeneration and normal choriogenesis (Cruz et al., 2006).

USP is a homologue of vertebrate RXR and heterodimerizes with EcR (Yao et al., 1992; Yao et al., 1993). In *D. melanogaster*, there is only one USP protein that functions as a repressor during postembryonic development, repressing gene activity *in vivo* when ecdysteroid titers are low (Schubiger and Truman, 2000; Ghbeish and McKeown, 2002). Reduction of maternal and zygotic USP functions results in embryonic lethality (Henrich et al., 1994; Ghbeish et al., 2001). By contrast, two isoforms of USP have been identified in insects belonging to the orders Lepidoptera, *Manduca sexta* (Lan et al., 1999); Diptera, *Aedes aegypti* (Wang et al., 2000) and *Chironomus tentans* (Vogtli et al., 1999). In *B. germanica*, when USP RNA was knocked down during the nymphal stage by using RNAi, the nymphs molted but arrested their development at the end of the nymphal stage, and were unable to molt into adults (Martin et al., 2006).

In the present study, we identified two isoforms each of EcR and USP in the red flour beetle, *Tribolium castaneum*. The developmental expression pattern of these two receptors was investigated using the real time quantitative reverse transcriptase PCR (qRT-PCR). Injection of dsRNA prepared using the common or isoform-specific region of EcR induced distinct phenotypes as well as changes in the expression pattern of 20E-regulated genes. In contrast, dsRNA prepared from only common region but not isoform specific region of USP caused developmental defects. These data showed that EcR but not USP isoforms play distinct roles during metamorphosis and EcRA plays a dominant role in transduction of ecdysteroid response in the red flour beetle, *T. castaneum*.

2. Materials and methods

2.1. Beetle strains and staging

Beetles were reared in whole wheat flour containing 10% yeast at 32°C. GA-1 strain kindly provided by Dr. Beeman of USDA was used in all the experiments. Newly ecdysed final instar larvae were separated based on the white head character and staged from that time onwards (Tan and Palli, 2008). To investigate the mRNA expression of selected genes, the final instar larvae, pre-pupae (the quiescent stage) and pupae were staged at 12 hr intervals.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from epidermis and midgut dissected from pooled 20 larvae or 30 pupae at each time point using TRI reagent (Molecular Research Center Inc., Cincinnati, OH), according to the manufacturer's instructions. The RNA was treated with DNase I (Ambion Inc., Austin, TX) and cDNA synthesis was performed using 2 µg total RNA and iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) in a 20 µl reaction volume.

2.3. Cloning of isoform-specific A/B domain for ecdysone receptor and RXR

The isoform-specific A/B domains for ecdysone receptor (EcR) and ultraspiracle (USP) were amplified by PCR using the isoform-specific primers (Table 2) derived from the genomic information in the Beetlebase and NCBI. PCR conditions were as follows: initial incubation of 94 °C for 2 min was followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min. PCR amplified fragments were gel-purified by resolving the PCR products on a 1% agarose gel. The purified PCR products were sequenced using Big-Dye version 1.1 Terminator Cycle sequencing kit and ABI sequencer (Applied Biosystems, Foster City, CA).

2.4. Double-stranded (ds) RNA synthesis

Genomic DNA was extracted from *T. castaneum* adults and purified using the DNeasy Tissue Kit (QIAGEN). DNA for *in vitro* transcription was amplified using the genomic DNA as a template. Primers for EcR or USP common and isoform-specific regions were designed by adding T7 polymerase promoter sequence at their 5' ends (Table 2). A 303 bp fragment for EcR common region, a 386 bp fragment for EcRA, a 228 bp fragment for EcRB, a 230 bp fragment for USP common region, a 97 bp fragment for USPA and a 436 bp fragment for USPB were PCR amplified. The dsRNA were transcribed directly from the PCR products using the MEGAscript RNAi Kit (Ambion Inc., Austin, TX) according to the manufacturer's protocol. For annealing the dsRNA, the reaction products were incubated at 75 °C for 5 min and cooled to room temperature. After the treatment with DNase, the dsRNA was purified using phenol/chloroform extraction followed by the ethanol precipitation.

2.5. Microinjection

The final larval instar larvae or the female pupae were anaesthetized with ether vapor for 4–5 min and aligned on a glass slide with double-sided tape on it. The dsRNA were dissolved in distilled water, and injected into larvae between the first and the second abdominal segment or pupae between the third and the fourth abdominal segments, using an injection needle pulled out from a glass capillary tube (Idaho technology). Nearly 0.8–1 µg (0.1 µl) dsRNA was injected into each larva or pupa. The dsRNA prepared from an 800 bp fragment from *male* gene of *Escherichia Coli* was used as the control. Injected larvae or pupae were removed from the slide and raised in whole wheat flour with 10% yeast at 32°C.

2.6. Ecdysone agonist application

To evaluate the effect of EcR knock-down on the expression of ecdysone response genes an ecdysteroid agonist, methoxyfenozide (Dhadialla et al., 1998) was used. Acetone containing technical grade methoxyfenozide (1 µg per larva) was topically applied on the dorsal surface of larvae on the second day of the final instar.

2.7. Semi-quantitative RT-PCR and quantitative real-time RT-PCR (qRT-PCR)

Semi-quantitative reverse transcription PCR (RT-PCR) was carried out to investigate the mRNA expression level of targeted genes after RNAi. RT-PCR conditions were as follows: initial incubation of 94 °C for 2 min was followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min. The relative mRNA expression of selected 20E-response genes was assessed

by qRT-PCR using MyiQ single color real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). All the qRT-PCR reactions were performed using the common program as follows: initial incubation of 95 °C for 3 min was followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, 72°C for 30 s. At the end of each cycle, a fluorescence reading determined the extent of amplification. Standard curves were obtained using a ten fold serial dilution of pooled cDNA from all the stages. Quantitative measurements were performed in triplicate and normalized to an internal control of *T. castaneum* ribosomal protein *rp49* mRNA for each sample.

3. Results

3.1. EcR and USP sequences

Sequence information on *Tribolium* EcR and USP available in the public database (<http://www.bioinformatics.ksu.edu/BeetleBase/>, <http://www.ncbi.nlm.nih.gov> and <http://www.hgsc.bcm.tmc.edu/>) was used to analyze the organization of these genes as well as to design primers to amplify isoform-specific regions. EcR isoform-specific regions were PCR amplified and the PCR products were sequenced. Analysis of sequences showed that EcR gene from *T. castaneum* codes for two isoforms that contain different amino terminal A/B domain and a common region containing CDEF domains. EcRA specific region contained 160 amino acids and showed the highest similarity with EcRA isoform cloned from *Leptinotarsa decemlineata* (Table 1) (Ogura et al 2005). The EcRA specific sequence from *T. castaneum* also showed a similarity with EcRA isoform-specific sequences identified in *Choristoneura fumiferana* (Perera et al 1999), *Bombyx mori* (Kamimura et al., 1997), *Aedes aegypti* (Wang et al., 2002), *Drosophila melanogaster* (Talbot et al., 1993) and *Locusta migratoria* (Saleh et al., 1998). The EcRB isoform-specific region contained 96 amino acids and these amino acids showed the highest similarity with the amino acids identified in the EcRB isoform of *Leptinotarsa decemlineata* (Ogura et al 2005). As shown in Table 1, the amino acids identified in the EcRB isoform-specific region of *T. castaneum* also showed a similarity with the amino acids identified in the EcRB isoforms of *Tenebrio molitor* (Mouillet et al., 1997), *Celuca pugilator* (Chung et al., 1998) and *Daphnia magna* (Accession # AB274824.1). EcR ORF contains seven exons, four of these exons code the common region present in both EcRA and EcRB isoforms (Fig. 1A). EcRA isoform-specific region is coded by the two exons and the EcRB isoform-specific region is coded by the one exon.

Sequences analysis of USP also identified two isoforms that are designated as USPA and USPB. USP ORF is coded by nine exons, of which five exons code the common region present in both isoforms, three, two exons code for USPA isoform-specific region and four exons code for USPB isoform-specific region (Fig. 1B). The USPA isoform contained 44 amino acids in the amino terminal end that are absent in the USPB isoform. As shown in Table 1, the 44 amino acid region of USPA specific region showed high similarities with the USPA isoforms identified in *Manduca sexta* (Jindra et al., 1997), *Aedes aegypti* (Kapitskaya et al 1996), *Chironomus tentans* (Vogtli et al., 1999), *Melipona scutellaris* (Accession # AY840093), *Apis mellifera* (The honey bee genome consortium, 2006) and *Scaptotrigona depilis* (Accession # ABB00308). The *T. castaneum* USPB isoform contained 188 amino acid insertions in the ligand binding domain. The N-terminal 60 amino acids of this insertion showed the highest homology with a conserved region present in neuralized and neuralized-like proteins belonging to a group of ubiquitin ligases and play a role in Notch pathway-mediated cell fate decisions during development of the *D. melanogaster* nervous system (Marchler-Bauer et al., 2007).

3.2. Developmental expression profiles of EcR and USP

To investigate the expression pattern of EcR and USP mRNA in the epidermis, which is responsible for cuticle synthesis, and in the midgut, whose remodeling is regulated by 20E and JH (Wu et al., 2006, Parthasarathy and Palli, 2007), we performed qRT-PCR by using the

cDNA synthesized using the RNA isolated from the tissues dissected from staged final instar larvae and pupae. EcR and USP isoforms showed distinct expression patterns in the epidermis and midgut (Fig. 2). In the epidermis, the EcRA mRNA showed two peaks of expression, one in the middle of quiescent stage and the other in the middle of the pupal stage (Fig. 2A). In the midgut, EcRA mRNA showed only one peak at the time when the larvae entered the quiescent stage (Fig. 2B). EcRB showed a more complicated expression pattern, and three peaks of EcRB mRNA were detected: at 72 hr after ecdysis into the final instar (AEFI), at 12 hr after entering the quiescent stage and at the beginning of the pupal stage (Fig. 2A). In the midgut, the EcRB mRNA showed only one peak at the beginning of the quiescent stage (Fig. 2B). In both the epidermis and midgut, EcRB mRNA levels increased at the end of pupal stage prior to adult emergence (Fig. 2).

In contrast, the developmental expression pattern of USPA and USPB during the final instar larval and pupal stages is similar. In both epidermis and midgut about 10-fold higher USPA mRNA was present when compared to the levels of USPB mRNA. In the epidermis, the USPA mRNA levels started to increase at the beginning of last instar larval stage and reached the maximum levels at 48 hr AEFI. Then, the USPA mRNA levels declined and stayed low throughout the pupal stage, except for a small rise at the beginning of the pupal stage (Fig. 2A). In the midgut, the USPA mRNA levels were low at the beginning of the final instar larval stage, began to increase after 48 hr AEFI, and reached the maximum levels by 72 hr AEFI (Fig. 2B). Then, the USPA mRNA levels declined and reached the minimum levels by 96 hr AEFI. Soon after the larvae entered the quiescent stage, the USPA mRNA levels increased again and reached the maximum levels by 12 hr after the larvae entered the quiescent stage. These high levels of USPA mRNA were maintained throughout the quiescent stage. The USPA mRNA levels were low at the beginning of the pupal stage, increased from 24hr AEP and reached the maximum by 48 hr AEP. These levels were maintained until 72 hr AEP. The USPB mRNA showed the similar expression pattern as the USPA mRNA in both the epidermis and the midgut, except for a few changes: in the epidermis, the increase in USPB mRNA levels during the early stages of final instar larvae continued until 72 hr AEFI and in the midgut, the USPB mRNA levels declined at 72 hr AEP prior to adult emergence.

3.3. Functional analysis of EcR and USP by RNA interference

To investigate biological functions of EcR and USP during development, especially during metamorphosis in *T. castaneum*, we injected dsRNAs prepared using isoform-specific regions of EcR or USP as templates into the final instar *T. castaneum* larvae. At five days after microinjection, semi-quantitative RT-PCR was performed to examine if the mRNA levels of targeted genes were reduced by RNAi. Total RNA extracted from whole body homogenates was subjected to semi-quantitative RT-PCR. The levels of EcR or USP mRNA were lower in insects injected with respective dsRNA than in control larvae injected with *malE* dsRNA (Fig. 3). Thus, the dsRNA targeting the EcR or USP mRNA caused the reduction in their mRNA levels, suggesting that microinjection of dsRNA was effective at silencing these genes in *T. castaneum*.

All the larvae injected with EcRA dsRNA arrested their development during the quiescent stage and died (Table 3 and Fig. 4A). None of them could progress through the larval stage when nearly 1 µg of dsRNA was injected into each larva. We also injected lower concentrations of dsRNA to determine if there is a dose-dependent effect. Most of larvae could not survive to the adult stage even when the amount of dsRNA injected into each larva was reduced to 0.04 µg. At this dose, a few larvae survived and became pupae and these pupae showed abnormal phenotypes in wing extension (Fig. 4B). Wings are formed during the larval-pupal metamorphosis and cover two-third of the abdomen of the pupa. Sclerotization of the forewings occurs in the pharate adult and the elytra are completely developed covering the hindwings. In

the adults developed from larvae injected with EcRA dsRNA during the final instar larval stage both the forewings and hindwings were short and because of under development of wings as a result the hindwings were exposed unlike in the normal adults. Compared to the lethal effect caused by EcRA dsRNA, the larvae injected EcRB dsRNA progressed in larval development and some of them became pupae. About 20% of injected insects (n=60) arrested development during the larval stage and showed development of compound eyes that normally occurs during the pupal stage (Table 3 and Fig. 4C). Nearly 60% injected animals (n=60) survived to the adult stage, and most of them exhibited defects in the wing development (Fig. 4D). Injection of EcR dsRNA prepared using the EcR common region (EcRC) as a template showed a similar effect observed in EcRA dsRNA injected insects (Table 3). Almost all injected larvae died during the quiescent stage.

Compared to the diverse phenotypes caused by the EcR dsRNA, dsRNA prepared using USPA and USPB isoform-specific regions showed no significant phenotypes. More than 80% of the injected larvae (n=50) survived to the adult stage, and no developmental defects were observed (Table 3). When the USP common region (USPC) dsRNA was injected, all the larvae died during the quiescent stage prior to pupation (Fig. 4A). The phenotypes observed in USPC dsRNA injected insects are similar to the phenotypes observed in either EcRA or EcRC dsRNA injected insects (Table 3). The fact that no developmental defects were observed after injection of USPA or USPB dsRNA was not due to the failure of RNAi, because the levels of the mRNA decreased after injection of respective dsRNA (Fig. 3). These data suggest that the USP isoforms may compensate each other's function during metamorphosis.

3.4. Effect of EcR RNAi on the expression of 20E response genes

To identify genes that are involved in the EcR action of *T. castaneum*, we investigated the expression of 20E-response genes in EcR dsRNA-injected animals by using qRT-PCR. The expression levels of ecdysone inducible early genes, broad (*br*), E74, E75, an ecdysone-inducible delayed-early gene hormone receptor 3 (HR3) and a nuclear receptor, which is known to be involved in ecdysone signal transduction, FTZ-F1, were investigated. We also investigated the effect of silencing of EcR isoforms on each other's expressions. The relative mRNA levels of these genes were compared with mRNA levels in cohorts injected with *male* dsRNA as a control. To determine the expression of these genes at the times of lower and higher ecdysteroid levels, the expression of these genes in dsRNA injected insects was monitored at 72, 90, 96 and 108 hr AEFI. Ecdysteroid levels were low at 72 hr and 96 hr but higher at 90 and 108 hr AEFI (Parthasarathy et al., 2008). When compared to their levels in control *male* dsRNA injected insects, the EcRA mRNA levels in the larvae injected with EcRA or EcRC dsRNA were lower at 90 and 108 hr AEFI (Fig. 5). The EcRB dsRNA injection did not affect EcRA mRNA levels. In contrast, when compared to its expression in control insects, the EcRB mRNA levels were lower in larvae injected with EcRA, EcRB or EcRC dsRNA (Fig. 5). The *br* mRNA levels in larvae injected with EcRA, EcRB or EcRC dsRNA were lower when compared to its levels in control insects. The expression of both E74 and E75 were lower in EcRA, EcRB or EcRC RNAi insects when compared to their levels in control insects. HR3 mRNA levels were higher in both EcRA and EcRB but not EcRC RNAi insects prior to the quiescent stage (108 hr AEFI). The injection of EcRA or EcRC dsRNA caused a reduction in FTZ-F1 mRNA levels at 90, 96 and 108 hr AEFI. In contrast, EcRB dsRNA caused an increase in FTZ-F1 mRNA levels prior to quiescent stage and a reduction in FTZ-F1 mRNA levels after the larvae entered the quiescent stage (108 hr AEFI, Fig. 5). These data showed that EcRA required for complete expression of EcRB and both isoforms of EcR play key roles in the expression of genes known to be involved in 20E signal transduction.

3.5. Ecdysone induction patterns of EcR isoforms

To investigate the distinct roles of EcR isoforms in *T. castaneum*, we performed an *in vivo* hormone induction experiment by application of an ecdysteroid agonist, methoxyfenozide, to the final instar larvae. After application, the mRNA levels of EcR isoforms were quantified using qRT-PCR. The mRNA levels of both EcRA and EcRB increased after the methoxyfenozide application. EcRA mRNA levels reached its peak at 6 hr after application while EcRB mRNA levels reached its peak at 12 hr after application, suggesting that EcRA may initiate ecdysteroid signal transduction in *T. castaneum* (Fig. 6).

4. Discussion

The major contribution from the current study is the discovery that the EcR but not USP isoforms have distinct functions during larval-pupal metamorphosis and EcRA is the key regulator of ecdysteroid response in *T. castaneum*. These conclusions are drawn based on data obtained from the mRNA developmental expressions, RNAi and hormone induction studies. QRT-PCR analyses of mRNA levels of EcR and USP isoforms showed distinct expression patterns of mRNA for EcRA and EcRB in both the epidermis and midgut, suggesting that these isoforms may have distinct functions during larval-pupal metamorphosis. This suggestion was confirmed by RNAi studies where either EcRA or EcRB or both were silenced. EcRA RNAi caused severe developmental defects and most larvae did not develop beyond the quiescent stage and died. In contrast, the EcRB RNAi caused less severe phenotypes and most larvae pupated, but the pupae showed developmental defects. Taken together, the developmental expression data and RNAi studies conclusively showed that EcR isoforms have distinct functions during larval-pupal metamorphosis.

In contrast to the EcR expression pattern, the expression pattern of USPA and USPB are similar in the last instar larval and pupal stages in both epidermis and midgut tissues, suggesting that these two receptors may be involved in similar functions. RNAi studies using USP isoform specific dsRNAs confirmed these suggestions. No developmental defects were observed when either USPA or USPB isoform specific dsRNA were injected into the final instar larvae. However, developmental defects similar to those seen in EcR RNAi insects were observed when dsRNA prepared using USP common region was injected into the final instar larvae. Taken together, the developmental expression data and RNAi studies conclusively showed that USP isoforms can compensate each others function at least during larval-pupal metamorphosis.

EcR RNAi studies in *T. castaneum* showed that the EcRA isoform plays a critical role during the larval stage. This is in contrast to the general belief based on the studies in several insects; these studies showed that EcRB isoform plays an important role in larval tissues, and EcRA isoform plays an important role in the development of adult structures. *Drosophila melanogaster* carrying mutations that inactivate all three isoforms of EcR are embryonic lethal (Bender et al., 1997). The EcRA mutants died during early to mid-pupal stages, suggesting that this protein is necessary for development of adult structures (Davis et al., 2005). The majority of EcRB1 and EcRB2 double mutants died during the early larval stage (Schubiger et al., 1998). The reduction in EcR levels through heat inducible dsRNA produced *in vivo* showed severe defects in molting, metamorphosis, programmed cell death of larval tissues and adult structures differentiation (Lam and Thummel, 2000). In contrast to what was observed in *D. melanogaster*, the injection of EcRA dsRNA into the last instar larvae of *T. castaneum* resulted in the death of injected larvae during the quiescent stage prior to pupation, and injection of EcRB dsRNA into last instar larvae caused only a few developmental defects during the larval stages. Most of the injected insects completed larval-pupal metamorphosis and showed some developmental defects during the pupal stage. These studies conclusively showed that EcRA is important for larval development. Then, why are the isoform specific-functions of EcR so different in *T. castaneum* compared to other insects? One possible explanation came

from qRT-PCR analysis of 20E-response genes in larvae injected with EcRA or EcRB dsRNA. These studies showed that the injection of EcRA dsRNA resulted in the reduction of both EcRA and EcRB mRNA levels. In contrast, the injection of EcRB dsRNA caused the reduction in EcRB mRNA but not EcRA mRNA suggesting that EcRA is required for the full expression of EcRB.

In addition, the qRT-PCR determination of mRNA levels of 20E-response genes in larvae injected with EcRA, EcRB or EcRC dsRNA showed that the changes in the expression of 20E-response genes are similar in insects injected with EcRA or EcRC dsRNA. For example, FTZ-F1 mRNA levels were reduced in larvae injected with EcRA or EcRC dsRNA compared to the FTZ-F1 mRNA levels in *malE* dsRNA injected larvae. In contrast, the larvae injected with EcRB dsRNA showed up-regulation of FTZ-F1 mRNA levels prior to entering the quiescent stage and caused a reduction after the larvae entered quiescent stage. These data suggest that EcRA and EcRB play distinct roles in regulation of FTZ-F1 expression.

In another experiment, the application of ecdysteroid analog methoxyfenozide induced the maximum expression of EcRA in 6 hr while the maximum expression of EcRB required 12 hr. This is in contrast to the induction pattern of EcRA and EcRB isoforms in other insects. For example, in the *Choristoneura fumiferana* midgut, fat body, and epidermis as well as CF-203 cells, EcRB mRNA was induced by 20E or a stable ecdysteroid agonist, tebufenozide, in one hour, and EcRA mRNA was induced in 3 hr (Perera et al., 1999). In the *Manduca sexta* day 2 fifth instar larval epidermis, both EcRA and EcRB mRNAs were induced by 3 hr of exposure to 20E (Jindra et al., 1997). However, in these experiments, the induction of EcRA and EcRB was tested only at 3 and 6 hr after adding 20E. Therefore, it is unknown whether there is a difference between EcRA and EcRB in response to 20E and whether EcRA or EcRB could be induced within 1 hr exposure to 20E. The data from RNAi and EcR mRNA induction studies showed that EcRA is required for the expression of EcRB. Therefore, EcRA RNAi likely caused severe defects in larval development by knocking-down the expression of both EcRA and EcRB.

Another interesting result is the increase of HR3 mRNA levels in larvae injected with EcRA and EcRB dsRNAs. These data suggest that EcRA and EcRB are involved in the suppression of HR3 expression at the time when the ecdysteroids are absent or at lower levels. The data presented here clearly showed that EcR is involved in the expression of the ecdysone response genes, including br, E74, E75, HR3 and FTZ-F1. In *D. melanogaster*, microarray analysis identified many ecdysone-response genes, including E74A, E75, Kr-h1, DHR3, DHR39, DHR78, FTZ-F1, Cyp18a1, L71, IMP-E2, IMP-L3, Fbp-2, Sgs-1 and urate oxidase, which are induced by 20E and depend on EcR for their expression (Beckstead et al., 2007). Taken together, the results of RNAi showed that the nuclear receptors of EcR and USP are essential for metamorphosis in *T. castaneum*. The EcRA isoform initiates ecdysteroid action by regulating the expression of the EcRB isoform and other early genes involved in ecdysteroid signal transduction.

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Fig. 1. Genomic organization of *TcEcR* and *TcUSP* genes. (A) *TcEcRA* and *TcEcRB* genomic organization. A1–A2, EcRA isoform-specific exons; B1, EcRB isoform-specific exons; C1–C3, C4, EcR common exons present in both isoforms. (B) *TcUSPA* and *TcUSPB* genomic structures. A1–A2, USPA isoform-specific exons; B1–B2, USPB isoform-specific exons; C1–C3, C5, USP common exons present in both isoforms. Exons mapped by comparison

of ORF and genomic sequences. Open boxes represent exon coding regions and solid boxes represent the regions used as a template to prepare dsRNA.

Fig. 2.

The developmental expression of EcR and USP mRNA in the epidermis and midgut. Insects that are in the final instar larval stage, quiescent stage and pupal stage were collected at 12 hr intervals. Total RNA was extracted from epidermis or midgut dissected from 20 larvae or 30 pupae at each time point. The levels of mRNA were determined by qRT-PCR using SYBR green I for detection and normalized to *T. castaneum rp49* ribosomal mRNA expression as an internal control. Mean+SE for three independently staged sets of larvae and pupae are shown.

Fig. 3.

Semi-quantitative RT-PCR analysis of RNA isolated from insects injected with EcR or USP dsRNA. The cDNA template was synthesized from total RNA extracted from larvae whole body homogenates which received *malE*, EcRA, EcRB, USPA or USPB dsRNA injection. The panels show the RT-PCR products for EcR or USP isoforms (top) and for *rp49* (bottom).

Fig. 4. *EcR* RNAi phenotypes. (A) RNAi larvae of *EcR* and *USP* arrested during the quiescent stage and underwent desiccation within 24 hr. (B) Low concentration ($0.04\mu\text{g}$) of *EcRA* dsRNA injected animals arrested during the quiescent stage, showing showed as a pupal-adult intermediate and defects in the wing development. Abnormal wing could not cover the dorsal side and animals subsequently died of dehydration. (C) Abnormal compound eye development in the *EcRB* RNAi larva. (D) *EcRB* RNAi animals showing abnormal phenotype exhibiting both pupal and adult structures.

Fig. 5.

The mRNA levels of 20E-regulated genes in larvae injected with *maleE*, EcRA, EcRB or EcRC (common region) dsRNA. The final instar larvae were injected with EcRA, EcRB or EcRC dsRNA at 12 hr AEFI and total RNA was extracted from whole body homogenates at 72 hr, 90 hr, 96 hr and 108 hr AEFI. The mRNA levels were determined by qRT-PCR and were normalized to *T. castaneum* rp49 expression. Mean $\pm$ SE (n=3) are shown. Top panel on the left shows ecdysteroid titers at 72, 90, 96 and 108 hr AEFI as reported in Parthasarathy et al 2008.

Fig. 6. Induction patterns of EcR isoforms. Methoxyfenozide was applied to the larvae on second day of the final instar larval stage and total RNA was extracted from larval whole body homogenates at 3 hr to 12hr after chemical application. The gene expression was determined by qRT-PCR and was normalized to *T. castaneum* rp49 expression. Mean $\pm$ SE (n=3) are shown. Lines with closed diamonds indicate EcRA expression and lines with open squares denote EcRB expression.

Table 1

Amino acid identity among selected EcRs and USPs.

	Number of isoform-specific amino acids	Percent identity
EcRA		
<i>Tribolium castaneum</i>	160	100
<i>Leptinotarsa decemlineata</i>	170	55
<i>Blattella germanica</i>	180	45
<i>Chroristonneura fumiferana</i>	90	43
<i>Aedes aegypti</i>	210	17
<i>Bombyx mori</i>	80	40
<i>Drosophila melanogaster</i>	210	27
<i>Locusta migratoria</i>	170	26
EcRB		
<i>Tribolium castaneum</i>	96	100
<i>Leptinotarsa decemlineata</i>	86	69
<i>Tenebrio molitor</i>	93	66
<i>Celaca pugilator</i>	120	33
<i>Daphnia magna</i>	100	16
USPA		
<i>Manduca sexta</i>	44	100
<i>Melipona scutellaris</i>	25	76
<i>Scaptotrigona depilis</i>	25	76
<i>Apis mellifera</i>	25	76
<i>Chironomus tentans</i>	20	75
<i>Aedes aegypti</i>	37	54

Table 2
Primers used in this study.

Gene	Forward 5'-3'	Reverse 5'-3'
For dsRNA*		
EcRC	CCTCCGGTTACCACTACAA	CTGGTTCAACCTTGATGACG
EcRA	ACATCAAGCACGAGATGA	ACATGGGTGAGGAGTAGCC
EcRB	CGCTCCAGAGGAGAGCAG	TGTCGGACTTGGCTAGAT
USPC	AGGGATAAAGTCGGTGCAA	CCCTCCAACATCTCCATTA
USPA	AAAAGAAAAACCGATGATGTCTGTG	TAGCGACATGTGCGGAGGTC
USPB	AAAGACACCGAACTGTGTGA	CGCTGGCCCAGTAATCGGAT
For isoform-specific region cloning		
EcRA	ATGTCTCGCTTGGATCC	GATACACCGCGTTCTTCGTT
EcRB	ATGTCTCGCTTGGATCC	GATACACCGCGTTCTTCGTT
USPA	ATGATGAAAAAAGAAAAACC	CTATGTCTTCAGGTCGACGT
USPB	ATGGGCAGCTTGGGAGCA	CGCTGGCCCAGTAATCGGAT
For RT-PCR and qRT-PCR		
EcRA	TACAGCCCCAACGAAAAGAT	GATACACCGCGTTCTTCGTT
EcRB	GACCTGGAGTTCTGGGACCT	CTTGGCTAGATGGTGGTTGC
USPA	AGCCGGAGTCTACCCAATTT	CGTATTTCCCGAAGGACTCA
USPB	GATGCAAGCACAGGATGCTA	GGACCAGGATTTTGACGTGT
RP49	TGACCGTTATGGCAAACCTCA	TAGCATGTGCTTCGTTTGG
BR	CACAACACTTCTGTCTGCGGTC	CACAGGGTGTTTGCAAGGAG
HR3	CCGTGCAAAAGTATGTGG	GTCGGCAGTATTGACATC
FTZ-F1	TGCGAGGAATCACAAACAAG	TGCAGTGCTTGTGGTAGAGG
E74	GCGTCTTCAAGCTGGTG	CAGCCTTTGACCGTCCAC
E75	CGGTCCTCAATGGAAGAAAA	TGTGTGGTTTGTAGGCTTCG

* T7 promoter sequence (TAATACGACTCACTATAGGG) at the 5' end of each primer

Table 3
Effect of dsRNA injected into the larvae on phenotypic changes

dsRNA	No. of injected larvae	No. of dead larvae	No. of dead pupae	No. of dead adults	No. of Normal Adults
<i>MalE</i>	40	3	2	2	33
EcRA	95	95	0	0	0
EcRB	60	12	6	7	35
EcRC	30	30	0	0	0
USPA	60	7	2	2	39
USPB	50	6	3	1	40
USPC	30	30	0	0	0