Steroid Receptor Co-activator Is Required for Juvenile Hormone Signal Transduction through a bHLH-PAS Transcription Factor, Methoprene Tolerant*

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Metamorphosis in insects is regulated by juvenile hormone (JH) and ecdysteroids. The mechanism of 20-hydroxycydsone (20E), but not of JH action, is well understood. A basic helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) family member, methoprene tolerant (Met), plays an important role in JH action. Microarray analysis and RNA interference (RNAi) were used to identify 69 genes that require Met for their hydro-remodelling of larval tissues, such as the midgut, is initiated by ecdysteroid action and Met is a member of this family. To determine whether other members of the bHLH-PAS family are required for the expression of JH-response genes, we employed RNAi to knockdown the expression of all 11 members of the bHLH-PAS family and studied the expression of JH-response genes in RNAi insects. These studies showed that besides Met, another member of this family, steroid receptor co-activator (SRC) is required for the expression of 15 JH-response genes tested. Moreover, studies in JH responsive Aag-2 cells revealed that Aedes aegypti homologues of both Met and SRC are required for the expression of the JH-response gene, kr-h1, and SRC is required for expression of ecdysone-response genes. These data suggest the steroid receptor co-activator plays key roles in both JH and 20E action suggesting that this may be an important molecule that mediates cross-talk between JH and 20E to prevent metamorphosis.

Juvenile hormones (JH)2 regulate many aspects of the life of an insect, including development, reproduction, diapause, caste determination, and polyphenism (1–5). The higher levels of JH during larval stages modulate ecdysteroid action to prevent metamorphosis, whereas lower levels or absence of JH at the end of larval or pupal stages allow ecdysteroids to promote metamorphosis (6). At the onset of metamorphosis, remodeling of larval tissues, such as the midgut, is initiated by ecdysteroid action in the absence of JH. However, exogenous application of JH or its analogs cause suppression of ecdysteroid-induced gene expression and blocks remodeling of larval tissues (7, 8). Thus, cross-talk between JH and 20-hydroxycydsone (20E, the most active form of ecdysteroid) determines the fate of larval tissues.

The mechanism of 20E but not of JH action is well understood. Methoprene tolerant (Met) was first identified in Drosophila melanogaster, mutant flies that are resistant to methoprene, a JH mimic (9). Met is a member of the basic helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) transcription factor family. Met is localized exclusively to the nuclei and ligand binding assays showed that it can bind to JH III with high affinity (10–12). RNAi-aided knockdown in expression of the met gene in the red flour beetle, Tribolium castaneum, which responds well to exogenous JH application, showed that Met is required for JH action in preventing larval-pupal metamorphosis, as well as precocious differentiation of adult structures (13, 14). In this insect, Met also plays an important role in JH regulation of vitellogenin synthesis in adult female beetles (15).

Members of the bHLH transcription factor superfamily regulate the expression of genes involved in cell proliferation, apoptosis, determination, and tissue differentiation during the development of animals and plants (16–18). All members of this superfamily contain a bHLH domain of 60 amino acids consisting of two regions: a DNA-binding basic region of 15 amino acids in length, and a helix-loop-helix region, which allows the formation of homodimer or heterodimer complexes (19–21). The bHLH transcription factors bind to a consensus hexameric DNA sequence called E-box (CANNTG), which is present in promoter regions of their target genes (22). The members of the bHLH transcription factor superfamily are classified into various families (PAS, period, per), aryl hydrocarbon receptor nuclear translocator (ARNT), and single-minded (Sim); HES, Hairy-Enhancer of split; USF/Myc/Upstream Transcription Factor; AS-C (Atonal, Mesp, Hand, p48, Shout, and Achaete-scute), and others) based on the presence of additional functional domains (23). We recently identified 11 members of the bHLH-PAS family in T. castaneum (24). RNAi analysis showed that knockdown in the expression of seven of 11 members of this family affected growth and development of T. castaneum. The inability to reach critical weight to undergo metamorphosis, failure to complete larval-pupal or pupal-adult ecdysis, and abnormal wing development are among the most common phenotypes observed in RNAi insects. Among bHLH tran-

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2 The abbreviations used are: JH, juvenile hormone; 20E, 20-hydroxyecdyson; Met, methoprene tolerant; SRC, steroid receptor co-activator; JHE, JH esterase; DMSO, dimethyl sulfoxide; bHLH, basic helix-loop-helix.
scription factors studied, the steroid receptor co-activator (SRC) and Met showed the most severe phenotypes (14, 24).

In this study, a combination of microarray, qRT-PCR, and RNAi methods were employed to study the function of bHLH-PAS family transcription factors in JH action. The data presented here showed that two members of the bHLH-PAS family, Met and SRC, are required to regulate the expression of JH-response genes. Two-hybrid and pulldown assays showed that SRC heterodimerizes with Met suggesting that this nuclear receptor co-activator could play an important role in JH action.

**EXPERIMENTAL PROCEDURES**

_Rearing and Staging of Beetles—_ _T. castaneum_ strain GA-1 beetles were reared on organic wheat flour containing 10% yeast at 30 °C, following the methods described previously (25). The newly molted final instar larvae were identified by white heads and staged from that time onwards.

_Double-stranded RNA Injection—_ Genomic DNA was extracted from _T. castaneum_ adults and purified using the DNeasy Tissue Kit (Qiagen). Fragments of genes coding for bHLH-PAS family members were PCR amplified using the primers reported previously (24), and these DNA fragments were used to prepare dsRNA, as described previously (24). Newly molted final instar larvae were anesthetized with ether vapor for 5 min and lined on a glass slide covered with double-sided tape. dsRNAs were injected into the ventral side of the first or second abdominal segment of the animals, using an injection needle made by the needle puller (Idaho Technology, Salt Lake City, Utah). About 0.8 to 1 μg (0.1 μl) dsRNA was injected into each animal. The dsRNA of _Escherichia coli_ maltase gene _(malle)_ was injected as a control. Injected larvae were removed from the slide after recovery and raised in whole wheat flour at 30 °C.

_Hormone Treatments—_ Hydroprene (ethyl (2E,4E,7S)-3,7,11-trimethyl-2,4-dodecadienoate, a gift from Wellmark International, Dallas, TX), a JH analog, was dissolved in cyclohexane to 2 μg/μl. JH III (Sigma) was dissolved in dimethyl sulfoxide (DMSO) or cyclohexane to 10 mM. Ponasterone A was purchased from Alexis Corporation (San Diego, CA) and dissolved in DMSO to make 10 mM solution. The final instar larvae, which were injected with dsRNA on the first day, were topically applied with 0.5 μl of 2 μg/μl of hydroprene solution, or 10 μl JH III solution, on the dorsum on the third day. The same volume of cyclohexane or DMSO was applied to the control larvae. The treated larvae were collected at 6 h after treatment and stored in a −80 °C freezer. 20-Hydroxyecdysone (20E, Sigma) was dissolved in distilled water to create a 100 mM solution, and 0.1 μl was injected into each insect. The same volume of distilled water was injected into the control insects.

_Microarray Analysis—_ Total RNA was isolated from the whole body of the treated larvae using spin columns (RNeasy, Roche Applied Science). The integrity of RNA was verified by an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA (>200 ng) from each of the three replicates was used to synthesize cDNA using the Agilent low RNA input fluorescent linear amplification kit (Agilent Technologies, Santa Clara, CA). The labeled cDNA were purified using RNase mini purification columns (Qiagen, Valencia, CA). Fluorescently labeled cDNAs (15 pmol) were hybridized to _T. castaneum_ microarrays. The microarrays were prepared by printing 60-mer oligonucleotides designed based on 15,008 genes selected from the 16,000 genes predicted by _T. castaneum_ genome annotations, and 736 control probe sets were put onto glass slides at Agilent Technologies. Data analysis was performed using GeneSpring program.

_RNA Isolation, cDNA Synthesis, and Quantitative Real Time PCR (qRT-PCR)—_ Total RNA was isolated from four larvae for each sample using the TRI Reagent (Molecular Research Center Inc., Cincinnati, OH). cDNA synthesis and qRT-PCR were performed as described previously (24) using the primers reported in Bitra et al. (24) and shown in Table 1. The mRNA levels were normalized using the internal control, the mRNA levels of RP49, a ribosomal protein.

_Constructs—_ Mammalian two-hybrid vectors (Promega), pBIND containing GAL4 DNA-binding domain, and pACT containing the VP16 activation domain were used to prepare GAL4 and VP16 fusion proteins of receptors and transcription factors. To prepare VP16:SRC(bHLH-PAS) or GST: SRC(bHLH-PAS) constructs, the DNA fragment coding for amino acids 29–370 of TcSRC were PCR amplified and cloned into pACT (Promega Corp.) and pGEX5.1 vectors (Amersham Biosciences), respectively. GST:SRC(LXXLL) was prepared by cloning the DNA fragment coding for amino acids 354–720 into the pGEX5.1 vector. GAL4:EcRDEF and GAL4:Met were prepared by cloning DNA fragments coding for amino acids 189–484 containing DEF domains of the TcEcRB isoform and complete open reading frame of TcMet, respectively, into pBIND vector. To prepare VP16:Hyopxia or VP16:Clock constructs, the DNA fragment coding for amino acids 2–875 of TcHypoxia and 2–576 of TcClock were PCR amplified and cloned into pACT (Promega Corp.).

_Cell Culture and Transfection—_ 3T3 cells were grown at 37 °C with 5% CO2 in a DMEM (Invitrogen) containing 10% fetal bovine serum. For transfection experiments, 50,000 cells/well were seeded in a 96-well plate. On the following day, the cells were transiently transfected with 166 ng each of receptor/transcription factor and 332 ng each of pFRLUC reporter construct, using a “Polyfect” transfection reagent (Qiagen). The plasmid Sport, containing the lacZ reporter gene, was co-transfected into cells and used for normalization. Aag-2 cells were cultured in Drosophila Schneider medium containing 10% FBS. These cells were transfected with Cellfectin (Invitrogen). Preliminary experiments showed that soaking these cells in dsRNA-containing medium for 72 h causes more than 90% knockdown in expression of the target gene. All RNAi experiments were performed by soaking cells in a dsRNA-containing medium for 72 h. For RNAi experiments, 40,000 Aag-2 cells were seeded into each well of 6-well plates and
cultured at 28°C overnight. The next day, the old medium was replaced with 1 ml of Drosophila Schneider medium containing 6 μg of dsRNA, and the cells were cultured for 6 h. At the end of this period, 1 ml of Drosophila Schneider medium containing 20% FBS was added to each well, and the cells were cultured for 3 days. The transfected cells were exposed to DMSO (1 μl/ml) or 1 μM JH III in DMSO (1 μl/ml) for 6 h.

Pulldown Assays—Pulldown assays were performed following the procedure described in the technical manual for the magneGST™ Pull-down system. Western blots were used to detect the pulldown proteins using the anti-V5 antibody and Enhanced Chemiluminescence (ECL) method (Pierce).

RESULTS
Identification of 100 Hydroprene-induced and 45 Hydroprene-suppressed Genes—Newly molted final instar T. castaneum larvae were injected with Met or malE (a gene coding for Maltsae in bacteria used as a control) dsRNA. On the third day after injection, the two groups of dsRNA-injected larvae were applied with hydroprene (a JH analog that works better than methoprene in T. castaneum) or cyclohexane (solvent control). Six hours after hydroprene application, total RNA was isolated from the whole body samples. The RNA was labeled and hybridized to T. castaneum custom microarrays. The signal for 11,757 of 15,008 probe sets was detected with at least one of the 12 RNA samples tested (three replicates for each of Met-Hyp, malE-Hyp, Met-CHX, and malE-CHX treatments). The spot intensity data for these probe sets were statistically analyzed using GeneSpring software (Agilent Technologies). The fold-difference in the expression (designated as “FD,” calculated by dividing the mean value of signal intensities of the hydroprene-treated samples with the signal intensities of the cyclohexane-treated samples) and the significance of difference (p value from t test) for 11,757 probe sets is shown as volcano plots (see Fig. 1, A and B). In the larvae injected with malE dsRNA, hydroprene induced the expression of 100 genes by 2-fold or more, with a p value of less than 0.01 (Fig. 1C, Table 2). Knockdown in the expression of met decreased hydroprene induction of 86 of 100 genes to less than 2-fold. Moreover, 37 of these 86 genes showed more than 50% reduction in their hydroprene-induced expression in Met RNAi larvae, compared with their expression in the control larvae (Fig. 1C, Table 2). The expression of these 37 genes derived from microarray data is shown in Fig. 1E. Hydroprene application also suppressed the expression of 45 genes by 2-fold or more, with a p value of less than 0.01. Knockdown in the expression of the met gene decreased the hydroprene suppression of 39 of 45 genes to less than 2-fold. Furthermore, 32 of these 45 genes showed more than 50% reduction in hydroprene-suppressed activity in Met RNAi larvae (Fig. 1D, Table 2). The expression of these 32 genes derived from microarray data is shown in Fig. 1F. In the functional annotation analyses, the 37 hydroprene-induced and 32 hydroprene-suppressed genes that require Met for their hydroprene regulation were preferentially associated with certain gene ontology terms identified using the Blast2Go program, revealing that the majority of these 69 genes participate in cellular or metabolic processes with binding or catalytic activity (Table 3).
pre or cyclohexane under identical conditions to those used for collecting RNA for the microarray analysis. Specific primers were designed based on the coding sequences of 16 randomly selected Met-regulated hydroprene response genes identified by the microarray experiments. The primers and RNA samples were used to quantify the mRNA levels of selected genes by qRT-PCR. Three control genes met (to measure knockdown efficiency), jhe (a known JH-response gene), and kr-h1 (Glean_12990, a known JH-response gene) were also included in the analysis. Met mRNA levels were reduced by more than 90% in the larvae injected with Met dsRNA, when compared with the levels in the larvae injected with malE dsRNA; this suggests that expression of the met gene was knocked down efficiently in Met dsRNA-injected larvae (Fig. 2A). In the malE dsRNA-injected larvae, the mRNA levels of both JHE and Kr-h1 increased by 2.5- and 6.5-fold, re-

**TABLE 2**

|                     | p < 0.0001 | p < 0.001 | p < 0.01 | p > 0.01 |
|---------------------|------------|-----------|----------|----------|
| **Hydroprene induced genes** |            |           |          |          |
| Hyp/CHX ≥ 2.0 (malE RNAi) | 7          | 36        | 100      | 231      |
| Hyp/CHX ≥ 2.0 (Met RNAi)  | 2          | 6         | 59*      | 437*     |
| **Hydroprene suppressed genes** |          |           |          |          |
| CHX/Hyp ≥ 2.0 (malE RNAi) | 0          | 1         | 45       | 380      |
| CHX/Hyp ≥ 2.0 (Met RNAi)  | 1          | 6         | 44*      | 554*     |

*a Includes genes that are induced or suppressed in Met RNAi insects only.

RCR Is Required for JH Action

Figure 1. Identification of hydroprene response genes. A and B, volcano plots of differentially expressed genes in control larvae injected with malE dsRNA and treated with either cyclohexane or hydroprene (A) or Met RNAi larvae treated with either cyclohexane or hydroprene (B). The p values of the t test were plotted against fold-suppression (left)/induction (right) (calculated by dividing the signal intensity of the samples isolated from hydroprene-treated larvae by the signal intensity of the samples isolated from the control larvae treated with cyclohexane) in gene expression for all the genes that showed a positive signal in at least one of the samples tested are shown. C, Venn diagram showing the distribution of three classes of genes: genes induced by hydroprene by 2-fold or more with a p value < 0.01; genes that showed reduction in hydroprene induction after Met RNAi; and genes that showed reduction in hydroprene induction by 2-fold or more after Met RNAi. D, Venn diagram showing the distribution of three classes of genes: genes suppressed by hydroprene by 2-fold or more with a p value < 0.01; genes that showed reduction in hydroprene suppression after Met RNAi; and genes that showed reduction in hydroprene suppression by 2-fold or more after Met RNAi. E and F, expression levels of 37 hydroprene-induced genes and 32 hydroprene-suppressed genes that require Met for expression are displayed using the Treeview program. The means of normalized signals from microarray data (n = 3) are displayed. The red color shows induction and the green color shows suppression of gene expression when compared with controls.
spectively, in the larvae treated with hydroprene, when compared with the levels in the larvae treated with cyclohexane (Fig. 2A). However, hydroprene did not induce such an increase in the mRNA levels of both these genes in Met dsRNA-injected larvae (Fig. 2A). These data suggest that the presence of Met is required for hydroprene induction of these two known JH-response genes. The mRNA levels of 9 of 11 genes tested were induced by hydroprene in the malE dsRNA-injected larvae, but not in the Met dsRNA-injected larvae (Fig. 2A). The other two genes (GLEAN_05078 and GLEAN_02187) tested showed different expression patterns in the qRT-PCR analysis, when compared with their expression patterns observed in the microarray analysis. Therefore, these two genes were not included in the further studies de-

### TABLE 3

69 genes that require Met for their hydroprene regulation

| Glean No. | Annotation | Fold-change malE | Fold-change Met |
|-----------|------------|------------------|----------------|
|            |            | Control | RNAi |
| ----------|------------|---------|-----|
| 1         | GLEAN_00303 | Synaptic vesicle protein | 4.45 | 1.62 |
| 2         | GLEAN_00639 | cg15920-isoform b | 3.04 | 0.78 |
| 3         | GLEAN_00688 | Glycerol-3-phosphate dehydrogenase | 6.31 | 1.27 |
| 4         | GLEAN_01474 | Protein containing du1703 | 2.81 | 1.06 |
| 5         | GLEAN_02187 | NA* | 12.68 | 1.47 |
| 6         | GLEAN_02552 | Cytochrome p450 | 2.81 | 1.07 |
| 7         | GLEAN_02843 | Cathepsin k | 4.88 | 0.79 |
| 8         | GLEAN_03155 | NA | 2.27 | 1.12 |
| 9         | GLEAN_03498 | pmp22 peroxisomal membrane | 2.52 | 1.21 |
| 10        | GLEAN_03733 | Solute carrier family member 35 | 6.86 | 1.50 |
| 11        | GLEAN_05078 | NA | 22.60 | 1.59 |
| 12        | GLEAN_05841 | Cytochrome c | 3.70 | 1.51 |
| 13        | GLEAN_05951 | cg13776-isoform a | 4.76 | 0.82 |
| 14        | GLEAN_05953 | Cathepsin b-like cysteine proteinase | 4.65 | 1.66 |
| 15        | GLEAN_06026 | Serine protease | 2.91 | 1.35 |
| 16        | GLEAN_06126 | Kruppel-like factor 5 | 3.13 | 1.35 |
| 17        | GLEAN_06326 | Multiple inositol polyphosphate phosphatase | 2.48 | 1.00 |
| 18        | GLEAN_06764 | Cytochrome p450 | 3.08 | 0.35 |
| 19        | GLEAN_06867 | Pol polyprotein | 2.53 | 1.10 |
| 20        | GLEAN_07245 | NA | 5.51 | 1.32 |
| 21        | GLEAN_08456 | Chromosome 6 open reading frame 206 | 6.14 | 0.51 |
| 22        | GLEAN_08469 | Juvenile hormone acid methyltransferase | 3.23 | 0.34 |
| 23        | GLEAN_08555 | NA | 2.10 | 0.97 |
| 24        | GLEAN_08636 | Adenylate kinase 7 | 5.76 | 1.61 |
| 25        | GLEAN_10586 | NA | 2.61 | 1.21 |
| 26        | GLEAN_11084 | NA | 3.30 | 1.02 |
| 27        | GLEAN_12232 | cg1213-isoform a | 4.89 | 1.19 |
| 28        | GLEAN_12587 | Isocitrate dehydrogenase | 2.64 | 1.20 |
| 29        | GLEAN_13069 | unc-5 homolog b | 2.90 | 0.40 |
| 30        | GLEAN_13081 | Heat shock protein 10 | 2.25 | 0.94 |
| 31        | GLEAN_13193 | Juvenile hormone esterase | 3.48 | 1.39 |
| 32        | GLEAN_13603 | Cytochrome p450 | 3.08 | 0.35 |
| 33        | GLEAN_13683 | Heat shock protein 10 | 2.25 | 0.94 |
| 34        | GLEAN_13957 | Multiple inositol polyphosphate phosphatase | 2.48 | 1.00 |
| 35        | GLEAN_14695 | Atdomain containing 3a | 2.49 | 1.11 |
| 36        | GLEAN_15457 | Hyperpolarization activated cyclic nucleotide-gated potassium channel | 3.30 | 1.53 |
| 37        | GLEAN_15693 | cg1435-isoform a | 3.31 | 0.37 |

### Suppressed

| Glean No. | Annotation | Fold-change malE | Fold-change Met |
|-----------|------------|------------------|----------------|
| 1         | GLEAN_00571 | kiaa1161 protein | 0.21 | 0.95 |
| 2         | GLEAN_01031 | Protease inhibitor-like protein | 0.46 | 1.17 |
| 3         | GLEAN_01282 | cg10433-isoform a | 0.43 | 1.29 |
| 4         | GLEAN_01570 | cg11642-isoform a | 0.26 | 1.44 |
| 5         | GLEAN_02832 | Leucine-rich transmembrane | 0.10 | 0.95 |
| 6         | GLEAN_03177 | Cat eye syndrome critical region protein 1 homolog precursor | 0.18 | 0.74 |
| 7         | GLEAN_04117 | Signal sequence delta | 0.50 | 1.53 |
| 8         | GLEAN_04205 | NA | 0.15 | 1.12 |
| 9         | GLEAN_04723 | Protein-disulfide isomerase associated 6 | 0.50 | 1.08 |
| 10        | GLEAN_05093 | Heat shock protein 68 | 0.01 | 0.91 |
| 11        | GLEAN_05096 | Heat shock protein 68 | 0.01 | 0.98 |
| 12        | GLEAN_05632 | cg14275-isoform a | 0.48 | 1.39 |
| 13        | GLEAN_06391 | Cys-loop ligand-gated ion channel subunit | 0.50 | 1.41 |
| 14        | GLEAN_07077 | Ets homologous factor | 0.39 | 1.19 |
| 15        | GLEAN_07620 | Gram-negative bacteria binding protein 3 | 0.39 | 1.16 |
| 16        | GLEAN_07955 | Leucine-rich transmembrane | 0.49 | 1.15 |
| 17        | GLEAN_08876 | Abnormal cell lineage family member (lin-41) | 0.47 | 1.37 |
| 18        | GLEAN_09289 | Alcohol dehydrogenase | 0.45 | 1.47 |
| 19        | GLEAN_09350 | NA | 0.20 | 2.36 |
| 20        | GLEAN_09465 | NA | 0.19 | 2.85 |
| 21        | GLEAN_10255 | Cytochrome P450 6a2(p450-b1) | 0.50 | 1.41 |
| 22        | GLEAN_10492 | at5g12010 f14f18_180 | 0.40 | 1.66 |
| 23        | GLEAN_11121 | Elongation of very long chain fatty acids (fenv1 sur4 yeast)-like 4 | 0.19 | 2.69 |
| 24        | GLEAN_11718 | Serine (or cysteine) proteinase clade b member 3a | 0.50 | 1.34 |
| 25        | GLEAN_13280 | Serine protease | 0.19 | 1.12 |
| 26        | GLEAN_13471 | NA | 0.29 | 0.61 |
| 27        | GLEAN_14022 | Leucine-rich repeat containing 15 | 0.40 | 1.61 |
| 28        | GLEAN_14153 | Inter-o-trypsin inhibitor heavy chain b4 | 0.43 | 1.52 |
| 29        | GLEAN_14955 | Chromosome 20 open reading frame 26 | 0.41 | 1.29 |
| 30        | GLEAN_15291 | Cytochrome p450 4c3 | 0.12 | 5.37 |
| 31        | GLEAN_15951 | NA | 0.27 | 0.65 |
| 32        | GLEAN_15979 | Aldehyde dehydrogenase | 0.39 | 1.08 |

*NA, not annotated.*
SRC Is Required for JH Action

Using the total RNA isolated from the JH III or cyclohexane-treated insects. The changes in the expression of genes observed in the JH III-treated larvae are similar to those observed in the hydroprene-treated larvae (Fig. 2A). The application of JH III increased the mRNA levels of nine hydroprene-induced genes and decreased the mRNA levels of four hydroprene-suppressed genes in the male dsRNA-injected larvae, but not in the Met dsRNA-injected larva (Fig. 2B). Interestingly, JH III induced expression of the jhe gene by 5.1-fold, which is much higher than the 2.6-fold induction of this gene observed after treatment with hydroprene. These data showed that the nine genes identified in the microarray experiments and confirmed by qRT-PCR as those induced by hydroprene are also induced by JH III; therefore, they could be up-regulated by JH in vivo. Similarly, the four genes identified in the microarray analysis and confirmed by qRT-PCR as those suppressed by hydroprene are also suppressed by JH III, and thus, they could be down-regulated by JH in vivo.

Identification of T. castaneum bHLH-PAS Family Transcription Factors Required for Regulation of JH-response Genes—The members of the bHLH-PAS family often function as heterodimers to regulate gene expression and Met is a member of this family. To determine whether any other members of the bHLH-PAS family are required for the expression of JH-response genes, we injected dsRNAs of all 11 members of this family individually into newly molted, final instar larvae, and the dsRNA-injected larvae were exposed to hydroprene for 6 h. The RNA samples isolated from RNAi larvae were used in qRT-PCR to quantify mRNA levels of all 11 members of the bHLH-PAS family and JH-response genes coding for JH esterase (JHE) and JH-inducible protein 03733 (Gleain_03733 abbreviated as G03733). Injection of dsRNA of each of the 11 bHLH-PAS family members knocked down the expression of the respective genes by 58–90% (Fig. 3A). Hydroprene induced the expression of both genes coding for JHE and G03733 in the larvae injected with dsRNA of control malE or all members of the bHLH-PAS family except Met and SRC (Fig. 3, B and C). In contrast, the expression of these genes was not induced by hydroprene in the larvae injected with either Met or SRC dsRNA (Fig. 3, B and C). Clock RNAi insects showed lower JH induction of these genes in some experiments, but not in all the experiments. Similarly, TcHypoxia RNAi insects showed higher JH induction of these genes in some experiments. Only Met and SRC RNAi insects showed lower JH induction of these genes in all the repeats. Thus, JH-response genes coding for JHE and G03733 require the presence of Met and SRC for their JH-induced expression.

SRC Is Required for JH Regulation of Gene Expression—Studies described in the previous section identified Met and SRC as the two bHLH-PAS family members required for regulation of JH-response genes coding for JHE and G03733. To determine whether SRC is also required for regulation of other JH-response genes, SRC dsRNA was injected into early final instar larvae, and the RNAi larvae were exposed to hydroprene for 6 h. The RNA samples isolated from RNAi larvae were used in qRT-PCR to quantify mRNA levels of 15 JH-response genes that require Met for their JH regulation. As shown in Fig. 3D, hydroprene induced the expression of 11
genes and suppressed the expression of four genes in the control larvae injected with malE dsRNA, but not in the larvae injected with the SRC dsRNA. The influence of SRC knockdown on hydroprene suppression of G07620 and G13280 genes is somewhat variable. In one experiment, hydroprene did not suppress the expression of these two genes in SRC RNAi insects, whereas in the other two experiments as shown in Fig. 3D, hydroprene induced expression of these two genes was reduced in SRC RNAi insects. The effect of SRC knockdown on the expression of 15 genes tested is similar to the effect caused by Met knockdown, described in the previous section. These data suggest that both Met and SRC are required for JH-regulated expression of these JH-response genes.

**SRC Heterodimerizes with Met**—Two-hybrid assays were conducted to determine whether SRC heterodimerizes with Met. Previous studies showed that the *D. melanogaster* ortholog of TcSRC, DmTaiman, heterodimerizes with DmEcR in regulation of invasive cell behavior in the ovary (26). Our previous studies showed that DmMet heterodimerizes with both DmEcR and DmUSP (27). Therefore, we conducted two-hybrid analysis to determine whether TcSRC heterodimerizes with TcMet or TcEcR. Steroid receptor co-activator proteins heterodimerize with steroid receptors through their LXXLL motif-containing regions as the heterodimerization surfaces. The bHLH-PAS family members heterodimerize with other members of this family using PAS domains as heterodimerization surfaces. The VP16:TcSRC(bHLH-PAS), GAL4:TcMet, and the luciferase reporter construct were transfected into 3T3 cells. The cells were exposed to DMSO or JH III for 48 h, and then the luciferase levels were quantified. The VP16:TcSRC(bHLH-PAS) heterodimerized with GAL4:TcMet in the presence of JH III caused a 3-fold increase in luciferase activity (Fig. 4A). Also, the heterodimer between VP16:TcSRC(bHLH-PAS) and GAL4:TcMet supported a JH III dose-dependent increase in the luciferase activity (Fig. 4B). These data showed that SRC heterodimerizes with Met. To determine whether any other HLH-PAS family mem-

**FIGURE 3. Met and SRC are required for JH action.** A, knock-down efficiency of gene expression after injection of dsRNA of bHLH-PAS family transcription factors. The dsRNAs for bHLH-PAS family transcription factors or malE (control) were injected into newly molted final instar larvae. Three days after injection of dsRNA, total RNA was extracted, and the relative expression, in comparison to ribosomal protein (RP49), was determined by qRT-PCR. The percent knockdown in the expression of each gene was determined by comparing mRNA levels in bHLH-PAS family transcription factor dsRNA-injected insects with mRNA levels in control insects injected with malE dsRNA. Mean ± S.D. of three biological replicates are shown. B and C, effect of knockdown in the expression of genes coding for bHLH-PAS family transcription factors on the expression of JH-response genes coding for JHE (B) and G03733 (C). Relative mRNA levels of JHE or G03733 in insects injected with control malE or bHLH-PAS family member dsRNA and exposed to solvent, cyclohexane (−) or hydroprene (+) are shown. Mean ± S.D. of three biological replicates are shown. Arrows point to the mRNA levels of JHE or G03733 in Met or SRC RNAi insects. D, SRC is required for hydroprene-regulated expression of JH-response genes identified by microarray analysis and confirmed by qRT-PCR. The mRNA levels of jhe, kr-h1, and nine JH-induced and four JH-suppressed genes were quantified in control larvae injected with malE dsRNA or in SRC RNAi larvae that were exposed to hydroprene or cyclohexane. Relative mRNA levels were calculated by comparing mRNA levels of these genes with the mRNA levels of RP49. Three biological replicates were performed, and the experiment was repeated twice with similar results. The means (n = 3) of relative expression levels in comparison to the RP49 mRNA levels are displayed using the Treeview program. The color bar is the same as displayed in the legend to Fig. 1E. E, SRC is required for 20E action. The mRNA levels of the 20E-response gene coding for hormone receptor 3 (HR3) were quantified in control larvae (injected with malE dsRNA), or SRC, USP, and EcR RNAi larvae injected with H2O or 20E. Relative mRNA levels were calculated by comparing mRNA levels of these genes with the mRNA levels of RP49. Mean ± S.E. of three biological replicates are shown.

**SRC Is Required for the Expression of 20E-response Genes**—To determine whether SRC is required for the expression of 20E-response genes, SRC, EcR, USP, or malE (control) dsRNA were injected into newly molted final instar larvae, and 20E was injected into these larvae at 3 days after dsRNA injection. At 6 h after 20E injection, total RNA was isolated, and the mRNA levels of 20E-response gene coding for HR3 were quantified. As shown in Fig. 3E, 20E induced the expression of HR3 in the control larvae, but not in larvae injected with SRC, EcR, or USP dsRNA. These data suggest that SRC plays a key role in 20E action as well.
bers heterodimerize with Met or SRC, we selected TcClock (Tc-Clock RNAi insects showed reduced induction of JH-response genes in some experiments) and TcHypoxia (TcHypoxia RNAi insects showed increased expression of JH-response genes). Neither TcClock nor TcHypoxia heterodimerized with TcMet (Fig. 4C) or TcSRC (Fig. 4D). These data suggest that TcClock and TcHypoxia may not have a direct role in JH action.

GAL4:TcEcRDEF and VP16:TcUSPDEF formed a heterodimer and supported an increase in reporter activity in the presence of a ecdysteroid, ponasterone A (Fig. 4E). Interestingly, the presence of VP16:TcSRC(LXXLL) dramatically increased ponasterone A response (Fig. 4E). These data showed that TcSRC interacts with EcR and USP and enhances ecdysone response as reported previously in D. melanogaster (26).

In pulldown assays, GST:TcSRC(bHLH-PAS) or GST: TcSRC(LXXLL) proteins were used as a bait to pull down a fusion protein of full-length TcMet containing His and V5 tags on either end and expressed in insect cells using a baculovirus expression system. As shown in Fig. 5A, GST: TcSRC(bHLH-PAS) but not the GST:TcSRC(LXXLL) protein

![FIGURE 4. SRC interacts with Met and EcR. A, SRC interacts with Met in a two-hybrid assay. DNAs of GAL4:TcMet, VP16:TcSRC(bHLH-PAS), or VP16 (control) along with the pFRLuc reporter construct, were transfected into 3T3 cells using the Polyfect lipid reagent. The transfected cells were exposed to DMSO (1 μl/ml) or 1 μl/ml JH III in DMSO (1 μl/ml) for 48 h. The cells were lysed, and the luciferase activity was measured. The luciferase activity was normalized based on the activity of the co-transfected β-galactosidase reporter construct, pSPORT (Invitrogen). Mean ± S.E. for three replicates are shown. Data were analyzed by analysis of variance and the bar marked with the star is significantly different from the control (p < 0.01). B, JH III enhances the interaction between Met and SRC in a dose-dependent manner. DNAs of GAL4:TcMet and VP16:TcSRC(bHLH-PAS) along with the pFRLuc reporter construct were transfected into 3T3 cells. The transfected cells were exposed to various concentrations of JH III for 48 h. The cells were lysed, and the luciferase activity was measured. Mean ± S.E. for three replicates are shown. The data were analyzed by multiple comparisons one-way analysis of variance. The data points are not significantly different from each other (p > 0.01). C, DNAs of GAL4:TcMet, VP16:TcSRC(bHLH-PAS), VP16:TcHypoxia, VP16:TcClock, or VP16 (control, pACT vector) along with the pFRLuc reporter construct were transfected into 3T3 cells. The transfected cells were exposed to DMSO or 1 μM JH III for 48 h. The cells were lysed, and the luciferase activity was measured. Mean ± S.D. for three replicates are shown. D, DNAs of GAL4:TcSRC(bHLH-PAS), VP16:TcSRC(bHLH-PAS), VP16:TcHypoxia, VP16:TcClock, or VP16 (control) along with the pFRLuc reporter construct were transfected into 3T3 cells. The transfected cells were exposed to DMSO or 1 μM JH III for 48 h. The cells were lysed, and the luciferase activity was measured. Mean ± S.D. for three replicates are shown. E, DNAs of GAL4:TcEcRDEF, VP16:TcUSPDEF, VP16:TcSRC(LXXLL), or VP16 (control) along with the pFRLuc reporter construct were transfected into 3T3 cells. The transfected cells were exposed to DMSO or ponasterone A for 48 h. The cells were lysed, and the luciferase activity was measured. Mean ± S.D. for three replicates are shown.

![FIGURE 5. SRC interacts with Met. A, SRC containing bHLH-PAS domains pulls down Met. GST:TcSRC(bHLH-PAS) and GST:TcSRC(LXXLL) proteins expressed in bacteria and purified using GST magnetic beads were used as a bait to pull down the HIS-TcMet-V5 fusion protein expressed in insect cells using a baculovirus expression system and purified using a nickel affinity column. The pulled down proteins were analyzed on a Western blot using antibodies made against V5 tag. B, SRC and Met interaction is specific. GST:TcSRC(bHLH-PAS) expressed in bacteria and purified using GST magnetic beads was used as a bait to pull down HIS-TcGATA-V5 (control) or HIS-TcMet-V5 fusion proteins expressed in insect cells using a baculovirus expression system and purified using a nickel affinity column. The pulled down proteins were analyzed on a Western blot using antibodies made against V5 tag.
was able to pull down TcMet. These data showed that SRC heterodimerizes with Met through its bHLH-PAS domain. To confirm specific interaction of SRC with Met, the GST: TcSRC(bHLH-PAS) fusion protein was used as a bait to pull down either TcGATA transcription factor or TcMet prepared using the baculovirus expression system. The GST: TcSRC(bHLH-PAS) fusion protein pulled down TcMet but not TcGATA (Fig. 5B). These data show the interaction to be between TcMet and TcSRC.

**DISCUSSION**

The major contribution of this study is the discovery that two bHLH-PAS family transcription factors are required for JH action. Metamorphosis is one of the most noticeable events in the life of an insect. Some insects undergo complete metamorphosis accompanied by a series of
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devolutional changes, including programmed cell death, cell proliferation, and differentiation. Studies on the molecular mechanism of JH action during the onset of metamorphosis are hampered by differences in the response to JH among the insects belonging to Lepidoptera, Coleoptera, Diptera, and other orders. Met has been proposed as a putative JH receptor involved in JH action since it was first identified in D. melanogaster (9). Its critical role in the regulation of metamorphosis has been shown in the red flour beetle, T. castaneum, where Met governs larval entry into larval-pupal metamorphosis and prevents premature expression of adult structures during the pupal stage (13, 14). We hypothesized that Met target genes in the JH-signaling pathway would be misexpressed when expression of the gene coding for Met is knocked down during the larval stages. As the systemic RNAi works very well in T. castaneum and these beetles respond well to JH, we used this model insect to test this hypothesis. Genome-wide analysis employing microarrays and RNAi identified 69 genes that require Met for their JH-regulated expression. Among the putative Met target genes, GLEAN_06126 and GLEAN_07077 coding for proteins with transcription factor activity, CLEAN_13683 coding for proteins with hormone binding activity, and GLEAN_13069, GLEAN_06391, GLEAN_11121, and GLEAN_04117 coding for proteins with signal transduction activity are promising candidates that may play key roles in JH signal transduction (Table 3). Further studies are underway to fully characterize the function of these newly identified Met target genes.

Members of the bHLH-PAS family often function as heterodimers in regulating the expression of target genes. In vertebrates, aryl hydrocarbon receptor (Ahr) binds to dioxin and other aryl hydrocarbons, translocates to the nucleus, and heterodimerizes with Trachealess and Single minded (both bHLH-PAS family members) and regulates transcription in the trachea and central midline, respectively (30). Two other members of this family, Cycle and Clock, also function as heterodimers to regulate circadian rhythms (31, 32). Based on these previous studies, we hypothesized that Met may heterodimerize with other members of the bHLH-PAS family and regulate JH-response genes. To test this hypothesis, we knocked down the expression of genes coding for all bHLH-PAS family members and quantified the expression of JH-response genes coding for JHE and G03733 in RNAi animals. These studies showed that the presence of only 2 (TcMet and TcSRC) of the 11 members of this family is required for the expression of genes coding for JHE and G03733. Subsequent studies showed that similar to Met, SRC is required for JH regulation of 15 JH-response genes. These data suggested that Met may heterodimerize with SRC and regulate expression of JH-response genes.

Two-hybrid experiments confirmed that TcMet interacts with TcSRC.

The closest D. melanogaster homolog of TcSRC is DmTaiman, a nuclear receptor co-activator. Previous studies showed an interaction between DmTaiman and DmEcR in 20E regulation of follicle cell migration (26). Recent studies of D. melanogaster identified a BTB domain transcription factor, AaFISC, and it interacts with a nuclear receptor, AaFTZ-F1, and regulates stage-specific expression of 20E-response genes during reproduction in the female mosquito (34). Data reported here showed that TcSRC interacts with TcMet. We previously reported physical interactions between DmEcR and DmMet, as well as between DmUSP and DmMet (27). However, these interactions are not hormone dependent. Genetic interactions between DmMet and the BTB domain protein DmBr have also been reported in the fruit fly (35). Taken together, the data presented here and in previous studies suggest that SRC interacts with a number of nuclear receptors and transcription factors, and this may be one of the key proteins that mediate cross-talk among signaling molecules to regulate various developmental processes. In fact, SRC RNAi data in T. castaneum support this hypothesis because knockdown in the expression of genes coding for SRC in T. castaneum affected growth, molting, metamorphosis, and reproduction; and the SRC RNAi insects died prematurely (24). Future studies on specific interactions between SRC and transcription factors, nuclear receptors and other proteins, and the influence of these interactions on various signaling pathways should help in understanding the function of this
important molecule. Model insects, *D. melanogaster* (for forward genetics studies because of the availability of powerful genetic tools and mutants), *T. castaneum* (for reverse genetics studies because of the functioning of systemic RNAi), and Aag-2 cells from *A. aegypti* (because of their response to JH and 20E) are good systems in which to conduct future studies on the functions of Met and SRC on the action of JH, 20E, and other signaling molecules.

Data presented in this paper and previous studies clearly showed that SRC plays important roles in both JH and 20E action. Based on these data, we propose a simple model (Fig. 7) for the function of SRC in JH and 20E action. One possibility is that the SRC interacts with Met in the presence of JH and promotes JH induction of JH-response genes. Also, SRC interacts with EcR in the presence of 20E and promotes 20E induction of ecdysone-response genes. However, in the presence of both 20E and JH, SRC may interact with both Met and EcR resulting in a decrease in expression of both JH- and ecdysone-response genes by respective hormones. Alternatively, SRC:Met and SRC:EcR/USP complexes may recruit different proteins in the presence of either JH or 20E when compared with the proteins recruited in the presence of both hormones. Future studies on this fascinating protein should reveal the molecular mechanisms of JH and 20E action and their cross-talk.

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