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Juvenile hormones antagonize ecdysone actions through co-repressor recruitment to EcR/USP heterodimers

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Abstract

Insect development is controlled by the combined actions of ecdysteroid and juvenile hormones. Transcriptional control by ecdysteroid hormones is mediated via two nuclear receptor superfamily members, ecdysone receptor (EcR) and its heterodimeric partner, ultraspiracle (USP). Although the ecdysteroid hormone 20-hydroxyecdysone acts as an EcR ligand and activates transcription through EcR/USP heterodimers, the activity of juvenile hormones, such as Juvenile hormone III (JH III), and methoprenic acid (MA) via USP remains unclear. Here, we demonstrate that juvenile hormones act as USP ligands and exhibit suppressive effects on ecdysone-dependent EcR transactivation. JH III- and MA-bound USP markedly repressed ecdysone-dependent EcR transactivation through shifting of the USP ligand-binding domain α-helix 12 without affecting EcR/USP heterodimerization or DNA binding. Moreover, transcriptional repression by USP ligands was attenuated by a histone deacetylation inhibitor. Our results suggested that juvenile hormones serve as USP ligands that antagonize EcR-mediated ecdysone actions through the recruitment of histone deacetylase complexes.

Keywords: Ecdysone receptor; Ultraspiracle; Juvenile hormone III; Co-activator

The orphan nuclear receptor ultraspiracle protein (USP) is the insect ortholog of the vertebrate retinoid X receptor (RXR). USP belongs to the nuclear receptor superfamily, a family of ligand-dependent transcription factors that regulate target gene expression through the binding of small, hydrophobic molecules [1–5]. Like RXR, USP also forms heterodimers with other nuclear receptors, including the ecdysone receptor (EcR), which then regulates insect development, molting, and metamorphosis in response to ecdysteroid hormones, such as 20-hydroxyecdysone [6–12]. EcR/USP is a functional unit that responds to 20-hydroxyecdysone, as demonstrated in vitro by transactivation assays [5,6,12] and in vivo using fly *usp* mutant lines [13–16]. Unlike its vertebrate homolog RXR, whose ligand is known to be 9-*cis*-retinoic acid (9cRA), no endogenous ligand has so far been identified for USP.

Juvenile hormones are a family of esterified sesquiterpenes that are thought to be candidate hormone ligands for USP [6,17–19]. It has been believed for some time that Juvenile hormones prevent metamorphosis by modulating ecdysteroid actions at the outset of the ecdysteroid hormone rise during molting. While the hypothesis that Juvenile hormones might act as NR ligands is based on structural homologies with vertebrate terpenes, as represented by retinoic acid, no direct evidence for the proposed function of Juvenile hormones has been reported.

The transactivation function of nuclear receptors in vertebrates has been shown to require a variety of common co-regulator complexes that in most cases

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show ligand-dependent interactions (directly or indirectly) with NR ligand-binding domains (LBD), particularly α-helix 12 (helix 12) located within the C-terminal region [1]. In *Drosophila*, it was reported that EcR transactivation function required Taiman (TAI), a homolog of human AIB1, a p160 family histone acetyltransferase (HAT) co-activator [20,21]. The methyltransferase TRR has also been reported to co-activate EcR transactivation function through its SET domain [22]. However, these co-activators appear to directly interact only with EcR, and to date, no co-activators that physically associate with USP have been identified. As USP is the indispensable partner of EcR in the heterodimer, it is unlikely that USP also recruits co-activators by ligand binding. Rather, we hypothesized that the USP ligand recruits a co-repressor to the EcR/USP heterodimer, thereby modifying the ecdysone-dependent transactivation function of EcR.

In the present study, we report that a juvenile hormone (JH III) and a synthetic juvenile hormone (MA) acted as USP ligands that repressed ecdysone-dependent transactivation without inhibiting heterodimerization or specific DNA binding of EcR/USP. Together with the findings that the histone deacetylase (HDAC) inhibitor TSA abrogated the suppressive effects of JH III and MA, our findings implied that USP ligand binding shifted the α-helix 12 region in the USP ligand-binding domain, allowing recruitment of an HDAC co-repressor complex that then attenuated ecdysone-induced transactivation.

Materials and methods

Plasmids. cDNAs encoding full-length ecdysone receptor B1 (EcR B1), ultraspiracle (USP), murine retinoid X receptor α (mRXRα), and *Renilla* luciferase (RL) were cloned into pAct5 to give protein expression under the control of the *Drosophila* actin 5C promoter. Four copies of hsp27 EcRE and eight copies of UAS were inserted into the promoter of the luciferase pGL3-basic vector (Promega) to generate 4xEcRE-TATA-luc and 17m8-TATA-luc, respectively. For two-hybrid assays in insect cells, GAL4-DBD- and VP16-AD-fusion expression vectors were constructed. GAL4-DBD and VP16-AD were amplified by PCR, digested, and ligated into pAct5 to give pActGAL and pActVP, respectively. The DEF region of EcR (aa 362–878) was cloned into pActGAL (GAL-EcR (DEF)). Likewise, the same region of USP (aa 230–508) and mRXRα (aa 206–467) were cloned into pActVP to give VP-USP(DEF) and mRXRα (DEF), respectively. The USP P498W/P499W mutant was generated by the substitution of the C-terminal region of USP by the oligonucleotide 5′-TCGAGCTG GAGGCGTGGTGGCCACCCGGCCTGGCGATGAAACTGGA GTAGT- 3′ at a *Xho*I site [19]. For expression of EcR DEF region GST-fusion protein, the DEF region of EcR was cloned into pGEX 4T-1. For generation of recombinant EcR B1/USP protein, FLAG-HA-tagged EcR B1 (FLAG-HA-EcR B1) and Myc-His-tagged USP (USP-Myc-His) were cloned into pFastBac (Invitrogen).

Chemicals. Muriateron A (MurA), 9-*cis*-retinoic acid (9cRA), and Trichostatin A (TSA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). JH III was purchased from MP Biomedicals (Irvine, CA). MA was purchased from Sigma–Aldrich (St. Louis, MO). Ligands were dissolved in ethanol or DMSO.

Cell culture. Schneider's line 2 (S2) cells were routinely maintained in Schneider's *Drosophila* medium (Gibco-BRL) supplemented with 10% fetal bovine serum (Gibco-BRL), 100 μg/ml penicillin, and 100 μg/ml streptomycin. *Spodoptera frugiperda* (Sf9) cells were routinely maintained in Sf-900 SFM (Gibco-BRL) with 100 μg/ml penicillin and 100 μg/ml streptomycin.

Transfection, luciferase assay, and S2 two-hybrid assay. S2 cells were seeded into 12-well plates and transfected at 50–60% confluence using Lipofectin (Invitrogen) in optimized medium using Opti-MEM (Gibco-BRL). For luciferase assays, cells were co-transfected with 500 ng 4xEcRE-TATA-luc as a reporter, 50 ng each expression vectors as indicated, and 1 ng pAct5-RL as an internal control. For two-hybrid assays in S2 cells, 500 ng 17m8-TATA-luc as a reporter, 50 ng GAL4- or VP16-fusion vectors, and pAct5-RL as an internal control were co-transfected. At 6 h after transfection, cells were re-fed with fresh medium containing either vehicle (ethanol) only or vehicle plus 1×10^{-6} M MurA, 7.5×10^{-6} M, JH III 1×10^{-5} M MA, 1×10^{-8} M 9cRA or 1×10^{-6} M TSA as indicated. Cells were harvested 24 h after treatment and assayed using the Dual-Luciferase reporter assay system according to the manufacturer's instructions (Promega) [23,24].

GST pull-down assays. GST-fused EcR(DEF) proteins were expressed in *Escherichia coli* BL21 and bound to glutathione–Sepharose 4B beads (Amersham–Pharmacia Biosciences). In vitro translated proteins were incubated with NET-N buffer (50 mM Tri–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, pH 8.0, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 mg/ml aprotinin). Binding was assayed in the presence or absence of 1×10^{-6} M MurA and/or 1×10^{-5} M MA. Bound proteins were separated by SDS–PAGE, lightly stained with Coomassie brilliant blue to verify equal protein loading, and then visualized by autoradiography [25].

Electrophoretic mobility shift assay. Generation of FLAG-HA-EcR B1 and USP-Myc-His expressing baculoviruses was performed according to the manufacturer's instructions (Invitrogen). Sf9 cells were infected with baculoviruses, cultured for 5 days, and harvested. Protein purification was performed using Ni–NTA agarose (Qiagen) and then anti-FLAG M2-agarose affinity resin (Sigma) according to the respective manufacturer's instructions [26]. Recombinant EcR B1/USP and double-stranded *hsp27* EcRE DNA (5′-TGAGCCCAGCGTCA GTATAAAAGCCGGCGTCAACGTCGCCC-3′) were used. Recombinant EcR B1/USP proteins and ligands 1×10^{-6} M MurA, 7.5×10^{-6} M JHIII, or 1×10^{-5} M MA were incubated for 30 min on ice in binding buffer (5 mM Tris–HCl, pH 8.0, 100 mM KCl, 6% glycerol, and 1 mM DTT), 1 mg poly(deoxyinosinic–deoxycytidylic) acid, and 0.3 mg BSA in a final volume of 20 μl. EcRE probe labeled with ^{32}P was then added and the mixtures were incubated for 30 min at room temperature. Entire reaction mixtures were loaded onto 3.5% polyacrylamide gels in TBE buffer and electrophoresed. Gels were dried under vacuum for 2 h and analyzed using an imaging analyzer (BAS1500, Fuji Film).

Results and discussion

Juvenile hormones antagonize ecdysone-dependent transactivation of EcR through the EcR/USP heterodimer

To investigate the molecular basis by which juvenile hormones prevent metamorphosis by modulating ecdysone actions, we examined the effects of a natural juvenile hormone (JH III) and a synthetic juvenile hormone (MA) on the ecdysone-dependent transactivation function of EcR. The synthetic 20-hydroxyecdysone analog Muristerone A (MurA), instead of 20-hydroxyecdysone, was used as a potent EcR ligand in a luciferase assay in

S2 cells transfected with a reporter plasmid containing an EcR-responsive element (EcRE) from the *hsp27* gene promoter, together with EcR B1 and/or USP expression plasmids. Expressed EcR/USP heterodimers displayed transactivation in response to MurA (Fig. 1, lane 8). However, this MurA-dependent EcR/USP transactivation was attenuated by the presence of JH III or MA (Fig. 1, lanes 20 and 24). Mouse RXR, orthologous to invertebrate USP, replaced USP function in MurA-induced transactivation, presumably as an EcR/mRXR heterodimer. However, in this case MA no longer exhibited a suppressive effect on EcR/mRXR heterodimers, rather behaved like 9cRA (9cRA) (Fig. 1, lanes 28 and 30) [27]. Thus, it appears that JH III and MA serve as ligands for USP and attenuate the ecdysone-dependent transactivation of EcR.

Juvenile hormone binding to USP does not prevent EcR/USP heterodimerization or DNA binding

To determine whether the transrepression shown by juvenile hormones was due to the prevention of EcR/USP heterodimerization, we assessed the ligand-dependent interaction between EcR and USP in a two-hybrid assay in S2 cells. As expected, the transcriptional activity of GAL4-fused LBD in EcR [GAL4-EcR (DEF)] was activated by MurA, while VP16-fused USP (DEF) [VP-USP (DEF)] robustly potentiated luciferase activity. These results confirmed the presence of functional and ligand-induced heterodimerization. Under these conditions, neither JH III nor MA showed inhibitory effects on EcR/USP heterodimerization (Fig. 2A, lanes 4, 6, and 8). While EcR/USP heterodimerization was not induced by 9cRA, when VP16-fused mRXRα(DEF) was transfected into S2 cells instead of VP-USP(DEF), EcR/mRXRα heterodimerization was clearly induced by 9cRA (Fig. 2A, lanes 17 and 18).

We then performed a GST pull-down assay using recombinant EcR and USP proteins to determine whether EcR/USR heterodimerization was blocked by juvenile hormones in vitro. While MurA weakly enhanced the interaction between EcR and USP, neither JH III nor MA had any effect on heterodimerization (Fig. 2B). EMSA analysis using ^{32}P-labeled EcRE was performed to determine the DNA-binding activity of EcR/USP heterodimers in the presence of juvenile hormones. We

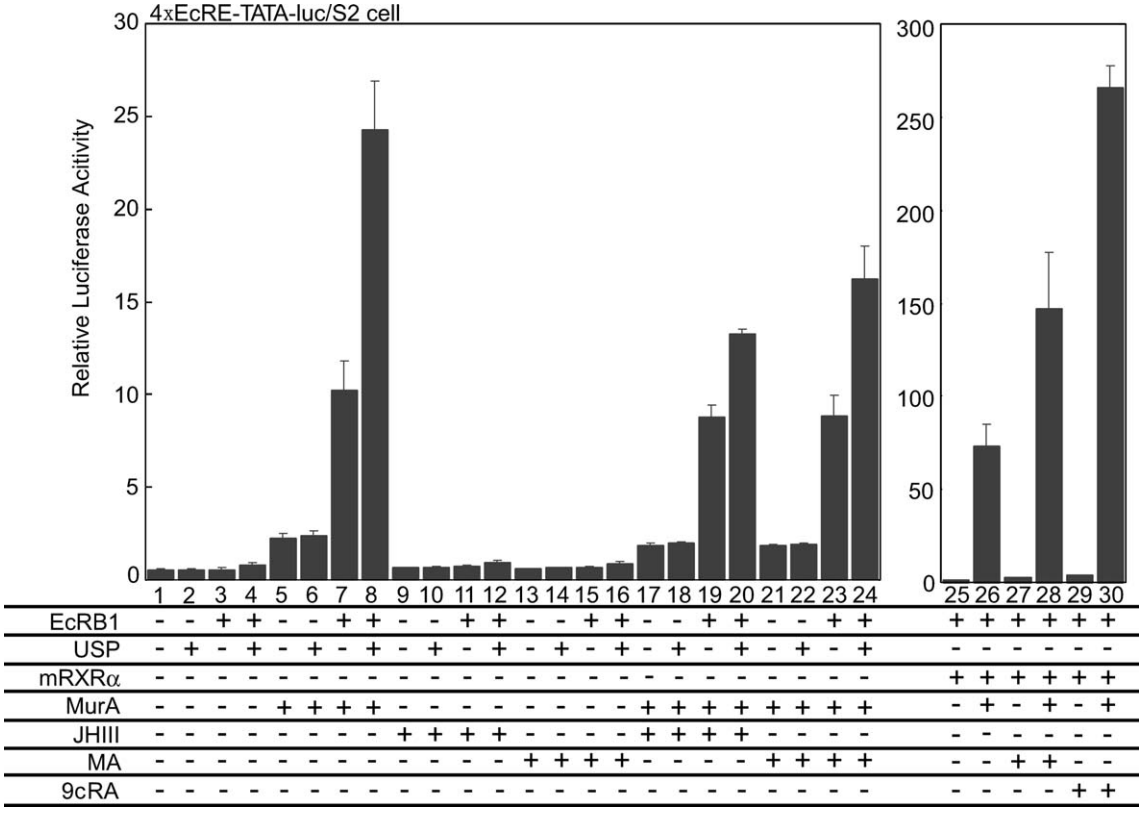

Fig. 1. Effect of JH III and MA on EcR B1/USP transactivation. Luciferase assays were performed as described in Materials and methods. Ligands were applied at the following concentrations: MurA 1×10^{-6} M, JH III 7.5×10^{-6} M, MA 1×10^{-5} M, and 9cRA 1×10^{-8} M. These conditions were used in all experiments unless otherwise stated. MurA induces EcR B1/USP transactivation (lanes 5–8) and in the absence of MurA, JH III and MA have no effect (lanes 9–12, 13–16). However in the presence of MurA, JH III and MA repress EcR B1/USP transactivation. For EcR B1/mRXRα, MurA also induces transactivation of EcR B1/mRXRα (lane 26), while RXR-ligands 9cRA and MA synergistically enhance transactivation (lanes 28 and 30).

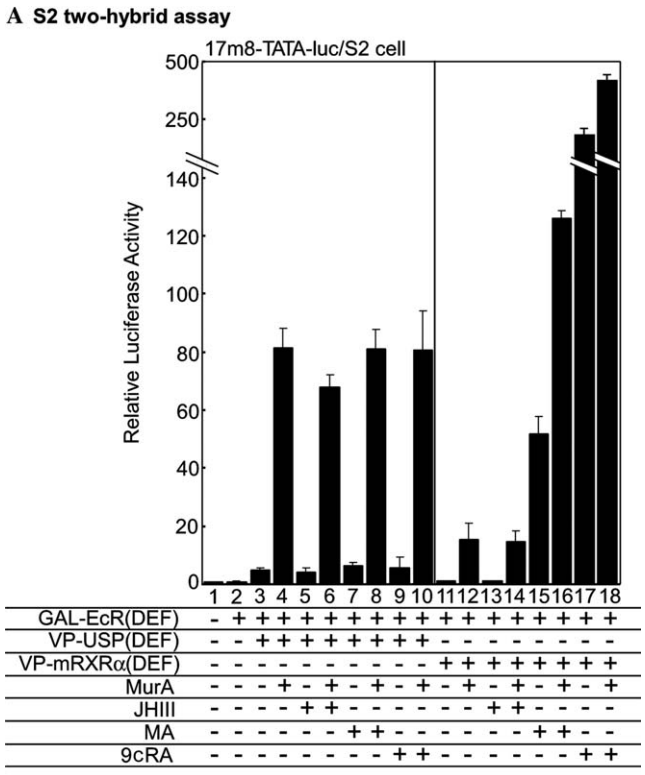

Fig. 2. (A,B) JH III and MA do not affect the formation of EcR/USP heterodimers. (A) S2 two-hybrid assays were performed as described in Materials and methods. MurA enhances the hetrodimerization of EcR (DEF) and USP (DEF) (lane 4). JH III and MA do not affect hetrodimerization, either in the absence or presence of MurA (lanes 6 and 8). For EcR/mRXRα, MurA and RXR-ligands 9cRA and MA also enhance heterodimerization (lanes 12, 13, 16 and 17), and synergistic effects are observed (lanes 14 and 18). (B) GST pull-down assays were performed as described in Materials and methods. GST-EcR(DEF)-fusion protein bound to glutathione–Sepharose 4B beads was used to pull down ^{35}S-labeled USP. Interaction between EcR (DEF) and USP is not affected by MA in the absence or presence of MurA.

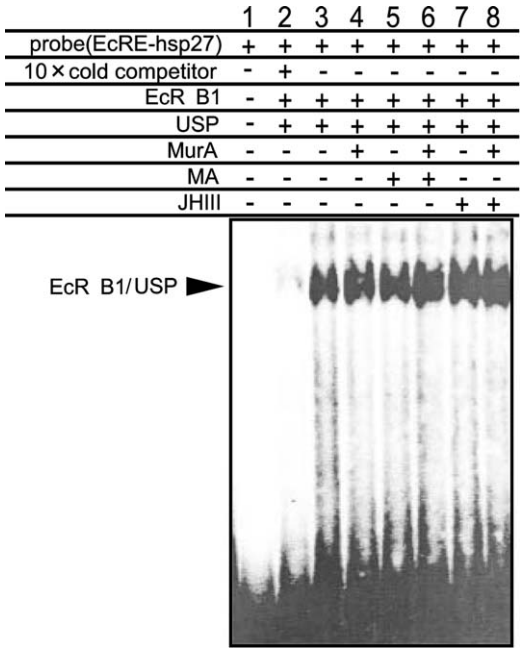

Fig. 3. JH III and MA do not affect the DNA-binding activity of EcR B1/USP. Purification of recombinant EcR B1/USP protein and electrophoretic mobility shift assays were performed as described in Materials and methods. ^{32}P-labeled EcRE from *hsp27*, an EcR/USP target gene, was used as a probe. JH III and MA do not affect the DNA-binding activity of EcR B1/USP regardless of the absence or presence of MurA, (lanes 6 and 8).

found that the juvenile hormones had no effect on DNA binding (Fig. 3, compare lanes 3, 4 with 5, 6 and 7, 8). These findings suggested that the transrepression mediated by juvenile hormones probably involved the recruitment of a co-repressor complex.

α-Helix 12 shifting in USP requires juvenile hormone-dependent transrepression

To evaluate whether USP indeed recruited a co-repressor in a ligand-dependent manner, we tested the α-helix 12 (H12) of USP. It has been well established that H12 serves as a physical interacting region for co-repressors as well as for co-activators. We tested the ligand response of a USP mutant that contained two amino acid substitutions (P498W/P499W), thought to alter the structure of the LBD [19], in the presence of juvenile hormones. We observed no suppressive effects of MA (Fig. 4B) or JH III (Fig. 4A) on the ligand response of the mutant, which suggested that USP H12 shifting induced by juvenile hormones may play a role in the repression of ecdysone-dependent EcR transactivation.

Transrepression by juvenile hormone-bound USP was abrogated by a histone deacetylase inhibitor TSA

Finally, we tested whether the juvenile hormone-mediated transrepression involved a histone deacetylase (HDAC)-containing co-repressor complex. As shown in Fig. 5 (lanes 8 and 12), when the HDAC inhibitor TSA was incubated with S2 cells, the juvenile hormone-induced transrepression of USP was markedly abrogated, while the transcriptional activity of unliganded EcR/USP was not affected by TSA, as expected (see Fig. 5 lanes 2, 6, and 10). Taken together, these results suggested that juvenile hormone-bound USP attenuates ecdysone-dependent transactivation through the recruitment of an HDAC co-repressor complex.

The transactivation function of EcR/USP is dependent on ecdysone binding, which then leads to the

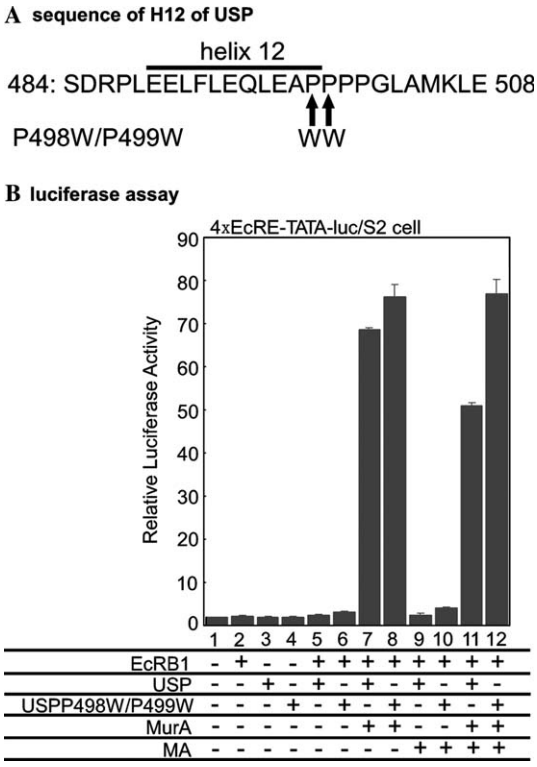

Fig. 4. (A) Sequence of USP helix 12. Amino acids targeted for mutation in USP P498W/P499W are highlighted. (B) Ligand-dependent conformational change of USP helix 12 is involved in the MA-dependent repression of EcR B1/USP. While USP P498W/P499 shows MurA-dependent transactivation of EcR B1/USP similar to wild-type USP (lanes 7 and 8), MA-dependent repression of EcR B1/USP transactivation in the presence of MurA is not observed (lanes 11 and 12).

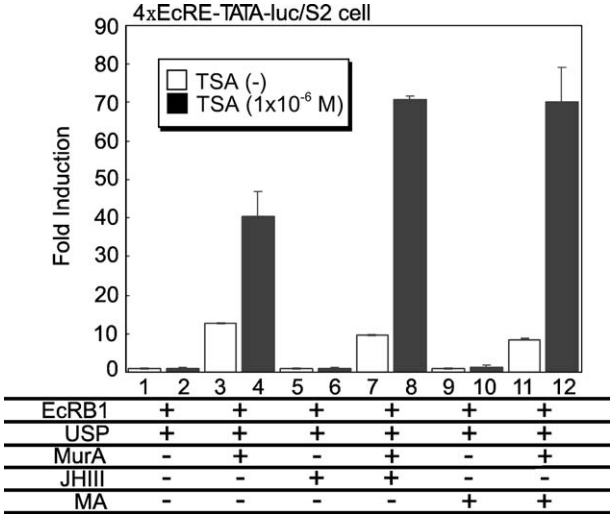

Fig. 5. JH III- and MA-dependent transrepression EcR B1/USP is released by TSA. TSA (1×10^{-6} M) enhances the MurA-dependent transactivation of EcR B1/USP (lane 2) and releases JH III- and MA-dependent transrepression (lanes 4 and 6).

recruitment of a number of co-activators such as TAI, NEJ, and TRR [20,22,28]. Here, we report that juvenile hormones antagonize ecdysone actions in the ligand-induced transactivation function of EcR/USP heterodimers. Our results indicate that JH III and MA act as ligands for USP and induce the recruitment of co-repressor complexes with HDAC activity through the shifting of α-helix 12 in USP. It was previously reported that while the LXXLL motif in TAI and TRR interacted with the EcR rather than the USP monomer, TAI could interact with USP when USP was heterodimerized with liganded EcR [20,28]. Therefore, it is possible that the USP H12 shifting due to juvenile hormone binding may lead to the dissociation of co-activators from ecdysone-bound EcR.

We found evidence that ligand-induced H12 shifting of USP was indispensable for transrepression. As the ligand-binding pocket of USP is large compared to other nuclear receptors [29,30], it is possible that the binding of unknown natural ligands caused USP to enter an inactive state. As the USP P498W/P499W mutant lost the transrepression activity in response to JH III or MA, it appears likely that juvenile hormones serve as USP ligands that recruit HDAC co-repressor complexes through H12 shifting. In vertebrates, NCoR and SMRT are known to act as nuclear receptor co-repressors through physical interactions between their CoRNR motifs and α-helices 3, 4, and 5 in unliganded LBDs [1]. Like NCoR and SMRT, a fly co-repressor containing a CoRNR motif could be recruited to liganded USP. To test this hypothesis, the identification of such co-repressor complexes is clearly required to help in elucidating the molecular basis of the juvenile hormone-induced antagonism of ecdysone actions.

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