Requirement of co-factors for the ligand-mediated activity of the insect ecdysteroid receptor in yeast

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ABSTRACT

In insects, a steroid hormone 20-hydroxyecdysone has an important role in regulating critical events such as development and reproduction. The action of 20-hydroxyecdysone is mediated by its binding to the ecdysteroid receptor (EcR), which requires a heterodimeric partner, ultraspiracle protein (USP), a homologue of the retinoid X receptor (RXR). The EcR–USP heterodimer represents a functional receptor complex capable of initiating transcription of early genes. Our goal was to establish a ligand-dependent transactivation system in yeast utilizing an insect EcR–USP heterodimer. This has been achieved using mosquito *Aedes aegypti* AaEcR–USP. Expression of AaEcR alone, but not USP, resulted in constitutive transcription of the ecdysone reporter gene coupled with the *Drosophila* heat shock protein-27 ecdysone response elements. Removal of the N-terminal A/B domain of AaEcR abolished its constitutive transcription. Constitutive transcription was also eliminated in the presence of its heterodimeric partner, AaUSPa, AaUSPb or mammalian RXR. This suggests that the A/B domain is essential for the EcR ligand-independent transactivation and its interaction with the yeast transcription complex. A ligand-mediated transactivation of Aa(ΔA/B)EcR–USP or Aa(ΔA/B)EcR–RXR heterodimers in response to an ecdysteroid agonist RH-5992 was observed only in the presence of GRIP1, a mouse co-activator. In the presence of a co-repressor, SMRT, Aa(ΔA/B)EcR–USP heterodimer exhibited a ligand-dependent repression activity. In addition, ligand-dependent transactivation systems for spruce budworm and fruit fly ecdysone receptors were also reported. This is the first report establishing the requirements of co-factors for a highly efficient ligand-dependent function of the insect EcR–USP in yeast. These findings open a way to study insect EcR–USP structure and function and to identify ligands that are specific for a certain group of insects, such as mosquitoes.
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INTRODUCTION

Ecdysteroids, the arthropod steroid hormones, have a crucial role in coordinating molting, metamorphosis and reproduction (Riddiford 1993, Thummel 1995). The action of ecdysteroids is mediated by the heterodimeric complex, consisting of two nuclear receptors, ecdysteroid receptor itself (EcR) and the insect homologue of the retinoid X receptor (RXR), ultraspiracle (USP). Upon binding the ligand, the EcR–USP heterodimer binds to a DNA ecdysone response element (EcRE) that is located in the promoter region of a series of ecdysteroid-responsive genes, and thereafter triggers the expression of a cascade of genes involved in regulating some key developmental events in insects. The formation of the EcR–USP heterodimer is required for both DNA and ligand binding (Yao *et al*. 1993, Kapitskaya *et al*. 1996). In addition to *Drosophila melanogaster* EcR (Koelle *et al*. 1991), several insect ecdysteroid receptors have now been cloned (Imhof *et al*. 1993, Cho *et al*. 1995, Fujiwara *et al*. 1995, Kothapalli *et al*. 1995, Swevers *et al*. 1995, Jindra *et al*. 1997, Verras *et al*. 1999).

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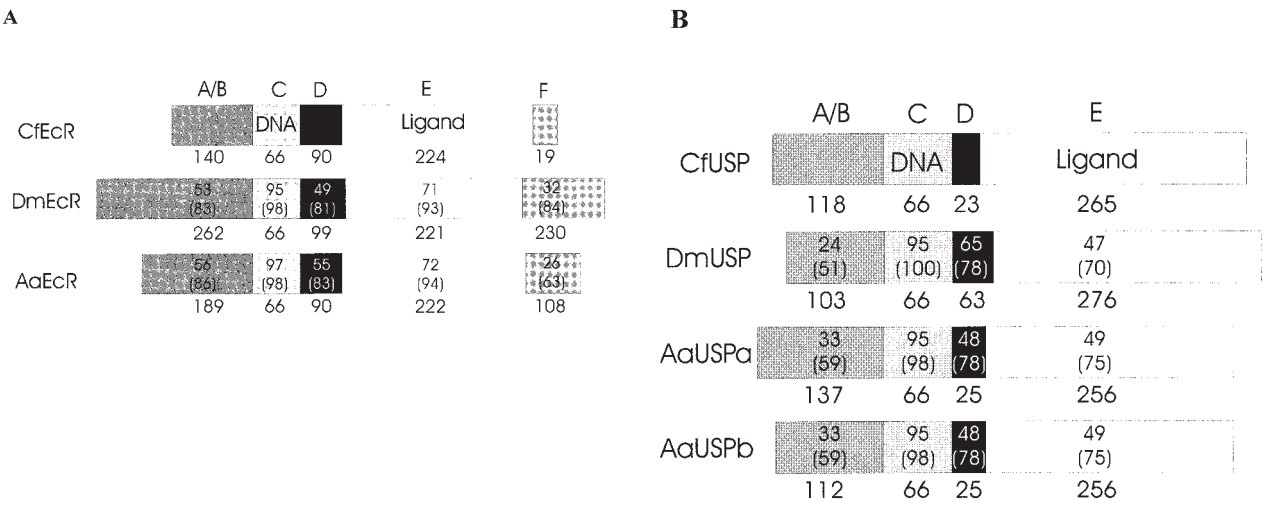

FIGURE 1. Domain comparison of insect EcR (A) and USP (B) receptors. Percent amino acid identity (top number) and percent amino acid similarity (bottom number) are indicated in each corresponding domain in comparison with CfEcR and CfUSP respectively. The number below each domain is its length in amino acids. (A) Alignment of CfEcR (Kothapalli *et al*. 1995), DmEcR-B1 (Koelle *et al*. 1991) and AaEcR (Cho *et al*. 1995). (B) Alignment of CfUSP (Perera *et al*. 1998), DmUSP (Henrich *et al*. 1990), AaUSPa and AaUSPb (Kapitskaya *et al*. 1996).

Likewise, in addition to *D. melanogaster* USP (Henrich *et al*. 1990, Oro *et al*. 1990, Shea *et al*. 1990), the heterodimer partner of EcR has also been cloned from several insects (Tzertzinis *et al*. 1994, Kapitskaya *et al*. 1996, Jindra *et al*. 1997, Perera *et al*. 1998).

The pleiotropic effects of ecdysteroids are reflected by the existence of several isoforms of both EcR and USP, which differ in their A/B domains. EcR isoforms were first identified in *Drosophila* (Talbot *et al*. 1993) and then in other insects (Fujiwara *et al*. 1995, Swevers *et al*. 1995, Kamimura *et al*. 1996, 1997, Jindra *et al*. 1996, Perera *et al*. 1998), including the mosquito *Aedes aegypti* (S F Wang, C Li & A S Raikhel, unpublished data). Unlike *Drosophila* USP, for which a single form of mRNA has been identified (Henrich *et al*. 1990, Oro *et al*. 1990, Shea *et al*. 1990), the mosquito *A. aegypti* (Kapitskaya *et al*. 1996, Wang *et al*. 2000*b*) and the tobacco hornworm *Manduca sexta* (Jindra *et al*. 1997, Lan *et al*. 1999) have been reported to have two isoforms.

The EcR partner can form a functional heterodimer with the USP protein from other insects, and even with mammalian RXR (Thomas *et al*. 1993, Yao *et al*. 1993, Verras *et al*. 1999, Wang *et al*. 1998). For example, Mediterranean fruit fly *Ceratitis capitata* ecdysone receptor (CcEcR) binds specifically to the *D. melanogaster* heat shock protein-27 (hsp27) EcRE as a heterodimer with DmUSP (Verras *et al*. 1999). For the mosquito, the EcR subunit has been shown to determine specificity of ligand binding (Wang *et al*. 2000*a*). Furthermore, *Drosophila* EcR has been shown to function with mammalian heterodimeric partners RXR (Yao *et al*. 1992, Hatzivassiliou *et al*. 1997).

EcRs or USPs have high homology in the corresponding DNA binding domains, but their A/B domains, which contain putative activation function (AF)-1, and ligand binding domains (LBDs), which contain putative AF-2 regions, share relatively low homology (Fig. 1; Kothapalli *et al*. 1995, Perera *et al*. 1998). Disrupting the ecdysteroid regulation cascade can be exploited as a new way for disruption of insect development. The low level of homology in the LBDs of EcR could be exploited to develop ligands that are specific for defined EcR. Subsequently, these compounds allow the disruption of the molting process in a pest-specific manner. The non-steroidal ecdysteroid agonist, RH-5992 that mimicks the action of ecdysteroid was used as a pesticide targeting lepidopteran larvae (Retnakaran *et al*. 1995). This compound mainly targets lepidopteran larvae, and is generally devoid of toxicity in non-lepidopteran species, including a wide range of important predators and parasites (Dhadialla *et al*. 1998).

A ligand-mediated transactivation system of EcR–USP and EcR–RXR has been developed in mammalian and insect tissue cultures (Thomas *et al*. 1993, Lan & Riddiford 1997). However, as there are many nuclear receptors expressed in mammalian and insect cells, the effect of ligand on the heterodimer might be compromised as a result

of the influence of other nuclear receptors. For example, in mosquito *A. aegypti*, expression of AHR38 can block heterodimeric formation of AaEcR–AaUSP and its activity as AHR38 competes for AaUSP (Zhu *et al*. 2000). Expression of the *M. sexta* USP-2 isoform can eliminate activation of MHR3 induced by the MsEcR-B1–USP-1 complex, as MsUSP-2 is competing with MsUSP-1 for MsEcR-B1 (Lan *et al*. 1999). In contrast to insect cells, yeast does not contain either nuclear receptors or any known homologues of mammalian co-activators. However, because of conservative general transcription machinery in eukaryotes, transactivation systems of mammalian nuclear receptors have been successfully reconstructed in yeast (see Butt & Walfish 1996). These systems have been used for structural analysis of various nuclear receptors, validation, and screening of pharmaceuticals (Butt & Walfish 1996, Butt & Chen 1999).

Several groups of investigators have tried in vain to reconstruct ligand-mediated transactivation of EcR–USP in yeast, because of lack of activity or high constitutive transcription activity (Dela Cruz & Mak 1997, Dela Cruz *et al*. 2000). Recently, we have reported the construction of a ligand-dependent transactivation system in yeast for spruce budworm *Choristoneura fumiferana* ecdysone receptor (Tran *et al*. 2001). This work describes the first example of yellow fever mosquito EcR–USP ligand-dependent transactivation system that responds to ecdysteroids and their analogue RH-5992, and its interaction with different co-activators. In addition to this, the ligand-dependent transactivation of *D. melanogaster* EcR–USP is also presented. The availability of a ligand-dependent transactivation systems for insect EcRs would provide an effective means to discover new chemicals, and validate and improve potential insecticidal candidates. Furthermore, this system could also provide a tool for structural and functional analysis of EcR with ecdysteroids, and with its partner USPs and other co-activators.

In this study, we report the reconstitution of a ligand-mediated transactivation system in yeast using the yellow fever mosquito *A. aegypti* EcR and USP. We have demonstrated that expression of the full-length of AaEcR alone can induce ligand-independent constitutive transcription of the reporter gene coupled with a promoter harboring the natural *Drosophila* hsp27 EcREs. Removal of the A/B domain of the AaEcR abolished constitutive transactivation activity of EcR, suggesting the important role of the A/B domain in ligand-independent transactivation. Co-expression of the A/B domain truncated EcR (AaΔEcR) with its heterodimer partners AaUSPa, AaUSPb or mammalian USP homolog RXRs failed to restore transactivation activity even in the presence of ligands. We tested the role of the following co-activators or co-repressors: GRIP1 (Hong *et al*. 1996), RIP140 (Cavailles *et al*. 1995), *Drosophila* CBP (Akimaru *et al*. 1997), mouse CBP (Chrivia *et al*. 1993), SMRT (Chen *et al*. 1996), SRC-1 (Onate *et al*. 1995), and N-coR (Horlein *et al*. 1995) in a ligand-dependent transactivation of insect EcR–USP complex. A ligand-mediated response was observed only when GRIP1, a mouse co-activator, was added to AaΔEcR–RXR or AaΔEcR–USP complexes. In contrast to the effect of GRIP1, the co-repressor SMRT exhibited ligand-dependent repression of constitutive activity. Different USPs such as mosquito USPa, USPb, USPs from *C. fumiferana* or *D. melanogaster*, or mammalian RXRs distinctively modulate the response of AaEcR to ligands. In addition to AaEcR–USP, we also reported ligand-dependent transactivation activity of spruce budworm *C. fumiferana* and *D. melanogaster* EcR–USP complexes in yeast. The transactivation responses to different compounds of these insect EcR–USPs in combination with series of cofactors are different, suggesting that insect EcR–USP activity is regulated by both ligands and co-activators/co-repressors. These findings open a way to study the insect EcR–USP complex and for discovering ligands for insect EcR and USP.

MATERIALS AND METHODS

Media, strains, plasmids and ligands

Standard yeast and *Escherichia coli* media have been prepared as described previously (Sherman *et al*. 1986, Sambrook *et al*. 1989). A yeast strain Y4727 *snq2::Δ pdr5::kanMX* mutant in the ABC transporter pathway was used as a host for transformation. The strain was derived from the yeast strain Y4727: *Mata his3-Δ200 leu2-Δ0 lys2-Δ0 met5-Δ0 trp1-Δ63 ura3-Δ0* (gift from Dr Jeff Boeke, Johns Hopkins University) by deleting the whole open reading frame of the SNQ2 and the PDR5 genes using the PCR-transformation technique as described previously (Storici *et al*. 1999). Yeast transformation was performed according to procedures described by Gietz *et al*. (1992). Yeast transformants with plasmids were maintained in correspondent drop-out selective media. Multicopy yeast–*E.coli* shuttle plasmids containing the full-length and A/B-domain-deleted receptor AaEcR and full-length AaUSPa and AaUSPb plasmids in addition to A/B-domain deleted receptor AaUSP (AaΔUSP) were constructed (see below). The

mammalian RXRα, RXRβ or RXRγ subtypes (Allegretto *et al*. 1993) were expressed in 2 μm multicopy plasmids under regulation of a CUP1 promoter with a LEU2 selective marker. The series of co-activators and co-repressors such as GRIP1 (Hong *et al*. 1996), RIP140 (Cavailles *et al*. 1995), *Drosophila* CBP (Akimaru *et al*. 1997), mouse CBP (Chrivia *et al*. 1993), SMRT (Chen *et al*. 1996), SRC-1 (Onate *et al*. 1995), and N-coR (Horlein *et al*. 1995) were also expressed as yeast 2 μm multicopy plasmids with a HIS3 selective marker. Construction of these plasmids is described below. The yeast expression plasmid with GRIP1 (Hong *et al*. 1996) was constructed as follows. The NsiI-BamHI (*GRIP1* gene with ADH1 promoter) fragment from the pGRIP812 (Walfish *et al*. 1997) was blunt-ended and cloned into the PvuII site of the pRS423 (Sikorski & Hieter 1989). The β-galactosidase reporter gene containing six copies of natural ecdysone response element (hsp27-EcRE) was also constructed (see below).

Ecdysteroid non-steroidal agonists RH-1, RH-2, RH-3, RH-4, RH-5, RH-6, RH-0345, RH-2485 and RH-5992 are derivatives of the original compound RH-5849 (Dhadialla *et al*. 1998). The structural formulae of the compounds RH-0345, RH-2485, RH-5849 and RH-5992 were published previously (Dhadialla *et al*. 1998). Muristerone A was purchased from Sigma. All ligands were dissolved in DMSO with a stock solution concentration of 500 μM. The final ligand concentration in the yeast cell culture was 10 μM.

Plasmid constructions

The plasmid pBRSS-6xEcRE-lacZ is a reporter construct carrying six copies of natural EcRE from the *D. melanogaster* heat shock protein-27 (*hsp27*) gene (Riddihough & Pelham 1987). The response elements are located in the upstream of the iso-1-cytochrome C (CYC1) promoter that is coupled with *E. coli* β-galactosidase gene (*lacZ*). This yeast–*E. coli* multicopy shuttle plasmid contains URA3 as a yeast transformation marker. Full-length or A/B domain-deleted versions of AaEcR were fused at the N-terminal in frame with human ubiquitin which was under CUP1 promoter (plasmids YEp–AaEcR and YEp–AaΔEcR, respectively). The 2 μm yeast expression plasmids YEp–AaEcR and YEp–AaΔEcR have been constructed, based on the plasmid YEpE12 (Graumann *et al*. 1996), which contains the TRP1 as a yeast selective marker, and ubiquitin-fused human estrogen receptor (ER) under CUP1 promoter. The YEpE12 plasmid was previously used for reconstruction of ligand-dependent transactivation of ER in yeast (Graumann *et al*. 1996). The following primers AaEcR-SalI and AaEcR-MluI were used for amplification of the full-length of AaEcR from cDNA (Cho *et al*. 1995). With AaEcR-SalI: 5′-GGAGTCGACCTTACATCTTGTCTTAAG ACTAAGAGGTGGTatgatgaaaagaagatggtcc-3′ (upper case indicates the nucleotide sequence belonging to human ubiquitin, the SalI site available in the ubiquitin is underlined, and lower case denotes the sequence belonging to AaEcR, starting from ATG). With AaEcR-MluI: 5′-AAGGACGCGTtgaacagaatgtcgtccgct-3′ (lower case indicates the nucleotide sequences belonging to 3′ terminal of the AaEcR, and the MluI restriction site is underlined). For amplification of the AaEcR with A/B domain deletion (Fig. 1) the following primers were used: AaEcR-MluI (above) and AaΔEcR-SalI: 5′-AGGAGTCGACCTTACATCTTGTCTTA AGACTAAGAGGTGGTatgcggcagcaggaggaactg tgtctg-3′ (upper case indicates the nucleotide sequence belonging to human ubiquitin, the SalI site available in the ubiquitin is underlined, and lower case denotes the sequence belonging to the DNA binding domain of the AaEcR starting from amino acid RQQEELCLV). After protein translation and ubiquitin cleavage, the arginine 'R' in the protein N-terminal would be exposed. That is proposed to be a signal for short-life protein (Bachmair *et al*. 1986), therefore, an additional methionine is added before the RQQEELCVL, to stabilize the protein. The DNA fragments were amplified in 30 cycles (96 °C for 30 s, 54 °C for 1 min, and 72 °C for 3 min) using high replication fidelity Deep Vent Polymerase (New England Biolabs). The PCR products for both full-length AaEcR and AaΔEcR were digested with SalI and MluI and subsequently subcloned into the SalI and MluI sites of the plasmid YEpE12 (Graumann *et al*. 1996).

Mosquito *A. aegypti* expresses two forms of USP (A and B isoforms) that differ only in the N-terminal region in the A/B domain (Fig. 1; Kapitskaya *et al*. 1996). The yeast expression vectors for A and B isoforms (pRS425-AaUSPa and pRS425-AaUSPb) and for AaUSP with deletion of the A/B domain (pRS425-AaΔUSP) were constructed as follows. The A and B isoforms of USP and ΔUSP were amplified from corresponding cDNAs using the following pairs of primers: AaUSPa-5′ and AaUSP-3′; AaUSPb-5′ and AaUSP-3′; and AaΔUSP-5′ and AaUSP-3′, respectively. AaUSPa-5′: 5′-AGGAGTCGACCTT ACATCTTGTCTTAAGACTAAGAGGTGGT atgctgaagaaggaaaaacc-3′; AaUSPb-5′: 5′-AGGA GTCGACCTTACATCTTGTCTTAAGACTAA GAGGTGGTatggatcccagcgatcgagg-3; AaΔUSP-5′: 5′-AGGAGTCGACCTTACATCTTGTCTTAAG

ACTAAGAGGTGGTatgtatccgccaaatcatccgctcagc-3′ (upper case indicates the nucleotide ubiquitin sequence, lower case denotes the nucleotide sequence of 5′-terminal of the AaUSPa and AaUSPb isoforms, starting from the first ATG codon, or starting from the DNA binding domain of the USP (MYPPNH) respectively, and the ubiquitin SalI site is underlined); and AaUSP-3′: 5′-AAGGACGCGTccacaagttgcttgttctagg-3′ (the MluI site is underlined, and lower case indicates the nucleotide sequence of the 3′ terminal of AaUSP cDNA. This primer is common for both the USPa and USPb nucleotide sequences). The PCR products were purified and digested with SalI and MluI and subsequently subcloned into the SalI-MluI sites of the yeast expression vector with the LEU2 marker pRS425–ERα. (The plasmid pRS425-ERα was constructed as follows: the BamHI–PmlI CUP1p–ERα–cyc_{ter} fragment from the YEpE12 plasmid (Graumann *et al*. 1996) was blunt-ended and then ligated into the PvuII site of pRS425 (Sikorski & Hieter 1989).)

The A/B domain-deleted version of CfEcR was fused at the N-terminal in frame with human ubiquitin which is also under CUP1 promoter (plasmids YEp-CfΔEcR). The multicopy yeast expression plasmid YEpCfΔEcR has been constructed based on the plasmid YEpE12 (Graumann *et al*. 1996) that contains the TRP1 as a yeast selective marker, and ubiquitin-fused human ER under CUP1 promoter. The following pair of primers, CfΔEcR-Sal I and CfEcR-Sac I, were used for amplification of the A/B domain-deleted CfEcR from the cDNA clone (Kothapalli *et al*. 1995). CfΔEcR-Sal I: 5′-GGAGTCGACCTTACATCT TGTCTTAAGACTAAGAGGTGGTatgcggcagca ggaggaactgtgtctg-3′ (upper case indicates the nucleotide sequence belonging to human ubiquitin, the *Sal* I site available in the ubiquitin is underlined, and lower case denotes the sequence belonging to the DNA binding domain of the CfEcR starting from amino acid RQQEELCLV); CfEcR-SacI primer: 5′-AAGGGAGCTCtaatctccc gcgcattc-3′ (lower case indicates the nucleotide sequences belonging to the 3′ terminal of the CfEcR, and the SacI restriction site is underlined). The A/B domain-deleted EcR (ΔEcR) started from the beginning of the DNA binding domain with amino acid sequence RQQEELCLV. After protein translation and ubiquitin cleavage, the arginine residue 'R' in the protein N-terminal that is a proposed signal for short life protein would be exposed (Bachmair *et al*. 1986), therefore an additional methionine was added before the RQQEELCVL to stabilize the protein. The DNA fragments were amplified in 30 cycles (96 °C for 30 s, 54 °C for 1 min, and 72 °C for 3 min) using high replication fidelity Deep Vent Polymerase (New England Biolabs). The PCR products for CfΔEcR were digested with SalI and SacI and subsequently recloned into the SalI and SacI sites of the plasmid YEpE12. The yeast expression vectors for spruce budworm *C. fumiferana* USP-CfUSP and CfUSP with deletion of the A/B domain (pRS425-CfUSP and pRS425-CfΔUSP respectively) were constructed similarly to the vectors with mosquito USP. Initially, the full-length CfUSP or CfΔUSP was amplified from a cDNA clone (Fig. 1, Kothapalli *et al*. 1995) using the following pairs of primers: CfUSP-5′ and CfUSP-3′ and CfΔUSP-5′ and CfUSP-3′, respectively. CfUSP-5′: 5′-AGGAGTCGACCTTACAT CTTGTCTTAAGACTAAGAGGTGGTatgtcaag tgtggcgaag-3′; CfΔUSP-5′: 5′-AGGAGTCGACC TTACATCTTGTCTTAAGACTAAGAGGTGG Tqatgtacccgcctaatcacccccctgagt-3′ (upper case letters correspond to the nucleotide sequence of ubiquitin, lower case letters denote the nucleotide sequence corresponding to the 5′-terminus of the CfUSP starting from the ATG or from the DNA binding domain of the CfUSP respectively, and the ubiquitin SalI site is underlined); CfUSP-3′: 5′-CCTTCCATGGgaatg tcaataatgcccgtg-3′ (the NcoI site is underlined, and the lower case letters indicate the nucleotide sequence of the 3′ terminus of CfUSP cDNA). The PCR products were purified and digested with SalI and NcoI and subsequently subcloned into the SalI-NcoI sites of a yeast expression vector containing a LEU2 selective marker, pRS425-ERα.

The DmUSP cDNA clone was used as a source for the plasmid construct. The N-terminal coding sequence of the DmUSP (Henrich *et al*. 1990, Oro *et al*. 1990) was amplified using two primers: DmUSP-AflII primer (5′-TTGTCTTAAGAC TAAGAGGTGGTatggacaactgcgaccagg-3′) and DmUSP-NcoI-primer (5′-agcaggtggaccatggacatgg-3′) (upper case letters indicate the sequence corresponding to ubiquitin, lower case letters indicate sequences corresponding to DmUSP, and the AflII and NcoI sites are underlined). The PCR fragment was digested with AflII-NcoI and subcloned into the AflII and NcoI sites of the plasmid YEpUbOCT1. The resulting plasmid contains the DmUSP cDNA sequence fused 5′ in frame with ubiquitin. Next, the NcoI-AflII II fragment of the DmUSP cDNA (Henrich *et al*. 1990, Oro *et al*. 1990) was cloned into the NcoI-Acc65I of the intermediate plasmid obtained above (both AflII and Acc65I sites were blunt-ended using Klenow DNA polymerase). The resulting plasmid, YEp-DmUSP encodes the full-length of the DmUSP amino acid sequence fused in frame with ubiquitin

sequences, expression of each being driven by the CUP1 promoter. The TRP1 selective marker is included to facilitate selection of transformants. The DmUSP cDNA sequence was also shuffled to another plasmid pRS425-ERα, containing the LEU2 with human ERa fused to ubiquitin under CUP1 promoter via the following the procedure. The SalI-MluI fragment containing a portion of ubiquitin and the entire coding region of the USP sequence from YEp-DmUSP was cloned into the SalI-MluI site of pRS425-ERα. The resultant plasmid, pRS425-DmUSP, contains a LEU2 selective marker. The USP sequence is fused in frame with ubiquitin and USP expression is driven by the CUP1 promoter. The yeast expression vector encoding the DmUSP with an A/B domain deletion (DmΔUSP, Fig. 1) was constructed as follows. The pRS425-DmUSP plasmid containing the full coding sequence of the DmUSP was used to amplify an N-terminally truncated DmUSP. First, the A/B domain deleted DmUSP was amplified using two primers: DmΔUSP-5′: (5′-AGGAGTCGACCTT ACATCTTGTCTTAAGACTAAGAGGTGGTat gtatccgcctaaccatccgctgagc-3′; upper case letters indicate the nucleotide ubiquitin sequence, lower case letters denote the nucleotide sequence of 5′-terminus of DmUSP starting from YPPNH, and the SalI site is underlined) and DmUSP-3′ (5′-AAGG ACGCGTcttttcggttagagcggatg-3′; (MluI site underlined, and lower case letters indicate the nucleotide sequence of the 3′ terminus of DmUSP cDNA). The PCR products were purified and digested with SalI and MluI and subsequently subcloned into SalI-MluI sites of the yeast expression vector with LEU2 marker, pRS425-ERα as described above. Similar to the plasmid YEpCfΔEcR, the plasmid YEpDmΔNEcR is the yeast expression plasmid with TRP1 marker, encoding the N-terminal truncated *D. melanogaster* EcR B-1 ecdysone receptor. The first 220 amino acids up to the sequence VNSSISS have been deleted and the resulting sequence is inserted into the yeast expression vector to produce a ubiquitin fusion protein under the control of a CUP1 promoter.

β-Galactosidase activity assay

A method to measure a β-galactosidase activity was developed to estimate a potency of compounds and to be simple enough to be applied in high-throughput screening. The transformed yeast cells with plasmids were grown overnight in selective liquid media at 30 °C and diluted in pre-warmed selective liquid media to 0·1 at an optical density of 600 nm (OD_{600}; $OD_{culture}$). $CuSO_4$ was added to the media to final concentration of 10 μM because all insect EcRs and USPs are under CUP1 promoter. One hundred microliters of the cell culture were spiked to each well of a 96-well microtiter plate. To each well, 2 μl ligand solution (diluted in DMSO) were added. For control wells, 2 μl DMSO were also added. The final concentration of the tested compounds in the media was 10 μM. Cells were incubated in the presence of a ligand in a shaker at 30 °C. After 4 h of incubation, 100 μl of 2 × 'Z' Sarcosine–O-nitrophenyl-β-D-galactopyranoside (ONPG) buffer (120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 20 mM KCl, 2 mM $MgSO_4$, 100 mM β-mercaptoethanol, pH 7·0, 0·4% lauroyl sarcosine, 4 mg/ml ONPG) were added to each well and the plate was further incubated at 37 °C. The 2 × 'Z' Sarcosine-ONPG buffer was freshly prepared or stored at −20 °C before use. After incubation at 37 °C for 1 h ($t_{incubation}$), the reaction was stopped by the addition of 100 μl quenching solution 0·5 M Na_2CO_3 and OD_{405} ($OD_{reaction}$) and β-galactosidase activity was measured in microplate reader (Biotek). As in all assays, the OD of the cultures was standardized as 0·1 and time for incubation with the β-galactosidase substrate ONPG was 1 h. The values presented in Figs 2–7 are equal to the median of optical density OD_{405} of at least eight independent reactions, multiplied by 1000.

RESULTS

Ligand-independent transactivation of AaEcR

Previous analyses using the *in vitro* insect cell line or *in vitro*-transcribed-translated receptors have shown that the formation of the EcR–USP heterodimer is required for both the DNA and ligand-binding activities (Yao *et al*. 1993, Kothapalli *et al*. 1995, Kapitskaya *et al*. 1996). In contrast, expression of *Drosophila* EcR alone in yeast has been shown to activate constitutive transcription of a reporter gene containing EcREs in its 5′-regulatory region (Dela Cruz & Mak 1997). In our experiments, we observed that expression of the full-length AaEcR alone induced transcription of the ecdysone reporter gene, irrespective of the absence or the presence of ligands (Fig. 2). This strong constitutive transactivation by the AaEcR was observed in the absence of USP (Fig. 2). The constitutive transcription activity of AaEcR was specific for a reporter gene harboring the EcRE. When AaEcR was co-expressed with a reporter plasmid YRpE2 (Lyttle *et al*. 1992) containing a reporter gene that was under regulation of the estrogen response element, no induced transcription was observed (data not shown). Even in the absence of copper induction, when expression

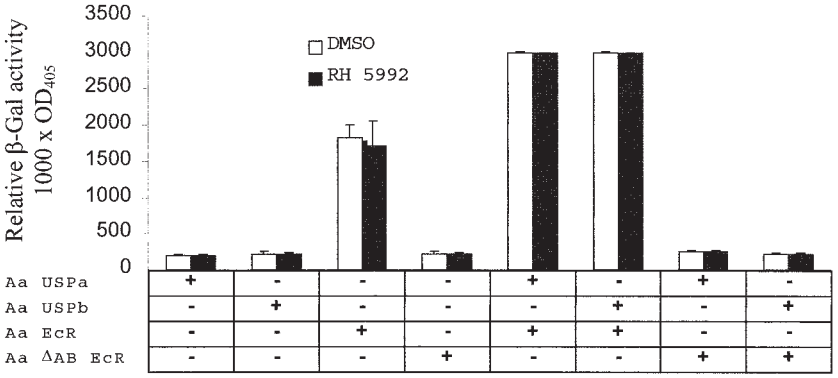

FIGURE 2. Transcriptional activation of *A. aegypti* (Aa) USP and EcR. The yeast strains carrying the reporter plasmid that contains six EcREs coupled with *E. coli* β-galactosidase (β-Gal) gene in the absence or the presence of different combinations of plasmids for expression of AaUSP a or b isoforms or AaΔUSP and EcR receptor. The relative transactivation activity in the absence or the presence of 10 μM RH-5992 was measured as described in Materials and Methods. The data are presented as a median of at least eight independent experiments, plus its standard deviation.

of AaEcR barely was observed in western blot, the constitutive transcription activity effect of AaEcR on the EcRE reporter gene was still observed (data not shown). Co-expression of AaEcR with either AaUSPa or AaUSPb resulted in stronger constitutive transactivation than was achieved by AaEcR alone (Fig. 2). However, the expression of either AaUSP isoform alone did not induce the reporter gene, clearly indicating that the presence of the full length of AaEcR was required for constitutive transactivation (Fig. 2). Surprisingly, removal of the A/B domain from AaEcR resulted in complete eradication of this constitutive transactivation activity. The constitutive transactivation ability of the truncated AaEcR was not restored by co-expression either with AaUSP or with mammalian RXRs (Figs 2 and 3). Thus the A/B domain of AaEcR presumably has a role as a ligand-independent transcription activator, at least in yeast.

Previously, Perera and coworkers (1999) showed that removal of the A/B domain of the *C. fumiferana* EcR does not affect either DNA or ligand binding activity. This suggests that the lack of the transactivation activity of AaΔEcR–USP could be due to the deficiency in communication between the heterodimer bound to EcRE and the yeast general transcription machinery. One of the possible reasons would be the lack of proper co-factors, which mediate such communication. Therefore we tested co-factors that could activate the ligand-dependent response of the AaΔEcR–USP or AaΔEcR–RXR heterodimers in yeast.

Reconstruction of ligand-dependent AaEcR–USP and AaEcR–RXR transcription in yeast

We speculated that AaΔEcR–USP and AaΔEcR–RXR could form a heterodimer with the ability to bind to the ligand but not initiate transcription. One possible reason for this inability to initiate transcription is the lack of co-factor(s) that serve as a 'bridge' between the heterodimer and the yeast general transcription complex binding to the TATA box of the CYC1 promoter. It has been shown previously that RXRs can interact with GRIP1, forming a protein link between ligand-activated nuclear receptors bound to cognate hormone response elements and the transcription initiation apparatus (Walfish *et al.* 1997). Therefore, the role of GRIP1 in the ligand-dependent transactivation assay for EcR–USP and EcR–RXR was tested. Transactivation of AaΔEcR–USP and AaΔEcR–RXR in the presence GRIP1 was observed in response to RH-5992 (Figs 3A and B). This suggests that GRIP1 can interact with both AaΔEcR–USP and AaΔEcR–RXR complexes. GRIP1 obviously acts differently than the A/B domain of EcR, because the full-length AaEcR initiates transcription in the absence of EcR ligands. We have noted that, in order to achieve ligand-dependent transactivation activity, it requires the presence of all three components: GRIP1, EcR and RXR or its counterpart, USP; without one of them, transactivation activity was not observed (Fig. 3).

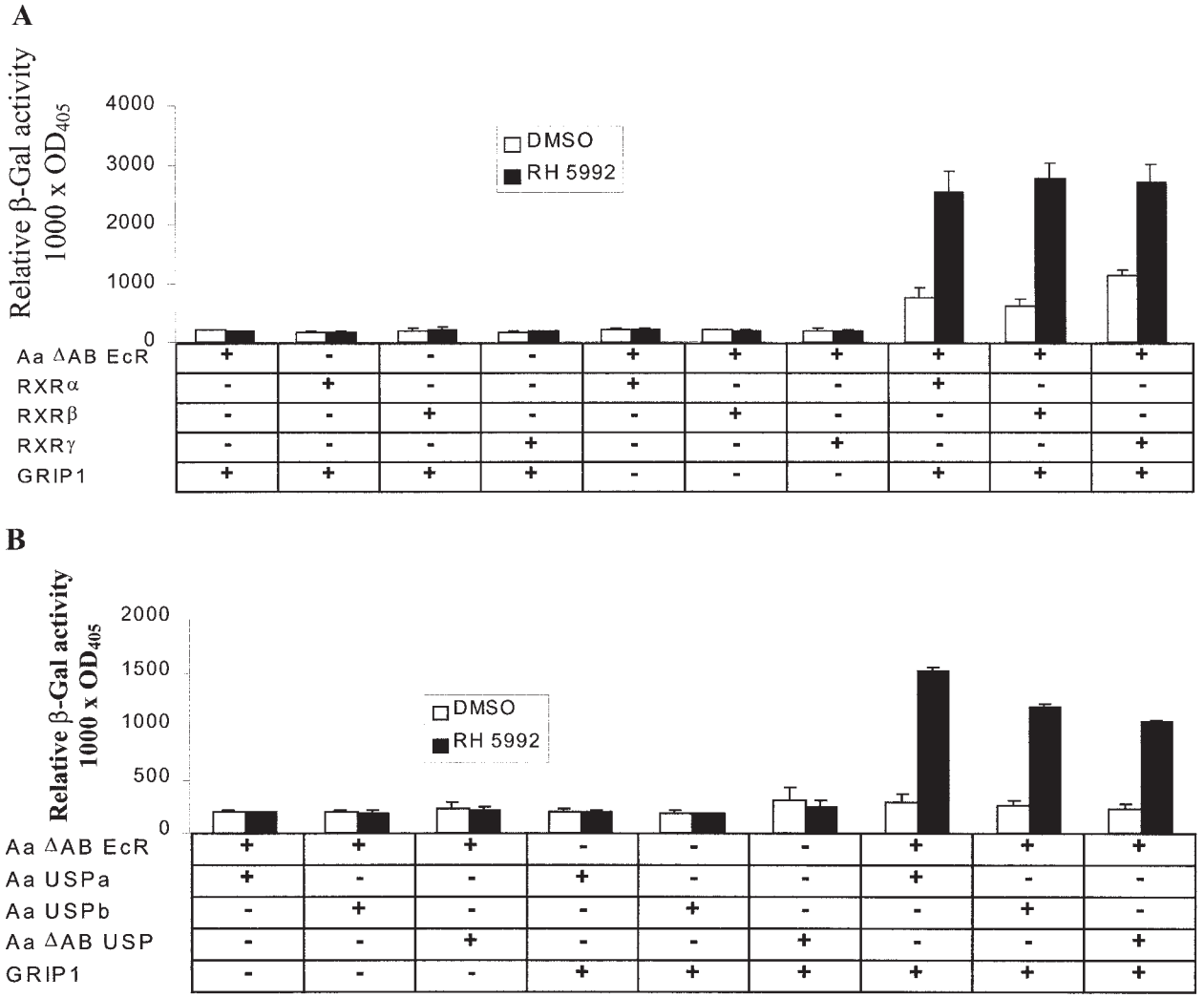

FIGURE 3. Transactivation of *A. aegypti* (Aa) EcR with A/B domain deletion in combination with mammalian RXRs or with AaUSPs in the absence and the presence of GRIP1. (A) Transactivation of Aa ΔUSP in combination with RXRs. (B) Transactivation of Aa ΔEcR in combination with different variants of mosquito USP. The yeast strains carrying the reporter plasmid that contains six EcREs coupled with *E. coli* β-galactosidase (β-Gal) gene in the absence or the presence of different combinations of plasmids for expression of AaΔEcR, RXRs or AaUSP receptors and GRIP1. The final concentration of RH-5992 was 10 μM. β-Galactosidase assays were performed as described in Materials and Methods. The data are presented as a median of at least eight independent experiments, plus its standard deviation.

Transactivation response to different ecdysteroid analogs

The ligand-dependent transactivation system in yeast for insect EcRs is derived from the following components: ecdysone response elements (DNA sequences to which EcR–USP binds), EcR itself, the partners of EcR (USP or RXR), co-activators, and the EcR ligands. A change in one in these components will affect the transactivation response. We investigated the effect of different ecdysteroid analogs in combination with the different RXRs and USPs, while keeping EcR, co-activator and the response element constant. The ecdysteroid analogs examined were different chemicals derived from the RH-5849 that has been thoroughly studied (Wing *et al*. 1988, Dhadialla *et al*. 1998). The derivatives of RH-5849 have a spectrum of activities relative to the specific insect EcR. We tested six proprietary compounds from the Rohm & Haas Company (coded as RH-1, RH-2, RH-3 RH-4 RH-5 and RH-6), four other compounds that have been published elsewhere (RH-0345,

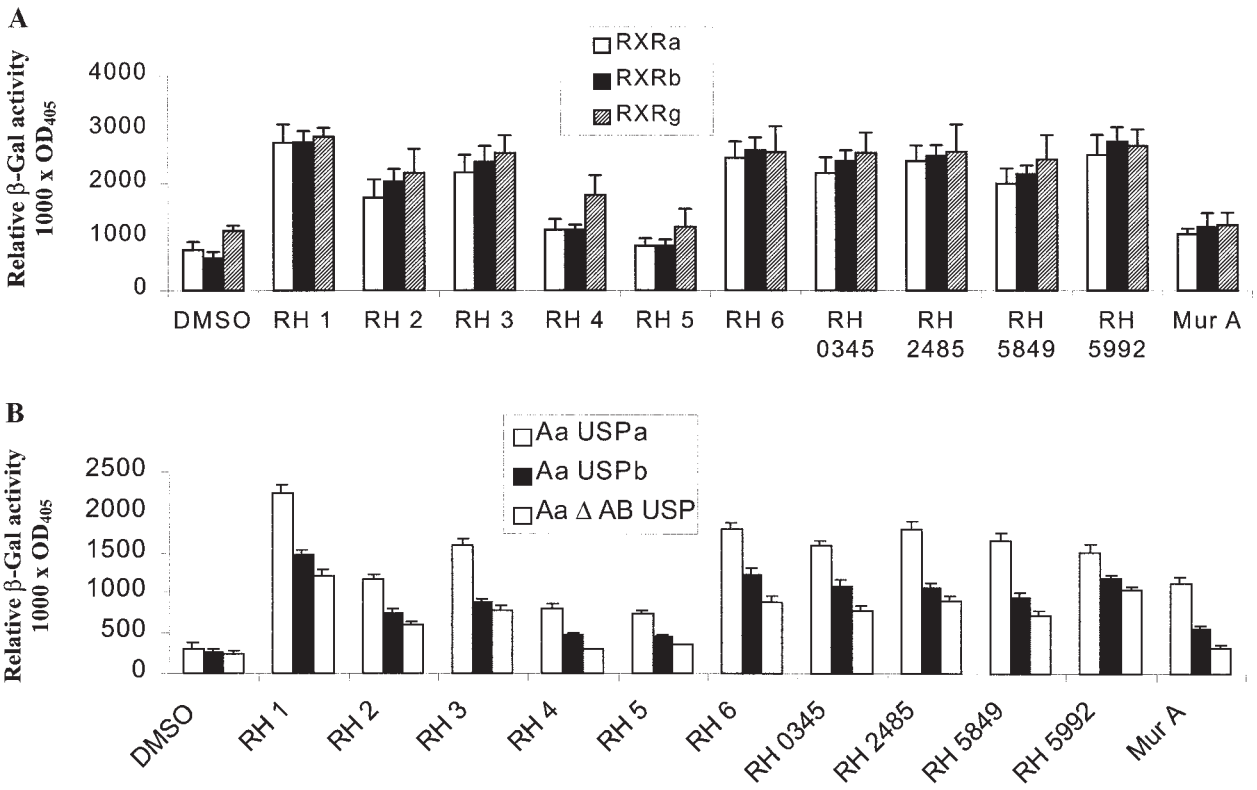

FIGURE 4. Transcriptional activation induced by different ecdysteroid agonists. (A) Transactivation activity of AaΔEcR–RXR (α, β or γ). (B) Transactivation activity of AaΔEcR–USP (a, b or ΔA/B variants). The yeast strains carrying the reporter plasmid that contains six EcREs coupled with *E. coli* β-galactosidase (β-Gal) gene in combination with the presence of different plasmids for expression of RXRs (α, β or γ) or USPs (AaUSPa, AaUSPb or AaΔUSP) and AaΔEcR and GRIP1. The final concentration of all ecdysteroid analogues was 10 μM. β-Galactosidase assays were performed as described in Materials and Methods. The data are presented as a median of at least eight independent experiments, plus its standard deviation.

RH-2485, RH-5849 and RH-5992; Dhadialla *et al*. 1998), and muristerone A (Mur A).

As shown in Fig. 3A, the RXR–AaΔEcR–GRIP1 complex induced a high basal transcription background; however, the presence of ecdysteroid compounds resulted in further increased transcription of the reporter gene. Different RXRs have different effects on transcription. The presence of the RXRγ results in the highest background. As shown in Fig. 4A, different EcR–RXRs responded differently to the test compounds. The weakest compounds are RH-4, RH-5 and Mur A.

Compared with the presence of RXR, that of AaUSP as a heterodimer partner of the AaEcR did not result in a high basal transcription background (Figs 3B and 4B). However, the heterodimers of AaΔEcR with different USPs (USPa, USPb, and ΔUSP) resulted in varying responses to the ecdysteroid compounds. The strongest response was observed for USPa and the weakest for ΔUSP (Figs 3B and 4B). The only difference between the USPa and USPb is in the N-terminal: the USPa is longer than USPb and there is some difference in the amino acid sequence of the N-terminal (Kapitskaya *et al*. 1996). The differences in the response of AaΔEcR–USPa and AaΔEcR–USPb (Fig. 4B) suggest that the A/B domain of the USP can affect the ligand-dependent response of the entire EcR–USP heterodimer. Although EcR receptor partners (USP or RXR) can enhance or reduce the response of the heterodimers to the ligands, overall we have observed consistent potency for different compounds using various RXRs or USPs as EcR partners (Fig. 5). For all EcR–RXRs or EcR–USPs, the compounds RH-4 and RH-5, in addition to Mur A, gave the weakest responses (Fig. 4).

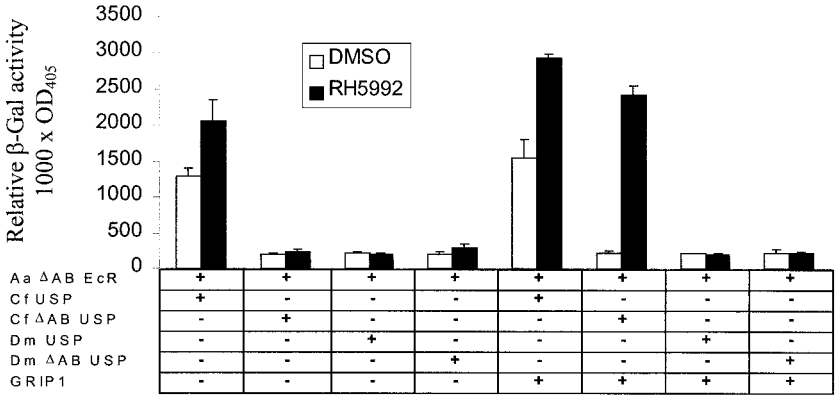

FIGURE 5. Transactivation of mosquito AaΔEcR in combination with different USP variants from spruce budworm and fruit fly in the absence and the presence of co-activator GRIP1. The yeast strains carrying the reporter plasmid that contains six EcREs coupled with *E. coli* β-galactosidase (β-Gal) gene in combination with the presence of different of plasmids for expression of USPs, AaΔEcR and GRIP1. The final concentration of RH-5992 was 10 μM. β-Galactosidase assays were performed as described in Materials and Methods. The data are presented as a median of at least eight independent experiments, plus its standard deviation.

Effect of different USPs in ligand-dependent transactivation of mosquito EcR

Although RH-5992 is a ligand for EcR, the receptor is able to bind to the ligand only in the context of a heterodimer with USP or RXR. As we have shown in Fig. 3, different RXRs have distinctive activities in combination with AaΔEcR. Three variants of mosquito USP also give different levels of response in combination with AaΔEcR. When EcR agonists were tested in the context of USPs, these variants gave similar responses; the differences were only in level of induction. The best result was observed for USPa (Figs 3 and 4B). As these variants differ from each other only in the A/B domain (AF-1), the differences in level of ligand induction suggested that the AF-1 domain may be working in concert with AF-2 in response to ligands. We further investigated how AaEcR responds to ecdysteroid ligands in combination with different USPs derived from spruce budworm *C. fumiferana* and fruit fly *D. melanogaster*.

As shown in Fig. 5, the presence of the full-length *C. fumiferana* USP, but not *D. melanogaster* USP, in combination with AaΔEcR resulted in constitutive transcription of the reporter gene. One must note that the expression of the AaΔEcR or CfUSP alone did not result in either constitutive or ligand-dependent transactivation. Surprisingly, deletion of the A/B domain of the CfUSP resulted in elimination of observed constitutive transactivation, suggesting that the AF-1 domain of the CfUSP modulates constitutive transcription of the reporter gene, but only in combination with EcR. In Fig. 5, we also showed that the presence of GRIP1 co-activators was absolutely required for the ligand-dependent response of AaΔEcR–CfΔUSP. In contrast to CfUSP expression, that of DmUSP in combination with AaΔEcR did not result in transcription induction. Even in the presence of GRIP1 the heterodimer complexes AaΔEcR–DmUSP and AaΔEcR–DmΔUSP failed to give a ligand-dependent response. We also observed that the heterodimer of AaΔEcR with CfΔUSP gave a stronger ligand-dependent response than the heterodimers of AaΔEcR with its own USP partners (Figs 3 and 5). These data suggest that USP can modulate the transactivation activity of the EcR and affect the EcR response to its ligands.

Role of other co-activators/co-repressors in the ligand-dependent response of insect EcR–USP complexes

A series of co-activators and co-repressors have been cloned from mammals and from *D. melanogaster*. They include: *Drosophila* CBP (Akimaru *et al*. 1997), mouse CBP (Chrivia *et al*. 1993), GRIP1 (Hong *et al*. 1996), SRC1A (Onate *et al*. 1995), RIP140 (Cavailles *et al*. 1995), SMRT (Chen *et al*. 1996), and N-coR (Horlein *et al*. 1995). Some of these enhance the ligand-dependent transactivation response in yeast-based systems (Joyeux *et al*. 1997,

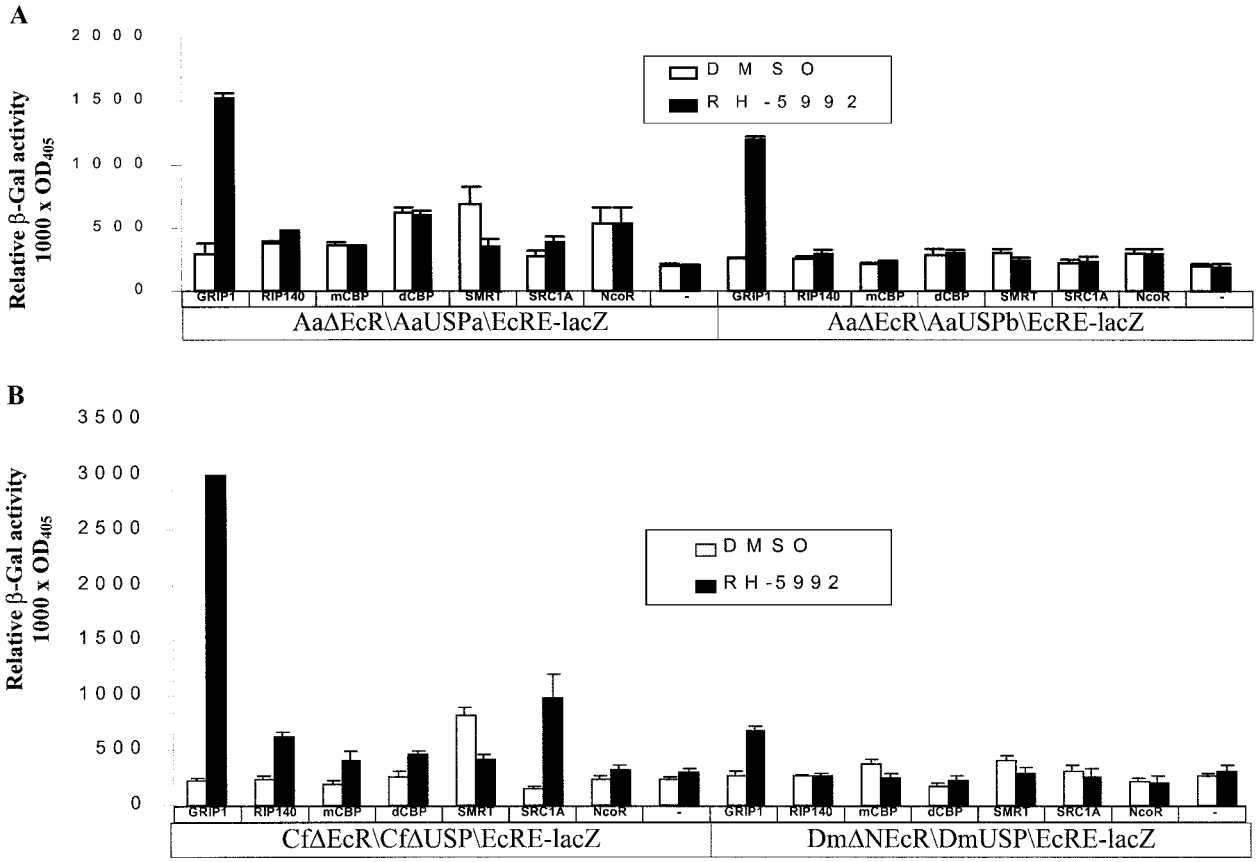

FIGURE 6. Roles of co-activators and co-repressors in transactivation of AaΔEcR. (A) Interaction of different transcriptional factors in combination with the heterodimers AaΔEcR–USPa or AaΔEcR–USPb. (B) Interaction of different transcriptional factors in combination with the heterodimers CfΔEcR–CfΔUSP or DmΔNEcR–DmUSP. The yeast strains carrying the reporter plasmid that contains six EcREs coupled with *E. coli* β-galactosidase (β-Gal) gene in combination with the presence of different of plasmids for expression of insect EcR and USPs in combination with co-activators/co-repressors. The final concentration of RH 5992 compound was 10 μM. β-Galactosidase assays were performed as described in Materials and Methods. The data are presented as a median of at least eight independent experiments, plus its standard deviation.

Walfish *et al*. 1997). Co-repressor-mediated ligand-dependent repression of nuclear receptors has not been observed in yeast to date. It is useful to know whether these co-activators/co-repressors are able to influence the activity of AaΔEcR–USPa and AaΔEcR–USPb in the presence and absence of ligands in yeast. Recently, we have reported a construction of ligand-dependent transactivation for spruce budworm CfEcR–USP in the presence of GRIP1 in yeast (Tran *et al*. 2001). In this study we tested the effect of other co-activators/co-repressors on CfEcR–USP. Although SRC1A belongs to the family of co-activator GRIP1, we did not observe activity similar to that observed for GRIP1 for mosquito EcR–USP (Fig. 6A). In contrast, co-activator SRC1A expressed was able to induce a ligand-dependent transactivation of CfΔEcR–CfΔUSP in yeast (Fig. 6B). (SRC1A expressed in the same plasmid was also able to enhance transactivation of the human thyroid hormone receptor, hTRβ, in response to tri-iodothyronine (P G Walfish, unpublished data).) Similar to mosquito AaΔEcR–USP, only GRIP1, but not SRC1A, gave an effect for *D. melanogaster* DmΔNEcR–USP (Fig. 6B). The small effect of RIP140 and CBP was observed when combined with CfΔEcR–CfΔUSP, but not with mosquito and fruit fly EcR–USP complexes (Fig. 6). (In other yeast transactivation systems, RIP140 was able to enhance the ligand-dependent response of ER (Sheeler *et al*. 2000).) This suggests that the co-activators GRIP1, SRC1A, RIP140 and CBP can distinctively interact with insect EcR–USP complexes in yeast. For the co-repressor N-coR, no effect was observed in

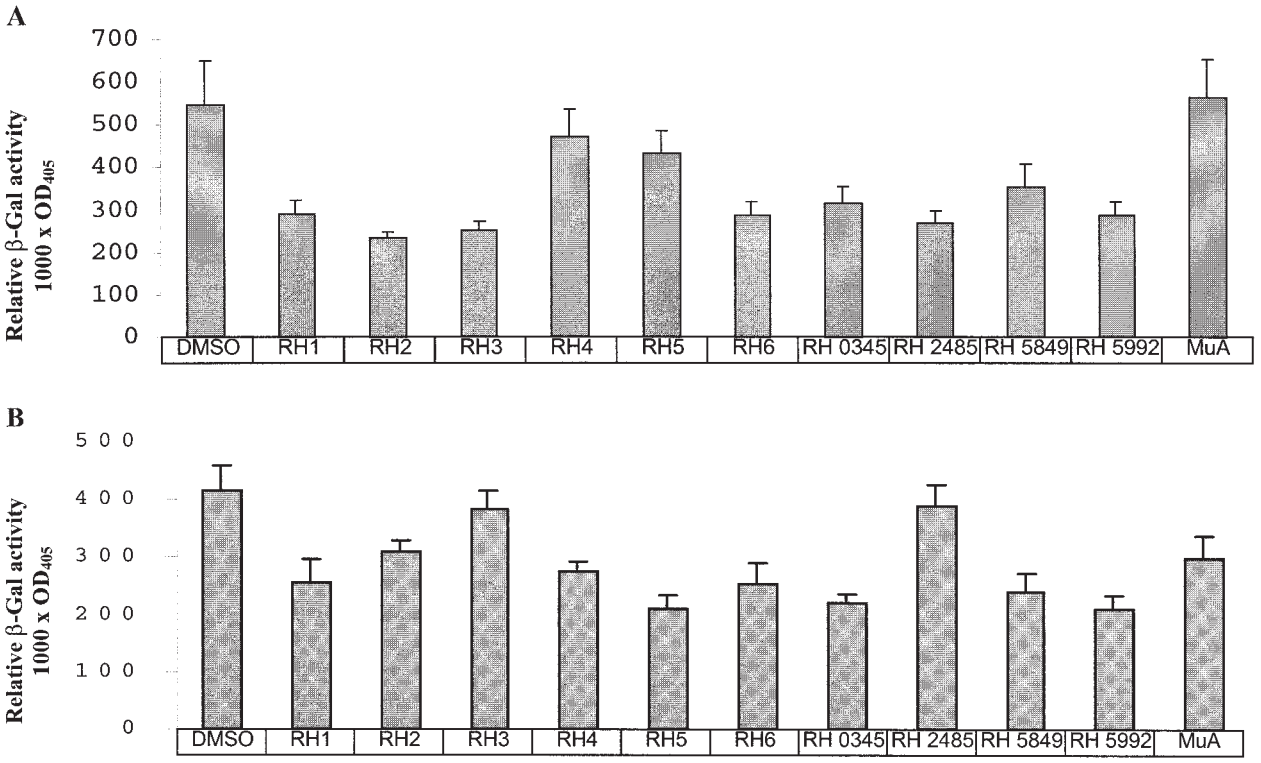

FIGURE 7. Roles of co-repressor SMRT in transactivation of insect EcR and USP receptors. (A) Impact of different ecdysteroidal analogs on transcription reduction of the AaΔEcR–USPa–SMRT complex. (B) Impact of different ecdysteroidal analogs on transcription reduction of the CfΔEcR–CfΔUSP–SMRT complex. The yeast strains carrying the reporter plasmid that contains six EcREs coupled with *E. coli* β-galactosidase (β-Gal) gene in combination with the presence of different of plasmids for expression of insect USP and EcR and SMRT. The final concentration of all ecdysteroid analogues was 10 μM. β-Galactosidase assays were performed as described in Materials and Methods. The data are presented as a median of at least eight independent experiments, plus its standard deviation.

either EcR–USP combination (Fig. 6). However, for the AaΔEcR–USPa and CfΔEcR–ΔUSP, the presence of co-repressor SMRT increased the level of transcription of the reporter gene (Fig. 6). When the ligand RH-5992 was added, the transcription of the reporter gene was reduced up to twofold (Fig. 6). We tested whether different ecdysteroid analogs can modulate the SMRT effect. As shown in Fig. 7, the more potent compounds are those with greater levels of ligand-dependent suppression. The effect is quite opposite to that observed in the presence of co-activator GRIP1 (Fig. 4). We have also noted that potencies of 11 test compounds for CfΔEcR–ΔUSP–GRIP1 and AaΔEcR–USPa–GRIP1 were different (Table 1); for the CfΔEcR–ΔUSP–SMRT and AaΔEcR–USPa–SMRT complexes these compounds also have different effects on transactivation reduction (Fig. 7). As shown in Fig. 2, AaEcR–USP induces constitutive transcription of the reporter gene. We tested whether SMRT is able to suppress the transactivation activity of the heterodimer. SMRT and AaEcR–USP were co-expressed in yeast in the presence of reporter gene. In the presence and absence of the ligand RH-5992, the constitutive transcription was not suppressed by SMRT (data not shown). Thus the ligand-dependent repression effect of SMRT was observed only in the context of AaΔEcR–USP, and not for AaEcR–USP.

DISCUSSION

Ligand-independent transactivation and the role of AF-1 in the ligand-dependent response

Overall, alignment of different insect EcRs and USPs revealed a high homology in their DNA binding domains. However, other regions such as A/B (AF-1) and D/E/F (AF-2) shared homology to a lesser extent, suggesting that these regions

TABLE 1. Comparison between data of transactivation activity in insect cells L57 and in yeast systems (fold induction)

	Fold induction		
	Insect cells	Yeast systems	
	CfEcR–DmUSP*	CfΔEcR–CfΔUSP*	AaΔEcR–AaUSPa
Compounds			
DMSO	1·0	1·0	1·0
Mur A	141	1·9	3·9
RH-1	54·0	6·5	7·6
RH-2	47·0	4·2	3·9
RH-3	30·0	5·1	5·3
RH-4	0·9	1·0	2·7
RH-5	21·0	6·7	2·5
RH-6	16·5	5·2	6·1
RH0345	23·6	5·0	5·3
RH2485	51·0	5·4	6·1
RH5849	27·7	3·4	5·6
RH5992	43·6	6·3	5·1

*Data from Tran *et al*. (2001) presented here for comparison. Fold induction over the background was obtained by comparing medians of transactivation activity induced by compounds and by DMSO. Data in the insect cells L57 and yeast assays were derived for all compounds at concentration 1 μM. The *Drosophila* L57 cell line lacking EcR was transfected with CfEcR and transactivation was measured using a chemoluminescent Tropix kit for β-galactosidase activity (Tran *et al*. 2001). The data in insect cell experiments are medians of three independent measurements; the data for yeast systems are presented as a median of at least eight independent experiments.

underwent significant divergence during evolution (Fig. 1). The divergence in the AF-1 and AF-2 domains of EcR and USP may modulate the EcR–USP complex in response to ecdysteroids. The evolutionary divergence in these domains in different insects can be exploited in designing ligands that may act in a species-specific manner.

Ecdysteroid-dependent induction of transcription in insects is not dependent solely on the EcR–USP complex, but also on a set of EcR–USP-interacting co-activators/co-repressors that might be expressed specifically in each tissue. In contrast to insect cells, yeast does not contain any nuclear receptors or homologues of co-activators/co-repressors. When EcR is expressed in yeast, the components of co-activators/co-repressors are absent. Also in contrast to what happens in insects, we have observed that expression of AaEcR alone in yeast, similar to what has been observed for DmEcR (Dela Cruz & Mak 1997), induces ligand-independent transactivation. Thus expression of DmEcR and AaEcR in yeast resulted in ligand-independent transcription of the EcRE reporter gene. However, expression of another EcR from spruce budworm (CfEcR) in the same expression yeast vector did not induce transcription of the reporter gene (Tran *et al*. 2001). Interestingly, both the fruit fly (*D. melanogaster*) and the yellow fever mosquito (*A. aegypti*) belong to the class Diptera, whereas spruce budworm is a lepidopteran. Co-expression of AaEcR and AaUSP further enhances the induction of transcription of the reporter gene (Fig. 2). These observations suggest that there exist co-repressors in insects that suppress constitutive transactivation activity of EcR. One of the possible candidates as a co-repressor is insect SMRTER, which has been identified as an EcR-interacting protein and a homolog of the mammalian co-respressors SMRT and N-coR (Tsai *et al*. 1999). Surprisingly, deletion of the A/B domain of EcR eliminates the constitutive transcription activity (Fig. 2). The ligand-dependent transactivation was observed only when a new co-activator (GRIP1) was added to the EcR–USP complex (Fig. 3). In the light of these observations, in the absence of ligand the transactivation activity of the A/B domain (AF-1) in EcR in insects may be masked by co-repressors, whereas this activity is not present in yeast cells because of a lack of co-repressor.

The role of the AF-1 domain in ligand-independent transactivation is well documented. For example, the AF-1 domain of ER can be activated by an EGF-triggered phosphorylation pathway (El-Tanani & Green 1997). The activity of the AF-1 domain is modulated by interaction with co-activators or co-repressors (Wilkinson & Towle

1997, Onate *et al*. 1998). We have also demonstrated that the full length of CfUSP can also induce ligand-independent transactivation. However, in contrast to AaEcR, the constitutive transactivation activity of CfUSP is dependent on the presence of EcR (Fig. 5). Deletion of the A/B (AF-1) domain of the CfUSP results in the elimination of constitutive transactivation. These observations suggest that the AF-1 domains of the AaEcR or CfUSP possess transactivation functions. Recently, we also demonstrated that deletion of the AF-1 domain of the CfEcR enhanced ligand-dependent transactivation in yeast, suggesting an inhibitory activity of the AF-1 domain of the CfEcR (Tran *et al*. 2001). As we have demonstrated above, AaEcR alone can induce transcription of the reporter gene harboring EcREs in the promoter (Fig. 2). The effect of the AaEcR is specific for the reporter with EcRE elements as expression of the AaEcR did not induce transcription of the reporter gene with estrogen response elements (data not shown). We can not exclude the possibility that yeast protein(s) exist that form heterodimers with EcR and bind to EcRE, even though a thorough search of the yeast protein database failed to find USP or RXR homologues. The functional EcR is commonly considered as a heterodimer consisting of two proteins, EcR and USP, yet our results clearly demonstrated that AaEcR alone, without USP, can function as a transactivator in yeast, in agreement with results obtained from DmEcR (Dela Cruz & Mak 1997). *In vitro* transcribed DmEcR (Vogtli *et al*. 1998) and AaEcR (S F Wang and A S Raikhel, unpublished data) are able to bind DNA without USP. It is also conceivable that the EcR homodimer is able to communicate with yeast basal transcription machinery independently of the ligand.

The A/B domains of EcR and USP have roles not only in ligand-independent or constitutive transactivation, but also in the ligand-dependent response. In Fig. 3, we demonstrate that, although mosquito USPa, USPb and ΔUSP in combination with its ΔEcR produce ligand-dependent transactivation, the efficacy or level of response was different for these variants. The best response to muristerone A was observed when AaΔEcR combined with AaUSPa. AaUSPa has an N-terminal sequence of MLKKEKPMLSVAAIIQAQGRWDRTL-PLAGL, whereas AaUSPb has a sequence MDPSDR. The remainder of the amino acid sequences are identical, except for one amino acid change (Kapitskaya *et al*. 1996). In the yeast transactivation assay, the the magnitude of the responses to ecdysteroid analogs are in the order AaΔEcR–USPa>AaΔEcR–USPb>AaΔEcR–AaΔUSP. There are at least two possible explanations for this. Firstly, as the A/B domain works in concert with the ligand-binding domain, deletion of the AF-1 domain or modification of the AF-1 domain can affect the potency of the ligands. Secondly, the AF-1 domain can interact with a co-activator (Ikonen *et al*. 1997, Wilkinson & Towle 1997), resulting in a modification in the AF-1 domain that can result in an increase or decrease in transactivation. In any case, we have observed the impact of N-terminal deletion on the activities of ligand binding and transactivation. In yeast, all three isoforms of *D. melanogaster* EcR showed ligand-independent transactivation of an ecdysone reporter gene, but the amount of activation was correlated with size of the N-terminal A/B domain present in the isoforms (Dela Cruz *et al*. 2000). Furthermore, we have observed ligand-independent transactivation of the AaEcR and CfUSP (the effect of the latter being dependent on the presence of EcR). It is interesting to note that, when the A/B domain of the AaEcR or CfUSP is fused, for example, with another CfEcR or DmUSP, the latter receptors do not exhibit ligand-independent transactivation. As we have noted, deletion of the A/B domain of the AaEcR results in elimination of constitutive transactivation even in the presence of the AaUSPa or AaUSPb; however, the presence of CfUSP leads to constitutive transactivation (Fig. 5). Recently, we have observed that CfUSP, when combined with DmΔEcR or CfEcR always induces constitutive transcription (Tran *et al*. 2001, and unpublished data). This raises the question of what happens if the A/B domain of the CfUSP is relocated to the N-terminal of EcR.

Response of EcR to its ligands is dependent on EcR partners

USP is the insect counterpart of the mammalian RXR. *In vitro* and *in vivo* data have shown that RXR could substitute for insect USP and form the heterodimer complex with EcR (Thomas *et al*. 1993, Yao *et al*. 1992, 1993, Suhr *et al*. 1998). However, in the absence of GRIP1 the heterodimer RXRα (β or γ)–AaΔEcR did not exhibit transactivation activity in response to the ligand RH-5992 (Fig. 3). In combination with GRIP1 co-activator, all RXR–EcR heterodimers increased the basal transcription background and started to respond to the ligand RH-5992. In contrast to RXRs, insect USPs either provoke constitutive transcription (in the case of CfUSP) or do not increase background even in the presence of GRIP1 (Figs 3B, 4B and 5). In examining three variants of mosquito USPs, we have observed that USP with different N-termini can have different levels of response to ligands, in

the following order: USPa>USPb>ΔUSP (Fig. 3B). For both DmUSP variants in combination with mosquito EcR, response to the ligand was not observed (Fig. 5). These results suggested that the A/B domain of USP could differentially modulate the transactivation function of EcR–USP. Thus the response of EcR to its ligands in transactivation is dependent not only on itself, but also on its partners. Modification in the AF-1 and divergence in the AF-2 of USP can affect the entire activity of the EcR–USP complex.

Role of co-activators/co-repressors in receptor transactivation in yeast

A series of nuclear receptors has been successfully reconstructed in yeast in the absence or the presence of co-activators such as RIP140, GRIP1 or SRC1 (Metzger *et al*. 1988, Mak *et al*. 1994, Butt & Walfish 1996, Hong *et al*. 1996, Joyeux *et al*. 1997, Walfish *et al*. 1997). The co-activator SRC1 has been shown to co-ordinate interaction between N- and C-termini of the androgen receptor that are necessary for transcriptional activity (Ikonen *et al*. 1997). GRIP1 has been shown to interact *in vitro* in a ligand-dependent manner with thyroid receptor, retinoic acid receptor and RXR (Hong *et al*. 1997, Walfish *et al*. 1997, Anafi *et al*. 2000). In this work, we observed that the presence of GRIP1 is required for ligand-mediated transactivation function of EcR–USP or EcR–RXR in yeast (Figs 3, 4 and 5). As shown in Fig. 3, GRIP1 co-activator protein markedly increased the ability of EcR–RXR heterodimers to transactivate the reporter genes harboring EcRE. For three subtypes of RXR receptors (α, β and γ) in combination with AaΔEcR and GRIP, increased basal transcription of the reporter gene was observed (Fig. 3A), suggesting that the interaction between EcR–RXR and GRIP does not require the presence of a ligand. As a USP homolog to RXR, we demonstrated that not only does EcR–RXR interact *in vivo* with GRIP, but EcR–USP also can interact with GRIP1 (Fig. 3B). This is first example of mammalian co-activator working in concert with insect nuclear receptors. Previously, GRIP1 was shown to interact with TR–RXR or VDR–RXR heterodimers (Walfish *et al*. 1997). Our results demonstrated that GRIP could interact *in vivo* with both EcR–RXR and EcR–USP in a transactivation assay. Although SRC1A belongs to the family of co-activator GRIP1, we did not observe activity similar to that observed for GRIP1 for mosquito EcR–USP (Fig. 6A). However, for other EcR–USP complexes from spruce budworm *C. fumiferana*, an effect of co-activator SRC1A expressed in the same plasmid was observed (Fig. 6B), suggesting that co-activators GRIP1 and SRC1A can distinguish insect EcR–USP complexes in yeast.

The *Drosophila* homolog of co-repressor SMRT, SMRTER, has been cloned and shown to interact with EcR (Tsai *et al*. 1999). Here we have demonstrated that SMRT can genetically interact with the insect EcR–USP complex in yeast (Figs 6 and 7). Consequently, we plan to investigate the interaction of *Drosophila* SMRTER with EcR–USP using this transactivation system. The ligand-dependent function of SMRT in combination with the mosquito and spruce budworm EcR–USP heterodimers is presented in Figs 6 and 7. The AaΔEcR–AaUSPa heterodimer alone did not induce transactivation of the reporter gene. The addition of SMRT to the heterodimer increased the basal transcription of the reporter gene. However, in the presence of ecdysteroidal analogs the acquired basal transcription was reduced (Figs 6A and 7A). The more potent the ligand, the stronger the reduction that is observed. A similar effect was observed for CfΔEcR–CfΔUSP. It has been proposed that co-repressors N-coR and SMRT repress transcription of targeted genes in the absence of ligands by interacting with nuclear receptors (Nagy *et al*. 1999). Upon the binding of nuclear receptors with ligands, co-repressors are released. In yeast two-hybrid experiments, in the absence of an EcR ligand, both SMRT and N-coR have been shown to interact with C-terminal of *D. melanogaster* EcR (Thormeyer *et al*. 1999). The combination of SMRT with DmEcR yielded a much greater expression (more than 30-fold) of lacZ reporter gene compared with the combination of N-coR with EcR (Thormeyer *et al*. 1999). This may explain why an effect of SMRT but not of N-coR was observed in our system (Fig. 6). Furthermore, Thormeyer *et al*. (1999) have shown that the presence of muristerone A leads to a decrease in the SMRT–EcR interaction *in vitro*. In another two-hybrid system mammalian assay, SMRT has been shown to interact with the heterodimeric DmEcR–USP complex (Tsai *et al*. 1999). Addition of hormone appears to disassociate SMRT completely from the heterodimer complex, therefore eliminating reporter activity (Tsai *et al*. 1999). Clearly, our data on SMRT in Figs 6 and 7, showing that addition of EcR ligand reduced the transactivation activity observed for EcR–USP–SMRT complexes, are consistent with the published data of Tsai *et al*. (1999) and Thormeyer *et al*. (1999). Tsai and coworkers (1999) have also shown that the semi-lethal mutation A483T in the DmEcR eliminates

EcR–USP interaction with SMRT. The alanine A483 residue is located within a highly conserved region of the LBD domain of *Drosophila* EcR (corresponding to A401 in *A. aegypti* EcR). Most probably, mutation A401T in the mosquito EcR will eliminate the SMRT effect that is shown in Fig. 7.

Nagy *et al*. (1999) reported the identity of complementary-acting signature motifs in SMRT and N-coR that are sufficient for receptor binding and ligand-induced release. The motif contains a hydrophobic core (PhiPhixxPhi) similar to that found in nuclear receptor co-activators. Surprisingly, mutations in the amino acids that directly participate in co-activator binding disrupt the co-repressor association (Nagy *et al*. 1999). It would be interesting to test the effect of mutant variants of mammalian SMRT in the system, to determine whether the mutant SMRT still could induce basal transcription as presented in Fig. 6. Recently, an EcR-interacting protein, SMRTER, which is structurally divergent from but functionally similar to mammalian SMRT and N-coR, has been isolated (Tsai *et al*. 1999). SMRTER repression activity is mediated by interaction with Sin3A, a repressor known to form a complex with the histone deacetylase Rpd3–HDAC. Mutant EcR that fails to interact with SMRTER results in a growth defect and lethality (Tsai *et al*. 1999). It would be useful to know whether insect SMRTER acts similarly mammalian SMRT in interacting with EcR–USP as presented in Figs 6 and 7.

Potential use of EcR transactivation assay in pesticide screening

One of the purposes in developing a ligand-dependent transactivation system for EcR in yeast is to use it for identifying EcR ligands. Species-specific EcR ligand potentially can be used as a pesticide that targets insect specifically. Figure 4 demonstrated that different ligands have different abilities to induce AaEcR transactivation in yeast. The availability of several EcR transactivation systems will enable the rapid identification and comparison of ligands that are specific for each EcR. The availability of EcR–USP transactivation system for mosquito, spruce budworm and fruit fly EcRs (Figs 3 and 5) allowed us to compare the potencies of different compounds in different insect EcR. Table 1 presented the fold induction of transactivation in spruce budworm and mosquito EcRs by different compounds. As shown in the Table, the compound RH-4 is inactive for CfEcR both in yeast and in insect cell lines transfected with CfEcR receptor; however, some activity is observed for this compound when AaEcR is used. In contrast, RH-5 is a weak ligand for AaEcR, but has a high potency for CfEcR transactivation. Furthermore, RH5849, the well-characterized ligand for insect EcR (Wing *et al*. 1988) has weak activity for CfEcR but strong activity for AaEcR transactivation. Among insect EcR and USP receptors, the ligand-binding domains are quite divergent, therefore it is possible that an EcR-targeting pesticide that is toxic to one type of insects is safe for others.

The yeast system reported here does not duplicate all aspects of the *in vivo* activity of EcR (e.g. the AF-1 region of EcR must be deleted), but it should prove to be a valuable tool for basic and applied studies. Further studies could involve the analysis of defined mutations analysis in which a specific amino acid of USP or EcR is changed, and investigation of how these mutations can affect responses to ligands and interactions with co-activators/co-repressors. The yeast system could be used to identify co-activators or co-repressors that act similarly to GRIP1 or SMRT respectively. We used the method for molecular profiling of several compounds possessing ecdysteroid-like properties. The results presented in Fig. 5 demonstrate that the method might serve as a system for screening of pesticides targeting EcR receptors. Searching for a USP ligand is of special interest. As we demonstrated, structural modification of USP or RXR affects the transactivation response to EcR ligand, therefore we believe that ligands of USP, if they exist, will also affect the transactivation response of EcR–USP to EcR ligand. Previously, Jones & Sharp (1997) demonstrated that juvenile hormone III (two JH III structures, monoepoxide and bisepoxide) is able to bind to and change the conformation of USP. We examined the agonist and antagonist properties of the JH1, JH2 and JH 3, in addition to phenoxycarb and methoprene, but did not see any agonist or antagonist activity of these proposed USP ligands. If firm conclusions are to be drawn on this subject, more intensive studies must be undertaken. We believe that the EcR–USP system could be used for studying ligands not only for EcR, but also for USP.

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