Species specific aspects of ecdysteroid receptor response to ecdysteroids and juvenile hormone

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Introduction

The functional ecdysteroid receptor

The functional ecdysteroid receptor is a heterodimer composed of the ecdysone receptor (EcR) and Ultraspiracle (USP). Insect development is largely driven by the interaction of these two nuclear receptors (Yao et al., 1992; 1993; Thomas et al., 1993), an interaction which is mediated by the insect molting hormone 20-hydroxyecdysone (20E) then further modulated by juvenile hormone III (JHIII). Ecdysteroids are the only endogenous class of insect steroid hormones and are capable of evoking a broad range of tissue specific transcriptional responses (Riddiford et al., 2000; Thummel, 2002). Both EcR and USP are members of a larger superfamily of nuclear receptors having a characteristic DNA binding domain (DBD) and ligand binding domain (LBD, Henrich et al., 1990; Oro et al., 1990; Shea et al., 1990; Koelle et al., 1991). The DBD consists of two cysteine-cysteine zinc fingers, which have highly conserved amino acid sequences. This moiety provides a dimerization interface capable of coordinating a series of cooperative structural transitions in the presence of a DNA response element that stabilize formation of the ecdysteroid receptor complex (Jakób et al., 2007). The DBD enables interaction of the ecdysteroid receptor complex with several defined DNA response elements to regulate the transcription of target genes. The most notable of these is a sequence in the promoter of the 27kDa heat shock protein (hsp27) of Drosophila melanogaster (Riddifough and Pelham, 1987). This inverted palindromic hsp27 ecdysone response element (EcRE) motif closely resembles that of the glucocorticoid receptor and the estrogen receptor (Hollenberg et al., 1985; Green et al., 1986). The LBD of both EcR and USP is a structural motif consisting of twelve alpha helices. The structure and function of this domain is highly conserved across members of the superfamily of nuclear receptors (Wurtz et al., 1996a,b). The LBD forms the dimerization interface for the EcR/USP heterodimer and the ligand binding pocket of EcR (Perlmann et al., 1996).

Mutational studies of EcR and USP have demonstrated that specific residues can be associated with such subfunctions as ligand binding, heterodimerization, and other protein-protein interactions. Mutations of specific residues of EcR D-domain and helix twelve residues reveal impaired ligand binding and heterodimer formation (Grebe et al., 2003).

EcR isoforms of Drosophila melanogaster

The diverse range of tissue specific transcriptional responses in D. melanogaster is the product of the three natural isoforms of EcR (A, B1, B2; Figure 1A). The EcR isoforms differ only in their N-terminal trans-activation domains, but are able to evoke different transcriptional responses (Hu et al., 2003; Ruff et al., 2009). Mutations of the N-terminal domain of EcR have demonstrated in an isoform specific manner the disruption of critical processes in larval development (Schubiger et al., 2005). The observation that certain EcR isoforms are expressed preferentially in specific cell types has led to the proposal that the isoforms exert control over specific cellular and tissue fates in the developing flies.
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(Robinow et al., 1993, Schubiger et al., 1998). The EcRA isoform has been principally associated with the remodeling of neurons during metamorphosis (Robinow et al., 1993; Truman et al., 1994; Davis et al., 2005) and is necessary for normal metamorphosis (Davis et al., 2005). The EcRB isoforms have been implicated in larval development (Bender et al., 1997; Schubiger et al., 1998, 2003). EcRB2 alone supports the proper development of the larval epidermis and border cells of the developing egg chamber (Cherbas et al., 2003). Ectopic expression of the B2 isoform in EcR null mutants can effectively rescue these mutants through larval development (Li and Bender., 2001), thereby indicating a distinct role of EcRB2 in the larval stage.

The EcR A and B isoforms in D. melanogaster are expressed via alternate promoters in the genome. EcRB1 and B2 isoforms arise from differential splicing of EcRB gene transcripts (Talbot et al., 1993). The EcR isoforms differ only in their respective N-terminal domains, but share a common DNA binding domain (DBD) and ligand binding domain (LBD; Figure 1A). Heterologous mammalian cell cultures, having no endogenous response to ecdysteroids, have been utilized to demonstrate the transcriptional capabilities of the EcR isoforms. Such studies have demonstrated that the D. melanogaster EcR isoforms are capable of mediating transcriptional activity in response to ecdysteroids, but do not produce equivalent responses (Mouillet et al., 2001).

![Figure 1](image).

Figure 1. Schematic representation of the EcR isoforms from A.) D. melanogaster and B.) L. decemlineata. The EcR isoforms of both species are distinguished by a unique N-terminal amino acid sequences (shaded) followed by a small conserved portion of the A/B (activation) domain. The EcR isoforms of both species share sequence identity in their respective C domains (DBDs), D domains (hinge regions), and E/F domains (LBDs).
The heterodimerization partner of EcR is Ultraspiracle (USP; Figure 2A) a protein that shares extensive sequence similarity with its vertebrate homologue, the retinoid X receptor (RXR; Henrich et al., 1990; Shea et al., 1990; Oro et al., 1990). Unlike many other insects, the *D. melanogaster* USP gene has no introns and codes only one variant of the protein which is expressed in all tissue types (Henrich et al., 1994). USP is commonly referred to as an orphan receptor although mammalian and insect cell culture studies have indicated that USP may be the juvenile hormone (JHIII) receptor (Jones et al., 2001; Sasorith et al., 2002). Further studies have demonstrated that USP is able to bind methyl farnesoate, an intermediate of the mevalonate pathway and precursor to JHIII, with nanomolar affinity, an affinity that is disrupted with the mutation of putative binding sites (Jones et al., 2006). More recent studies have shown that the crustacean RXR can induce reporter gene activity in response to methyl farnesoate in the presence of EcR (Wang and LeBlanc, 2009). However, the exogenous application of juvenoids to *D. melanogaster in vivo* does not support this finding (Zhou and Riddiford, 2002; Dubrovsky et al., 2004; Wilson et al., 2006).

Mutations of USP indicate that the receptor has both repressive and inductive functionalities (Ghbeish et al., 2001; Schubiger and Truman, 2000; Przibilla et al., 2004). A DBD minus fusion protein of the *Choristoneura fumiferana* USP from an earlier study demonstrated increased transcriptional activity indicating a potentially repressive role for the USP DBD (Schubiger and Truman, 2000; Henrich et al., 2003). Null mutants of USP in *D. melanogaster* are unable to develop past the first or second larval instar (Perrimon et al., 1985; Oro et al., 1992) while disruption of normal USP function blocks metamorphosis (Hall and Thummel, 1998; Henrich et al., 2000).

Earlier studies have shown that *D. melanogaster* USP is able to heterodimerize with EcR in mammalian cell culture, but unable to mediate transcriptional activity in response to ecdysteroids. Therefore, the endogenous USP activation domain has been replaced in this study with the viral protein 16 (VP16) activation domain of the herpes simplex virus to create VP16-USP fusion proteins thereby maintaining transactivational activity of the EcR/USP heterodimer in the heterologous mammalian cell culture system. Three variations of the VP16-USP construct were utilized (Figure 2A. VP16-USPI includes a six amino acid region from the C-terminal end of the *D. melanogaster* A/B domain that is highly conserved among insect species along with the DBD, hinge region, and LBD of *D. melanogaster* USP (aa 98-507). The construct VP16-USPII (aa 104-507 in *D. melanogaster*) does not include this conserved region, but is otherwise identical to VP16-USPI. A third construct, VP16-USPIII, includes only the hinge region and LBD of USP fused to the VP16 activation domain (aa 170-507 in *D. melanogaster*). The equivalent constructs were also created for this study with USP from the Colorado potato beetle *Leptinotarsa decemlineata* (Figure 2B).
Similarities of EcR and USP across two insect species

Two isoforms of EcR (A and B) have been identified in the genome of *Leptinotarsa decemlineata* (Colorado potato beetle; Figure 1B). Both *L. decemlineata* EcR and USP share sequence identity for every DBD residue that is conserved among other insect species (Ogura et al., 2005). These similarities indicate that the functional ecdysteroid receptor complexes of both species should be able to recognize and interact with the canonical hsp27 EcRE. Additionally, the EcR isoforms of both species share a region of sequence identity on the C-terminal sides of their respective activation domains (Figure 1A,B). Despite these similarities, the N terminal domains of the EcR isoforms of these two species are divergent sharing only a few small sequence motifs between EcRA and EcRB isoforms (Ogura et al., 2005). Likewise, the two EcR proteins are divergent in their LBDs sharing only about 67% homology (Henrich et al., 2003; Fig. S1, Beatty et al., 2009). The two species share even less sequence identity in their respective USP LBDs with less that 39% homology (Henrich et al., 2003; Fig. S2, Beatty et al., 2009).

The ecdysone and juvenile hormone interaction

The natural ligand that stabilizes the EcR/USP interaction is 20-hydroxyecdysone (20E). This small hydrophobic molecule, like other steroids, is derived from cholesterol and functionally resembles retinoic acid and vitamin D3 (Yao et al., 1992). *D. melanogaster* EcR is capable of ligand binding and interacting with DNA in the absence of USP, although both interactions are considerably increased by the presence of USP (Lezzi et al., 2002; Grebe et al., 2003; Azoitei and Spindler-Barth, 2009; Braun et al., 2009). In the absence of ligand, EcR and USP have the ability to interact and heterodimerize at
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a basal level (Lezzi et al., 2002). In the presence of ligand, the heterodimerization of these two proteins is stabilized allowing the functional ecdysteroid receptor to effectively mediate transcriptional events. In developing *D. melanogaster* larvae ecdysone titers increase preceding important physiological and morphological changes. Studies in other insects indicate that when JHIII is also present, larval-larval molting is preserved. Therefore JHIII has been implicated in the regulation of early insect development although its exact role in *D. melanogaster* development is not clear. The presence of 20E alone has been associated with metamorphic development (reviewed in Riddiford, 1996).

Cell culture studies have shown that JHIII can effectively modulate the ecdysteroid induced transcriptional response. This synergistic effect, known as potentiation, reduces by about 10-fold the concentration of 20E necessary to achieve a maximal inductive response while JHIII alone is unable to evoke a transcriptional response above basal levels (Henrich et al., 2003). Similar JHIII mediated effects have been observed in vivo (Dubrovsky et al., 2004) and in insect cell cultures (Fang et al., 2005).

**Ecdysone agonists**

A number of phytocompounds that act as steroidal agonists and synthetic nonsteroidal agonists of ecdysone have been identified (Elbrecht et al., 1996; Dinan et al., 2001). Two potent phytoecdysteroids, muristerone A (murA) and ponasterone A (ponA) are well characterized for the ability to induce ecdysteroid regulated transcriptional events. MurA is commonly used to assess the activity of ecdysteroid responsive systems because it is much more slowly metabolized by cells than 20E. PonA is utilized because of the relatively high affinity of EcR for this ligand. The respective properties of these ligands make them capable of evoking a stronger ecdysteroid mediated transcriptional response from *in vitro* studies.

Although the basic mechanisms of molting and development are conserved among insect species, nonsteroidal agonists of ecdysone demonstrate order specific toxicity. The diacylhydrazines, interact with the functional ecdysone receptor of certain insects having particular toxicity in Lepidopteran and to a lesser extent in Dipteran species. The diacylhydrazine methoxyfenozide (RH2485) binds to the Lepidopteran ecdysteroid receptor with 400 times the affinity of 20E, the natural insect molting hormone, while *D. melanogaster* EcR is only able to bind methoxyfenozide with about half the affinity of 20E (Carlson et al., 2001). Recent crystallographic studies of the LBD from the Lepidopteran *Heliothis virescens* indicate structural differences in the ligand binding pocket that may account for the increased binding affinity of diacylhydrazines to EcR in insects of this order (Carmichael et al., 2005). The potency of these nonsteroidal agonists has increased interest in this class of compounds as species-specific insecticides. There are currently four commercially available diacylhydrazines as insecticides. Of these, halofenozide (RH0345) is marketed to control Coleopteran and Lepidopteran insect larvae while tebufenozide (RH5992), methoxyfenozide (RH2485), and chromafenozide target lepidopteran species specifically (Dhadialla et al., 1998; Nakagawa et al., 2005). Members of another
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potent group of ecdysone agonists, tetrahydroquinolines (THQ), have demonstrated Dipteran specific activity when applied to D. melanogaster S2 insect cell culture lines with the same compounds failing to evoke a response in Bm5 cells from the Lepidopteran Bombix mori (Soin et al., 2010).

Motivation and specific aims of this work

The functional ecdysteroid receptor of D. melanogaster is a model for general nuclear receptor function. Because EcR and USP both share extensive sequence homology with their respective vertebrate counterparts, characterization of functional aspects of specific residues and motifs within the proteins can lead to an increased understanding of how this superfamily of nuclear receptors mediates transcriptional events in vivo. This work aims to describe specific functions of EcR and USP through ligand and DNA binding analysis as well as the transcriptional capabilies of wildtype and mutant constructs as tested in a well-defined heterologous mammalian cell culture system (Yao et al., 1992; No et al., 1996; Mouillet et al., 2001). Site-directed mutagenesis of specific residues within these domains offers the possibility to examine specific subfunctions that these residues perform within their respective domains. Of major importance is understanding how isoform specific N-terminal domains exert unique effects on the overall activity of the receptors despite complete LBD sequence identity. As an extension, characterization of isoform specific functionality can facilitate a better understanding of how tissue specific effects can be elucidated in vivo. The mechanisms of hormone signal transduction that ultimately evoke tissue specific transcriptional events occur broadly in all insect species and most arthropods where ecdysteroid action is mediated by the ecdysone receptor and its heterodimeric partner USP. Although these similarities exist, the structural and functional properties of EcR and USP vary among insect species, thereby creating a basis for species-specific characterization of these proteins. By comparing the transcriptional activity of the ecdysteroid receptor complexes from two major insect orders, species-specific variation can be measured in the presence of 20E and agonists. Nonsteroidal agonists such as diacylhydrazines demonstrate in vivo toxicity and major differences in receptor affinity in an order-specific fashion (Soin et al., 2001; Palli et al., 2005). This variation in the functional attributes of EcR and USP across insect species provides the basis for presumed differences in ecdysteroid mediated developmental events. The fact that all ligand dependent activity is mediated through binding of steroids or nonsteroidal agonists to the EcR LBD and that the affinity of this interaction is further increased by the presence of USP, indicates that these differences may be exploited to screen a range of compounds which may act in a species-specific manner to disrupt ecdysteroid mediated developmental events. This characterization of the USP LBD from D. melanogaster first analyzed site directed mutations of this domain in a yeast two-hybrid system. The study then aimed to employ a heterologous cell culture system to analyze the effect of a subset of these mutations on the mechanisms by which the unique N terminal domains of the D. melanogaster EcR isoforms mediate ecdysteroid inducible transcriptional responses. The final aim was to characterize the differences of the EcR isoforms and USP from D. melanogaster and L. decemlineata with the idea to develop a heterologous mammalian cell culture
system as a tool to screen potential insecticidal candidates based on their ability to induce or disrupt ecdysone mediated transcriptional activity. The characterization of the EcR and USP from *D. melanogaster* and *L. decemlineata* was performed in the following three stages.

Project A:
**Functional analysis of the ligand binding domain of USP from *Drosophila melanogaster***

The purpose of this study was to identify how mutations of the USP LBD affect overall ecdysteroid receptor function. Earlier studies utilized site-directed mutations in an EcR LBD/ Gal4 activation domain (AD) or DBD fusion protein to characterize the ligand binding and dimerization properties of this domain in a yeast two-hybrid assay system (Lezzi et al., 2002; Grebe et al., 2003). It has been well established that the LBD of EcR demonstrates autonomous function, with the LBD alone maintaining the ligand binding capabilities normally associated with the full length receptor (Yao et al., 1993). EcR is able to bind ligand in the absence of USP, however ligand binding increases tenfold in the presence of USP (Lezzi et al., 2002; Grebe et al., 2003). This points toward a distinct role for USP in stabilizing the EcR/USP heterodimer therefore modulating the activity of the functional ecdysone receptor. Crystal structure studies of the USP LBD from *D. melanogaster* (Clayton et al., 2001) and *Heliothis virescens* (Billas et al., 2001) indicate a fixed antagonistic position of the LBD helix 12. Residues of helix 12 have a hydrophobic interaction with residues in the loop between helix 1 and helix 3 to effectively lock the helix into this antagonistic position. It has also been demonstrated that a phospholipid interacts strongly with residues in the USP ligand binding pocket to further stabilize the antagonistic position of helix 12 (Billas et al., 2001). Recent studies utilizing Förster Resonance Energy Transfer (FRET) have demonstrated that USP exerts an influence on the heterodimer complex that can overcome the effect of a mutation (K497E) that disrupts the salt bridge between helix 12 and helix 4 of EcR (Tremmel et al., 2010). This salt bridge maintained by amino acid residues K497 and E648 of EcR is thought to play an important role in stabilizing the antagonistic position of helix 12 in this receptor. This study utilized the yeast two-hybrid system used previously to characterize the effects of site-directed mutations on fusion proteins of the EcR LBD (Grebe et al., 2003). By analyzing the effect of site-directed mutations in helix 12 and in the USP LBD phospholipid binding pocket on the antagonistic position of this helix in USP fusion proteins, this study aims to explore the modulatory role of USP as a partner in the functional ecdysteroid receptor.

Project B:
**Characterization of the EcR isoforms from *Drosophila melanogaster***

The EcR isoforms of *D. melanogaster* display unique transcriptional capabilities (Hu et al., 2003), but differ only in their N-terminal transactivation domain (Talbot et al., 1993). Previous studies have used heterologous mammalian cell culture to assess the activity of the EcR/USP heterodimer. Mammalian cell cultures are an ideal tool for analyzing the activity of EcR and USP because they have no endogenous response to ecdysteroids. Ecdysone inducible activity is achieved by cotransfection of
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Chinese hamster ovary (CHO) cells with EcR and USP in plasmids with mammalian constitutive expression promoters. Earlier cell culture studies have demonstrated that all EcR isoforms are capable of mediating ecdysteroid inducible transcriptional responses, but do so in an isoform specific manner. The goal of this study was to characterize how the three EcR isoforms (A, B1, and B2) mediate the activity of the functional ecdysone receptor. Furthermore, site-directed mutations in the EcR LBD that had affected receptor function in the yeast two-hybrid system (Lezzi et al., 2002; Grebe et al., 2003; Bergman et al., 2004) were analyzed to characterize their effect on isoform specific ecdysteroid mediated transcriptional activity. This characterization of the ecdysone receptor isoforms is intended to utilize the heterologous mammalian cell culture system as a tool to develop hypotheses for receptor function as a precursor to in vivo analysis.

Project C: Characterization of ecdysteroid receptors from diverse insect species

The functional ecdysteroid receptor coordinates important morphological and physiological changes throughout insect development in response to ecdysteroids and juvenoids. The basic mechanisms of these developmental events are conserved among insects, even though EcR and USP exhibit species-specific differences. Heterologous cell culture studies and biochemical analysis of EcR and USP have defined functional differences that may underlie species-specific ecdysone mediated developmental profiles (Mouillet et al., 2001; Billas et al., 2003; Beatty et al, 2006; Graham et al., 2007). As an extension, these profiles may serve as the basis to develop a screening system to identify compounds that alter the natural ecdysone receptor response. These studies characterized the EcR isoforms (A and B) and USP from the Coleopteran *L. decemlineata* alongside the *D. melanogaster* EcR isoforms (A, B1, and B2) and USP. *L. decemlineata* is a major crop pest that quickly develops a resistance to insecticides and is known to be susceptible to a variety of ecdysone agonists. The purpose of this study was to characterize the EcR isoforms and USP of these two species in a parallel fashion in order to demonstrate differences in their receptor function when treated with 20E and agonists, with the idea to develop a screening system for potential insecticidal compounds based on their ability to induce and potentiate ecdysteroid inducible transcriptional activity.

A fourth project was introduced describe the nature of the interaction between EcR and USP. The resulting publication serves as both a literature review and to introduce new findings on the transcriptional capabilities of the ecdysteroid receptor in the heterologous cell culture system.

Project D: Utility of heterologous cell culture systems in characterizing the heterodimeric partnership of EcR and USP

Cell culture systems have demonstrated great utility in the characterization of the roles of EcR and USP in the functional ecdysteroid receptor. This book chapter is meant to provide an overview of the
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functional characteristics of the ecdysteroid receptor derived from these studies. Previous studies have demonstrated that EcR and USP maintain functional attributes associated with ligand binding and transcriptional capabilities in a variety of cell culture systems (Lezzi et al., 2002, Grebe et al., 2003,2004; Bergman et al., 2004, Przibilla et al., 2004; Beatty et al., 2006). Furthermore, these studies allow mutational analysis of specific subfunctions associated with certain amino acid residues. The heterologous cell culture systems offer a general platform to characterize unique isoform specific functional attributes of EcR an USP and to better define the roles of these nuclear proteins in diverse insect species. Such studies contribute to a better understanding of the mechanisms by which the functional ecdysteroid receptor interact with a variety of ligands, including ecdysteroids and nonsteroidal ec dysone agonists to mediate transcriptional events. As an extension, the heterologous cell culture system is useful in demonstrating the potentiation effect with JHIII (Henrich et al., 2003) and a number of intermediates of juvenile hormone as well as interactions with other cofactors. These findings can be applied to develop hypotheses for ecdysteroid receptor function that can be tested in vivo.
Results and discussion

Project A: Characterization of the *Drosophila melanogaster* USP LBD

Publication:


The majority of the USP mutations in this study demonstrated a severe reduction in their ability to induce expression of the reporter gene. These mutant USP fusion proteins retained a portion of DNA binding and transactivation properties that facilitate ligand binding by the EcR LBD. However, USP LBD mutants with a deletion of helix 12 (ΔH12) maintain the ability to dimerize but eliminate the capability of the EcR/USP heterodimer to bind hormone (I, Figure 3A). Electrophoretic mobility shift assays (EMSA) demonstrate a disruption of ΔH12 DNA binding (I, Figure 3B). This mutant also failed to induce reporter gene activity in the yeast two-hybrid assay (I, Table 1). Because ΔH12 mutants retain dimerization capability, it can be inferred that this mutant causes a moderate conformational change that subsequently affects DNA binding.

The amino acid L490 has been implicated in the interaction with the loops between helix 1-3 and the stability of the antagonistic position of helix 12. A mutation of this residue, L490R, and a second position, E493K, not associated with the antagonistic position of helix 12 demonstrated severe reduction in reporter gene activity, but retained dimerization and DNA binding capabilities (I, Figure 3). The effect of L490R indicates that, in fact, the antagonistic position of helix 12 is critical for inducible reporter gene activity without disrupting ligand binding activity of EcR. Recent findings by Tremmel et al. (2010) have confirmed that when the antagonistic position of EcR helix 12 is disrupted by mutation of residues in the EcR LBD, the USP LBD exerts a compensatory effect, which maintains the ligand binding and transcriptional capabilities of the receptor complex. Because ligand binding is contingent on dimerization (Grebe et al, 2003), it can be inferred that at least partial dimerization capability is maintained in USP constructs with these two mutations. In fact, EMSA analysis demonstrates a reduction in DNA binding of about half for L490R. This reduction indicates that dimerization may also be reduced by about half in this mutant. The DNA binding characteristics and therefore dimerization properties were nearly normal for E493K.

A second set of mutations included residues that form a putative salt bridge with the hydrophilic end of bound phospholipid moiety of USP. Mutation of residue S376, conserved among USP sequences, and K379 resulted in the severely reduced transcriptional activity in the yeast two-hybrid assay and elimination of hormone inducibility when treated with 25µM murA (I, Table 1). The mutations retained reduced hormone binding (I, Figure 5A) and weak DNA binding in the presence of hormone (I, Figure 5B). It is important to note that S376A was able to evoke strong levels of induction in the presence of
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80μM (13.9 fold induction) and 150μM (51.6 fold induction) murA thereby indicating retention of ligand binding capability although ligand affinity is reduced in this mutant.

The hydrophobic residues (L281, W318, L322, I323, and V326) of the USP ligand binding pocket that interact with the phospholipid were also analyzed in this study. Mutations at all positions involved in hydrophobic interactions with the phospholipid affected ligand binding to the heterodimer (I, Table 1., Figure 5A.). Transcriptional activity was eliminated in mutations to all but one of these residues. Substitutions I323A and I323V were the exception. Mutations at this position resulted in slightly lowered ligand binding capabilities but superinduction in the yeast two-hybrid assay when treated with MurA (I, Table 1., Henrich et al., 2000). The singular effect of mutation at this residue implies a distinct role for this residue. Both the alanine and valine substitutions remove the larger isoleucine side group and potentially relieve steric constraints in the USP ligand binding pocket. In fact, substitution with a negatively charged group (I323D) or with a much larger side chain (I323F, data not shown) result in the abolition of transcriptional activity. Similarly, position L322 seems to be more affected by the size of the side chain than the introduction of a charged residue. Substitution to a smaller sidechain in L322G resulted in the elimination of receptor function, but the longer sidechain in L322R retained DNA binding.

Mutations of amino acid residues of the USP ligand binding pocket that have no direct interaction with a putative ligand revealed that specific functions can be attributed to specific amino acid positions. The mutation D349S reduced hormone binding to EcR while retaining the ability to dimerize. DNA binding was only slightly lower in this mutant but there was a complete elimination of transcriptional activity in the two-hybrid assay. The mutation E392A diminished ligand binding of the heterodimer and reduced activity in the two-hybrid assay (I, Figure 6A). An expected result, as this residue is thought to play an important role in USP function because it is highly conserved among known USP sequences.

A super-shift indicated heterodimerization in the presence of hormone for L410F, I414A, and L415F but DNA binding in the absence of hormone was reduced (I, Figure 6B). These mutations also caused reduced binding of hormone to EcR (I, Figure 6A.) with complete elimination of transcriptional activity. This result indicates that residues of the USP ligand binding pocket play an important role in the inductive capabilities of the heterodimer.

One mutation of the USP dimerization interface in helix 10, P463D, destroyed all receptor function except DNA binding. This indicates that dimerization coordinated by the USP LBD is not necessary for DNA binding of the fusion protein in the yeast two-hybrid system.

This study reveals that mutations in USP behave differently in the yeast two-hybrid assay than analogous EcR mutations (Lezzi et al., 2002; Grebe et al., 2003). In this case, modification of the USP LBD fused to the Ga4 DBD affects the functionality of the fusion protein in the transcriptional assay. The ability of the fusion protein to bind DNA depends on both the presence of helix 12 and its unique antagonistic position.

Transcriptional activities do not always correspond to the ligand binding and DNA binding data for some mutations such as S376A, I323A, and I323V. The fact that the superinductive mutations I323A and I323V demonstrate reduced ligand binding properties, may indicate that the receptors are able to
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compensate through heightened dimerization capability. Alternately, the receptors may interact with endogenous comodulators in the yeast two-hybrid system to modulate transcriptional responses. Other mutations of USP such as E493K, which retains the normal receptor functions of dimerization, ligand binding and DNA binding, may be unable to interact with other factors necessary for transactivation. Mutations such as the superinducers and others that cause a reduction of normal activity point to the active role that USP plays in transactivation of the EcR/USP heterodimer.

Project B:
Characterization of the ecdysteroid receptor isoforms of Drosophila melanogaster

Publication:

Previous studies have demonstrated EcR isoform specific capabilities to mediate transcriptional activity in response to ecdysteroids and potentiation with JHIII in both D. melanogaster cell cultures (Hu et al., 2003) and heterologous mammalian cell culture systems (Mouillet et al., 2001). The fusion construct VP16-USPI, containing six conserved amino acids of the USP activation domain, produced only minor differences in the ability to mediate transcriptional activity compared to VP16-USPII when cotransfected with EcR in preliminary studies (II, data not shown). As a result it was presumed that these conserved residues of the USP N-terminal domain have little effect on ecdysone responsive transcriptional activity and VP16-USPII and VP16-USPIII constructs were used for other experiments. The EcRB1 isoform demonstrated higher basal transcription and murA induced levels of activity around four fold higher than the other isoforms (II, Figure 1). Western blotting revealed that EcRB1 exhibited a weaker immunostaining signal than EcRB2 (II, Figure 2a). The fold inducibility of EcRB2 in the presence of murA was nearly comparable to EcRB1 although the basal transcriptional levels of this isoform were low and more closely resembled EcRA (II, Figure 1). The strongest potentiation response, as measured by fold induction over basal activity, was generated by EcRB2 when treated with 0.1µM murA and 80µM JHIII. The EcRA isoform generated the weakest profile of murA inducible transcriptional activity. This isoform also generated the weakest immunoblotting signal, leading to the hypothesis that its low inducibility could be a product of lower receptor concentration. Recent studies have demonstrated that EcRA degrades at a significantly higher rate than the EcRB isoforms in a heterologous cell culture system (Schauer et al., 2011). However, to test the theory that the amount EcRA transfected into the cell could have a rate-limiting effect on transcriptional activity, a range of EcRA encoding plasmid from 10ng to 500ng was transfected into the cells. The results indicated that as little as 10ng of EcRA could evoke a maximal transcriptional response (II, Figure 3).
Experiments with the VP16-USPIII construct, lacking the USP DBD and analogous to a Choristoneura fumiferana construct that demonstrated increased activity in an earlier study (Henrich et al., 2003), revealed impaired basal and murA induced transcriptional levels when paired with EcRA and EcRB2. Furthermore, these isoforms failed to produce a potentiation response with the VP16-USPIII construct. The EcRB1 isoform, however, maintained both an ecdysteroid inducible transcriptional response to murA with the USP DBD deficient fusion protein. This isoform alone was able to evoke potentiation with JHIII when paired with the VP16-USPIII fusion construct, although at a significantly lower level equal to about half the activity produced with a max dosage of 1µM murA (II, Figure 1). Western blots probed with a VP16 antibody confirmed expression of VP16-USP variants, generating bands of predicted size and demonstrating the same relative concentrations of these proteins (II, Figure 2b).

Two site-directed mutations of EcR that had impaired certain aspects of receptor function in earlier yeast two-hybrid assays (Lezzi et al., 2002; Grebe et al., 2003; Bergman et al., 2004) were tested with the EcR isoforms to analyze whether the effects on fusion proteins are the same as on whole receptors. The site-directed mutation EcRB2(K497E) caused a significant elevation of basal transcription compared to EcRB2 wild–type when paired with VP16-USPII (II, Figure 4). In contrast, the EcRA and EcRB1(K497E) mutants displayed no significant increase in basal transcription levels. EMSA analysis confirmed that EcRB2(K497E) has a higher level of affinity for an hsp27 EcRE than the mutant EcRA and EcRB1 isoforms in the absence of hormone (II, Figure 6). Although differences in receptor concentration could be a possible explanation, it is not likely because the K497E mutant isoforms demonstrate murA induced transcriptional activity that exhibits the same isoform specific pattern of transcriptional response as the wild type EcR isoforms. More specifically, although EcRB2 generates the strongest western immunoblot signal of the EcR isoforms, EcRB1 evokes the strongest transcriptional activity in response to murA. The residue K497 is situated in helix 4 of the EcR LBD and has been implicated in mediating ecdysteroid dependent transcriptional activity via formation of a salt-bridge with helix 12 (Wurtz et al., 1996a,b). These results of this study are comparable to results from earlier yeast two-hybrid assays with K497A and K497E mutants that strongly increased ligand induced transcriptional activity. These mutations were shown to have reduced ligand binding capabilities in fusion proteins (Grebe et al., 2003) therefore, the increased basal activity in K497E mutants may indicate the loss of a specific function associated with this amino acid position. Consistent with this explanation, deletion studies of the EcRA N-terminal domain have demonstrated that amino acids 15-29 of this domain are responsible for the proper positioning of LBD helix 12 in EcRA K497E mutants (Tremmel et al., 2010). This result indicates that the EcR N-terminal domain shares an intramolecular interaction with the LBD of this receptor that contributes to the overall conformation of the heterodimer complex.

In order to test whether the increased basal activity of EcRB2(K497E) is the product of increased affinity to dimerize with USP, a competition experiment was performed. A range of quantities of the plasmid encoding EcRB2(K497E), from 0ng to 500ng, were cotransfected into cell cultures with a constant 100ng of wild-type EcRB2 and VP16-USPII. Under these conditions, the basal level of transcriptional activity reached a level intermediate to both wild-type EcRB2 and the K497E mutant (II,
Figure 5). The explanation for this result is that EcRB2(K497E) is unable to displace the non-mutant EcRB2 in the EcR/USP heterodimer because the affinity of the mutant for USP is lower. The mutation A483T is a larval lethal mutation in vivo, associated with the physical interaction of EcR and a corepressor, SMRTER (Tsai et al., 1999; Carney and Bender, 2000). This mutation did not significantly affect the basal or murA induced levels of transcription in EcRA or EcRB2 mutants and basal levels of activity for the EcRB1 mutant were unaffected. However, EcRB1(A483T) demonstrated a significantly reduced murA induced transcriptional activity (II, Figure 4).

The residue M504 was selected for mutation because this is a consensus site for ligand binding in D. melanogaster EcR (Wurtz et al., 1996a,b). Yeast two-hybrid analysis of this mutant revealed that it has normal dimerization affinity in the absence of ligand, but is unable to bind ligand and exhibits no elevation in dimerization affinity in response to ligand (Grebe et al., 2003). Consistent with these earlier results, M504R produced normal basal levels of transcription in this study, but was unable to evoke murA induced transcriptional activity (II, Figure 4). Competition assays were performed by cotransfecting a range of EcRB2(M504R) quantities (0-500ng) with a constant quantity (100ng each) of EcRB2(K497E) and VP16-USPII to establish the affinity of the M504R mutant to form a heterodimer with USP. Because the basal transcriptional activity of M504R mutant is preserved, activity of the mutant cannot be distinguished from the non-mutant EcRB2 in the absence of ligand. Therefore, EcRB2(K497E), which exhibits increased basal levels of transcriptional activity with the EcRB2 isoform, was used as a proxy for non-mutant EcRB2 in this experiment. The EcRB2(M504R) mutant was able to offset the high basal transcriptional levels of EcRB2(K497E) indicating that this mutant isoform has a higher affinity for interaction with USP in the absence of hormone (II, Figure 5).

This study was intended as a starting point to assess the in vivo function of EcR and USP in D. melanogaster in order to understand the unique roles of EcR and USP in the transcriptional processes that mediate development. These results indicate that EcR is able to perform both general and isoform specific functions.

This study demonstrated that the USP DBD is necessary to both ecysteroid mediated and JHIII potentiated responses for EcRA and EcRB2. However, the isoform specific response of EcRB1 with VP16-USPIII is an indication that the role of USP is not equivalent for the three EcR isoforms. The EcRB1 isoform is able to maintain both basal and ligand induced transcriptional activity in the absence of the USP DBD, a result consistent with previous studies (Schubiger and Truman, 2000; Ghbeish et al., 2001), suggesting a mechanistic interaction with the hsp27 EcRE different than that of EcRA and EcRB2 with the implication of a distinct role for EcRB1 in in vivo development.

The three EcR isoforms display distinct transcriptional capabilities that are a product of their distinct N-terminal domains. The most distinct isoform specific interaction occurred with EcRB2(K497E). Analysis of the mutation by EMSA indicates that the mutant receptor is able to bind to the hsp27 EcRE with a high affinity in the absence of hormone. The relative mobility of this complex observed in this interaction is equivalent to that of the non-mutant receptor complex in the presence of ligand.
This finding indicates that the K497E mutation is not disrupting a cofactor interaction as predicted. Because K497E demonstrated about normal dimerization capabilities in competition studies, it is likely that the high basal transcriptional activity observed in the yeast two-hybrid assay was the product of increased DNA binding affinity of the EcR/USP complex. It is conceivable that the K497E mutation disrupts a ligand-dependent activation function (AF2) with helix 12. Such a disruption may result in a conformation of the receptor that interrupts a corepressor interaction in a manner normally evoked by ligand. In other words, the mutation enables a conformation of the receptor that elucidates constitutive activation leading to increased levels of basal transcriptional activity. An alternative explanation proposed by Tremmel et al. (2010) is that intramolecular contacts between a nuclear receptor interaction motif in the EcR N-terminal domain modulate the position helix 12 of the EcR LBD. The isoform specific effect may be the result of the smaller N-terminal domain of EcRB2 interacting with K497E to create a unique conformation of the protein. In fact, the EcRB2 AB domain lacks the nuclear receptor interaction motif that is thought to coordinate the interaction of the N-terminal domain with the LBD (Tremmel et al., 2010).

A number of subfunctions can be defined as the sum of receptor functionality. The characterization of subfunctions associated with specific residues of EcR may lead to the underlying basis for their effects on developmental processes. This study represents a systematic approach for the analysis of isoform specific mutational effects in EcR to develop a basis for subsequent analysis in D. melanogaster.

Project C:
Characterization of ecdysteroid receptors from diverse insect species

Publication:


Manuscript:

(V) Beatty JM, Spindler-Barth M, Henrich VC. A cross species comparison of EcR and USP in the functional ecdysone receptor.

The properties of the three D. melanogaster EcR isoforms (DmEcRA, DmEcRB1, and DmEcRB2) paired with the VP16-DmUSP fusion proteins from an earlier study (II) were compared to the EcRA and EcRB isoforms of L. decemlineata paired with VP16-LdUSP fusion constructs analogous to those
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used in the earlier study. Preliminary studies were conducted to characterize the basal transcriptional levels as well as the ecdysoid induced responses to a range of steroidal and nonsteroidal agonists. The quantitative levels of transcriptional activity observed for the heterodimers of each species differentiate themselves in an isoform specific manner. That is to say, both the basal and ligand induced transcriptional activity produced by a heterodimer is greatly influenced by the isoform specific N-terminal domain of the EcR heterodimeric partner (II; III; V).

Effect of steroidal and non-steroidal ecdysone agonists on the transcriptional activity of same species EcR/USP heterodimers

An isoform specific pattern of transcriptional activity is observed for receptors of both species and can be summarized as follows: D. melanogaster heterodimers are generally more responsive to murA while L. decemlineata receptors demonstrate a significantly greater transcriptional response to RH2485. The transcriptional activity evoked by 20E and makA with heterodimers from both species was relatively subdued, but isoform specific (III, Figure 2A).

The D. melanogaster isoforms exhibited a pattern of ligand induced transcriptional response similar to that observed in earlier studies (III, Figure 2A). A maximum response was evoked with 2.5µM murA in all cases. The D. melanogaster isoforms also responded to the nonsteroidal agonist methoxyfenozide (RH2485), but a much lower response was observed with 20E and makisterone A (makA). The transcriptional response of the LdEcR/LdUSPII dimer was much different than that of the DmEcR/DmUSP II complex (III, Figure 2B). The L. decemlineata constructs responded much more robustly to RH2485 with LdEcRA/LdUSP heterodimer demonstrating up to a 25-fold induction. However, the L. decemlineata response to murA and 20E was substantially weaker than that of D. melanogaster (III, Figure 2A). MakA evoked the lowest levels of induction in both species. The relative levels of transcriptional activity demonstrated by the ecdysone agonists occurred in an isoform specific and species specific manner.

Western blots were performed to verify the expression of the EcR proteins and were probed with the EcR specific antibodies. The immunobots indicate relative differences in the concentrations of D. melanogaster EcR isoforms, a result that has been observed in previous studies (II; Figure 2A; III, Figure 2C). The differences in transcriptional activity are not the result of differences in receptor concentration, but reflect the species specific and isoform specific differences in the transcriptional capabilities of these proteins.

In order to more carefully examine the potency of these ecdysteroids across species, a series of dose response experiments were performed with the natural ecdysteroid agonist murA, makA, and ponA (II, Figure 3A-C) and with the nonsteroidal agonists RH2485, halofenozide (RH0345), RH5849, and tebufenozide (RH5992). MurA was more potent in D. melanogaster receptors, producing a maximal effect in the range of 1-10µM (III, Figure 3A). A maximal response with LdEcR/LdUSP II required concentrations of murA of at least 50µM (III, Figure 3B,C). However, the L. decemlineata receptors achieved a higher maximum fold induction (in excess of 30-fold) in response to 50µM murA. PonA
evoked a maximum response in both species at a concentration of 1 µM, while makA failed to evoke a strong response in either species with concentrations as high as 50 µM (III, Figure 3A-C). Dose response profiles of the nonsteroidal agonists reveal that the maximal inductive response of the LdEcR/LdUSP111 heterodimer to these compounds is much higher than the D. melanogaster receptors. All RH compounds, except RH5849, demonstrated a maximum inductive response (over 10-fold) at 10 µM with the L. decemlineata heterodimer (III, Figure 4B,C). RH2485 and RH5992 demonstrated about the same level of response, with RH0345 having an intermediate response, and RH5849 evoking the lowest levels of activity (III, Figure 4B,C). The D. melanogaster receptors exhibited a fold induction of less than 10-fold in the presence of nonsteroidal agonists as concentrated as 50 µM (III, Figure 4A).

A construct of DmUSP with a deletion of the DBD (DmUSP111) was able to produce activity with the DmEcRB1 isoform, but forms a mostly inactive heterodimer with DmEcRA and DmEcRB2 (II, Figure 1; III, Figure 7A). More specifically, the DmEcRB1 DBD alone mediates DNA binding in the absence of the USP DBD. In the case of DmEcRA and DmEcRB2, the DBD of both dimerization partners is required to evoke DNA binding and therefore a transcriptional response. This result indicates the isoform specific nature of the interaction between EcR and USP. The analogous LdUSP111 construct when paired with LdEcRA and LdEcRB exhibited severely reduced transcriptional capabilities (III, Figure 7B).

EMSA analysis was performed in order to analyze the interaction of the heterodimers from both species with the cognate hsp27 EcRE. The EMSA gels reveal that DmEcRB2/DmUSP111 interacts with the hsp27 EcRE in the absence of ligand. The DmEcRB1/DmUSP111 heterodimer complex exhibits a weak band under the same conditions (III, Figure 5; V, Figure 5A). An ecdysteroid induced increase in band intensity and therefore an increase in DNA binding to the radiolabeled hsp27 EcRE was observed for heterodimers containing DmEcRB1 or DmEcRB2 with DmUSP111 (III, Figure 5; V, Figure 5A). The DmEcRB2/DmUSP111 complex exhibits a faintly visible band in the EMSA gel in the absence of ligand (V, Figure 5A). The intensity of this band increases only slightly with the addition of ligand. However, the DmEcRB1/DmUSP111 complex retains basal ability to interact with the hsp27 EcRE. This interaction is increased in the presence of ligand resulting in two distinct complexes (V, Figure 5A).

The LdEcRA/LdUSP111 heterodimer exhibits two distinct complexes capable of DNA binding in the absence of ligand. There is a slight increase in the intensity of both complexes in response to 2.5 µM murA (III, Figure 5; V, Figure 5B,C). A heterodimer of LdEcRB/LdUSP111 interacts with the hsp27 EcRE to produce a single band in the EMSA gel. In the presence of ligand, this complex demonstrates increased DNA binding and equivalent relative mobility to the unliganded complex (III, Figure 5; V, Figure 5B,C).
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**Effect of steroidal and nonsteroidal ecdysone agonists on the transcriptional activity of cross species EcR/USP pairings**

Cross-species comparisons were conducted to further elucidate the properties of EcR and USP from *D. melanogaster* and *L. decemlineata* (III, Figure 7B; V). In every case where a DmEcR isoform is paired with LdUSPII the ligand induced transcriptional activity in response to murA (V, Figure 1A) and RH2485 (V, Figure 2A,B) is at least as great as that of the same species DmEcR/DmUSPII heterodimer. However, the DmEcR/LdUSPII heterodimers mediated a significantly reduced transcriptional response in the presence of 20E (V, Figure 3A).

The DBD deficient LdUSPIII is unable to evoke a transcriptional response with DmEcRB1 (III, Figure 7C), unlike DmUSPIII, which maintains basal and ligand induced activity with this isoform (II, Figure 1). This experiment also revealed that DmUSPIII forms a less active heterodimer with both *L. decemlineata* EcR isoforms (III, Figure 7C). When an LdEcR isoform is paired with DmUSPII, the resulting heterodimer is unable to mediate the level of ligand induced transcriptional activity achieved with LdEcR/LdUSPII receptors. This finding holds true for all of the ligands tested in this study. It is interesting to note that although ligand inducibility is compromised in the cross species LdEcR/DmUSPII heterodimers, the basal levels of transcriptional activity are higher than those associated with the same species LdEcR/LdUSP receptor (V, Figure 3B).

**The effect of JHIII on transcriptional activity in same species EcR/USP pairings**

The term potentiation refers to an effect by which the presence of JHIII effectively reduces, by about tenfold, the concentration of ecdysteroid necessary to achieve a maximal transcriptional response from the functional ecdysteroid receptor. The potentiation response of *D. melanogaster* receptors to JHIII in the presence of ecdysteroids is well characterized (II, Figure 1). This study asked whether the potentiation response also occurs in *L. decemlineata* receptors and if potentiation occurs when receptors of both species are subjected to sub-maximal dosages of a nonsteroidal agonist in conjunction with JHIII. When murA was replaced with a submaximal dosage of RH2485 along with JHIII, no potentiation response was observed for DmEcRB2/DmUSPII or either LdEcR/LdUSPII heterodimer (III, Figure 6B). Only the DmEcRB1/DmUSPII heterodimer is capable of mediating a potentiation response with RH2485 and JHIII (V, Figure 2A). Treatment with 20E in combination with JHIII is capable of evoking a slightly higher transcriptional activity in DmEcR/DmUSPII heterodimers than that observed with 20E alone (V, Figure 3). The LdEcR/LdUSPII heterodimer was able to evoke a slight potentiation response when treated with a sub-maximal dosage of murA (III, Figure 6A) or 20E and JHIII (V, Figure 3). This low level potentiation disappears when LdEcR/LdUSPII heterodimers are treated with RH2485 and JHIII (III, Figure 6B; V, Figure 2C).
The effect of JHIII on transcriptional activity in cross species EcR/USP pairings

Cross species DmEcR/LdUSPII heterodimers retain the ability to be potentiated to maximal transcriptional activity by murA and JHII that is normally associated with same species D. melanogaster heterodimers (V, Figure 1A). Potentiation by JHIII is also observed with the DmEcR/LdUSP heterodimers in the presence of 20E, although not to maximum level of transcriptional activity evoked by 10μM 20E (V, Figure 3). When the cross species DmEcRB2/LdUSPII receptor is exposed to RH2485 and JHIII no significant potentiation of transcriptional activity is observed (V, Figure 2B). In contrast, the simultaneous presence of JHIII and RH2485 can potentiate the transcriptional of the DmEcRB1/LdUSPII complex (V, Figure 2A).

When LdEcR isoforms are paired with DmUSPII the induction by murA and 20E is diminished. Because the cross species LdEcR heterodimers are not as sensitive to ecdysteroids at these concentrations, potentiation is more difficult to discern. Despite this observation, a submaximal dosage of murA and JHII is able to evoke a higher level of transcriptional activity than 0.1μM murA alone with the LdEcR/DmUSPII heterodimers (V, Figure 1B). Similarly, only low level potentiation is observed when the LdEcRB/DmUSPII receptor is exposed to 20E and JHIII (V, Figure 3). No potentiation was observed when LdEcRB is paired with DmUSPII in the presence of RH2485 and JHIII (V, Figure 2C).

This study provided a controlled characterization of L. decemlineata and D. melanogaster EcR and USP demonstrating differences in transcriptional activity, ligand responsiveness, and potentiation with JHIII. The results of this study are largely consistent with biochemical and in vivo findings from previous studies of the receptors from both species, supporting the usefulness of mammalian cell culture as a tool to assess the characteristics of EcR and USP across insect species. The relative differences in observed receptor transcriptional capabilities suggest that cell culture can be used to predict the toxicity of candidate insecticidal compounds between species. The quantitative differences in transcriptional activity among EcRs of different insect species likely play an important role in coordinating in vivo developmental events. The quantitative levels of transcriptional activity are also dependent on USP as was observed with the effect of cross species heterodimers in this study.

Recent studies have shown that the presence of an hsp27 EcRE a full length DmUSP increases ligand binding and interaction with DNA (Azoitei and Spindler-Barth, 2009). DmUSP exerts a stabilizing influence on the antagonistic position of helix 12 in EcR LBD mutants. A result which indicates that USP plays more than a passive role in the EcR/USP heterodimer (Tremmel et al., 2010). In contrast, a DBD deficient USP in the presence of DNA causes an isoform specific reduction in ligand binding (Azoitei and Spindler-Barth, 2009). The DBD and its C-terminal extension, the D domain, are known to evoke DNA binding but are also implicated in transactivation of the heterodimeric complex (Hatzivasiliou et al., 1997). In fact a portion of the D domain, the T-box (with the amino acid KREAVQEER in DmUSP; V, Figure 7) of the USP ortholog RXR is known establish a series of sequences specific contacts with DNA that facilitate dimerization and conduct the heterodimeric partner with the correct orientation to the appropriate half-site of the DNA response.
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element (Rastinejad et al., 2000). The D domain of DmUSP contains a unique glycine rich region that is not found in the amino acid sequences from other species (V, Figure 7). Regions such as these with low structural complexity and high flexibility are thought to facilitate regulatory functions and be involved in cofactor interactions (Jakób et al., 2007). Such features may explain how USP of both species modulate the effect of the ecdysteroid receptor complex to produce the species specific effects on transcriptional activity observed in the cross species and same species heterodimers of these studies.

Interaction of the ecdysteroid receptor complex with ligand and the nature of this interaction facilitates potentiation with JHIII and other juvenoids. The exact mechanisms of potentiation are unclear, but the potentiation effect has been observed with other compounds known to interact with basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) transcription factors (Oberdorster et al., 1999). One such factor in D. melanogaster, methoprene tolerant (MET), is known to bind JHIII (Ashok et al., 1998; Miura et al., 2005). Mutations to MET block the normally toxic effects of methoprene in D. melanogaster (Ashok et al., 1998) and MET is known to interact with EcR and USP (Li et al., 2007). Although the exact role of JHIII in modulating ecdysteroid activity is unclear this modulation could be related to the shape conferred on the EcR ligand binding pocket by the ligand. It is known that the confirmation of the EcR ligand binding pocket is much different in the presence of nonsteroidal ecdysone agonists (Billas et al., 2003), therefore preventing interactions with cofactors which affect potentiation. This change in the conformation of the EcR LBD may explain the loss of potentiation when D. melanogaster and L. decemlineata receptors are treated with RH2485 and JHIII. The demonstration of such differences among receptors from different insect species establishes the utility of the heterologous cell culture system as a tool to assess the effect of candidate insecticidal compounds on receptor function.

Project D:
Utility of heterologous cell culture systems in characterizing the heterodimeric partnership of EcR and USP

Publication:


Utility of heterologous cell cultures to assess EcR and USP function

The heterologous mammalian cell culture system is useful to reconstitute the transcriptional activity of the functional ecdysteroid receptor in cells that have no endogenous response to ecdysteroids. Chinese hamster ovary (CHO) cells are utilized because they express no detectable level of RXR
(Nieva et al., 2009), the mammalian ortholog of USP, which can from a transcriptionally active heterodimer with EcR (Christopherson et al., 1992; Henrich et al., 2003). The cells also have no detectable levels of the vertebrate EcR ortholog FXR, which can dimerize with RXR and mediate transcriptional activity in response to juvenile hormone (Kitareewan et al., 1996). This heterologous system relies on cotransfection of plasmids encoding EcR and USP under the control of constitutive mammalian promoters into the CHO cells. A third plasmid vector expressing a reporter gene under the control of the canonical hsp27 ecdysone response element (EcRE; Riddihough and Pelham, 1987; Dobens et al., 1991) is also transfected into the cells. Also transfected into the cells is a vector encoding a constitutively expressed reporter gene such as β-galactosidase to normalize both cell mass and transfection efficiency. The transfected cultures are challenged with cell culture medium containing ligands that may act as ecdysone or juvenoid agonists. After an incubation period, the cells are harvested and the cellular contents are processed for subsequent reporter gene assays. The expression of reporter gene is a direct indication of transcriptional activity mediated by the functional ecdysteroid receptor. Previous studies have demonstrated that USP alone is unable to mediate an ecdysteroid inducible response in CHO cells. However, the expression of EcR alone is able to evoke a relatively low level of basal and induced transcriptional activity via the hsp27 EcRE.

The primary advantage of the heterologous mammalian cell culture system is that it provides an environment for the assembly of an ecdysteroid inducible reporter gene assay with no interference from endogenous EcR and USP. Because all ecdysteroid inducible transcriptional activity in mammalian cell culture is the product of exogenously expressed proteins, the system can be utilized to characterize the functional properties of structurally modified EcRs and USPs. Insect cell cultures do not afford this same property because they typically express some endogenous level of EcR and USP as well as cofactors that influence ecdysteroid mediated transcriptional responses. The functional effects of endogenously expressed proteins in insect cell cultures cannot be differentiated from the effects of exogenously expressed EcRs and USPs. As an extension, when EcR and USP of one insect species such as *L. decemlineata* are expressed in a cell culture line from another insect species such as *D. melanogaster* S2 cells the functional characteristics of nuclear receptors from both species are difficult to differentiate. The heterologous cell culture system has demonstrated species specific receptor characteristics that correspond to *in vivo* studies of ligand responsiveness (Graham et al., 2007a,b). Similarly, cell culture studies of site directed mutations to the EcR LBD have confirmed putative ligand binding sites predicted by structural studies and receptor modeling (Billas et al., 2003).

**Comparison of EcR/USP characteristics across cell systems**

Studies of EcR and USP expressed in different heterologous cell culture systems indicate that the functional properties of these nuclear receptors are mostly independent of the cellular context (Lezzi et al., 2002; Grebe et al., 2003, 2004; Bergman et al., 2004; Przibilla et al., 2004; Beatty et al., 2006,
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2009). Such studies are useful in producing hypotheses concerning biological mechanisms that can be tested in subsequent in vivo studies.

The yeast two-hybrid system examines the interaction of a fusion protein consisting of the Gal4 activation domain (AD) to the EcR LBD and a second fusion protein consisting of the Gal4 DBD with the USP LBD (Lezzi et al., 2002). When the Gal4 components of this system are brought together by interaction of the EcR and USP LDBs the complex is able to mediate transcription of a LacZ reporter gene under the control of a UAS promoter element. This system tests the interaction of the LBD from EcR and USP without the possibility of other interdomain interactions.

Site directed mutations of EcR and USP Gal4 fusion proteins have been tested in the yeast two-hybrid system (Grebe et al., 2003,2004; Bergman et al., 2004; Przibilla et al., 2004). Mutations of the EcR LBD corresponding to in vivo larval lethal point mutations (Bender et al., 1997), demonstrate reduced ligand affinity (Grebe et al., 2003) and a reduction in basal and induced transcriptional activity (Bergman et al., 2004). Mutations in the USP LBD generally cause a reduction in ponA binding (IV, Figure 14.1) and loss of transcriptional activity (Przibilla et al., 2004). These findings indicate that USP modulates the ligand binding affinity of EcR by reducing ligand dissociation thereby stabilizing the EcR/USP complex. When the corresponding mutations of whole receptors are tested in CHO cells, there is little effect on transcriptional activity indicating that the interaction of the USP LBD with the natural USP DBD is different than with the Gal4 DBD in the yeast two-hybrid system. This points to an important role for USP interdomain signalling in modulating the transcriptional capabilities of the EcR/USP heterodimer.

Mutations of EcR tested in the yeast two-hybrid system behave similarly to the corresponding mutations of full length EcRs tested in CHO cells indicating the consistency of mutational effects across heterologous cell systems. The highly conserved residue M504 in helix 5 of the LBD has been implicated with ligand binding in structural studies of Heliothis zea EcR (Wurtz et al., 2000; Billas et al., 2003). The substitutions M504A and M504R have little effect on basal transcriptional levels in yeast two-hybrid assays and CHO cell cultures but severely affect ligand dependent transcriptional activity in these systems (Grebe et al., 2003, Bergman et al., 2004; Beatty et al., 2006). Wild type EcR is able to translocate into the nucleus of CHO cells at a basal rate in the absence of ecdysteroids and at an elevated rate In the presence of ecdysteroids. The basal rate of EcR translocation into the nucleus is completely disrupted by the mutation M504R, although the M504A mutant retains the same rate of translocation into the CHO cell nucleus as wildtype EcR in the absence of ecdysteroids. Both amino acid substitutions were unable to translocate into the nucleus of CHO cells at the elevated rate observed with wildtype EcR. Because the residue M504 has been associated with ligand binding, the failure of these mutants to produce an elevated rate of translocation indicates that this process is functionally dependent on ligand binding to EcR and subsequent heterodimerization with USP (Nieva et al., 2005).

The residue K497 occurs in helix 4 of the EcR LBD and is highly conserved across insect species. The mutations K497A and K497E both reveal elevated levels of reporter gene activity in yeast two-hybrid studies in the absence of hormone and a slight increase in ligand induced reporter gene activity (Bergman et al., 2004). Both substitutions disrupt the formation of a salt bridge between helix
[^4 and helix 12 and reduce ligand binding in the Gal4 fusion proteins (Grebe et al., 2004). Mutation of the residue in helix 12 that forms the salt bridge with K497 reveals only a slight reduction in ligand binding. This result indicates that the impairment of ligand binding in K497 mutants is not the product of disruption of the salt bridge with helix 12.

To further examine the functional properties of the lysine residue at this position, the effect of the mutation K497E on the activity of the three D. melanogaster EcR isoforms was tested with USP in mammalian cell cultures. Competition studies where wild type EcR and the corresponding K497E mutant are cotransfected into CHO cells with a limited quantity of USP indicate that the K497E mutant has lower affinity for dimerization with USP (Beatty et al., 2006). EcRB2 K497E alone produced higher basal levels of transcriptional activity consistent with the increased basal levels of reporter gene activity seen in the yeast two-hybrid assay. EcRA and EcRB1 K497E were unable to evoke a significant increase in basal transcriptional activity in CHO cell cultures. The isoform specific effects of K497E likely underlie an interaction between the N-terminal specific domain of EcRB2 and this residue.

Studies have shown that EcR interacts with ligand to mediate transcriptional responses in the absence of USP. Although ligand affinity is increased by the presence of USP (Bergman et al., 2004; Grebe et al., 2004), the K497E mutant demonstrates elevated transcriptional activity over wild type EcR when no USP is present (IV, Figure 14.2). This result indicates a role for the residue K497 and the interaction with helix 12 in the ligand dependent activation function (AF2).

**The effect of juvenile hormone on EcR and USP**

There is growing evidence that juvenile hormone (JH) exerts multiple modes of action on a range of gene targets. Recent studies, incubating dissected D. melanogaster salivary glands with JH, have characterized a variety of responses that may be associated with gene targets of juvenile hormone (Beckstead et al., 2007).

Many similarities exist between USP and its vertebrate ortholog RXR although cell culture studies have revealed that the two proteins are not functionally equivalent (Billas et al., 2001; Clayton et al., 2001). Unlike D. melanogaster USP, RXR is unable to mediate a transcriptional response in the presence of 20E (Henrich et al., 2003) and is unable to facilitate translocation of EcR into the nucleus of CHO cells (Nieva et al., 2005). Despite these differences, RXR is activated by the JH analog methoprene and methoprene acid. This finding has brought forth the possibility that USP is the JH receptor. Insect cell cultures have demonstrated that a USP homodimer is able to mediate transcriptional activity in response to JH via a DR12 response element (Jones et al., 2001) and molecular modeling indicates that the USP ligand binding pocket is capable of binding JH (Sasorith et al., 2001). Methyl farnesoate has demonstrated nanomolar affinity binding with the USP LBD, an interaction that is disrupted by the mutation C472A (Jones et al., 2006). The mutation C472A in full length USP coexpressed with EcR in CHO cell cultures affected neither ecdysteroid inducible transcriptional responses nor potentiation mediated by JHIII or methyl farnesoate.
Results and discussion

In mammalian and insect cell cultures JH and analogues are unable to mediate a transcriptional response with EcR and USP via an hsp27 EcRE (Henrich et al., 2003; Fang et al., 2005). In CHO cell cultures JH alone is unable to mediate transcriptional activity, however in the presence of JH a tenfold lower dosage of ecdysteroid is able to achieve a maximal transcriptional response (Beatty et al., 2006). This potentiation effect is characteristic of other intermediates of the mevalonate pathway and precursors of JH such as farnesolic acid, farnesyl diphosphate, and methyl farnesoate (IV, Figure 14.3). The components of this pathway including JHIII are dependent on available nutrients (Belles et al., 2005) and may provide a sensing mechanism in which nutritional state and 20E titers determine the nature of molts in developing larvae. The broad action yet low-level affinity of these intermediates for the EcR-USP heterodimer may act as a trigger to maintain larval-larval molting during the larval feeding stages when 20E titers are low.

There appear to be multiple modes of action that underlie the potentiation effect on the EcR/USP heterodimer and the high affinity interaction of JH and methyl farnesoate observed with USP in the absence of EcR. In the presence of EcR, JH demonstrates a low affinity interaction that may involve the recruitment of cofactors that interact with the EcR/USP heterodimer to mediate transcriptional responses. RXR, the vertebrate ortholog of USP, has demonstrated differences in ligand affinity in the presence and absence of heterodimeric partners (Desvergne, 2007).

**Cell cultures reveal species specific aspects of EcR and USP**

The protein sequence and isoform expression of EcR and USP vary broadly across insect species, yet these differences presumably result in the divergent profiles of development observed in these insects. Chimeric constructs that combine receptor domains of EcRs and USPs from different species have been studied in order to describe the molecular basis for differences in receptor function. When a chimeric construct of the *D. melanogaster* USP with the *Chironomus tentans* LBD substituted for the natural *D. melanogaster* LBD is introduced into *usp* mutant flies, the chimeric USP rescues larval development but is unable to perform the functions essential to metamorphosis (Henrich et al., 2000). This result indicates a distinct function for the *D. melanogaster* USP LBD that the corresponding *C. tentans* USP LBD is unable to perform.

A repressive role has been proposed for the USP DBD based on the effect of *in vivo* mutations on developing *D. melanogaster* larvae. Mutations of conserved residues within the *D. melanogaster* USP DBD had no effect early inducible genes but resulted in premature expression of other genes indicating a repressive function of the DBD had been disrupted (Schubiger and Truman, 2000; Ghbeish et al., 2001). Studies in mammalian cell cultures have also demonstrated domain specific interactions with the USP DBD when paired with the *D. melanogaster* EcR isoforms. A *Choristoneura fumiferana* USP construct with a deleted DBD (USP-ΔDBD) exhibited greatly increased basal and ecdysteroid inducible transcriptional activity when coexpressed with full length *D. melanogaster* USP (IV, Figure 14.4; Henrich et al., 2003). In contrast, an equivalent *D. melanogaster* USP-ΔDBD was able to dimerize with all three EcR isoforms, but only EcRB1 completely retained the ability to mediate
transcriptional activity in response to ecdysteroids. When paired with the USP-ΔDBD, the EcRA and B2 isoforms exhibited severely reduced basal and ecdysteroid induced transcriptional activity (Beatty et al., 2006).

The functional differences of EcR and USP are difficult to assess with in vivo studies. Mammalian cell culture studies allow the careful dissection of molecular and domain specific subfunctions that underlie the roles of EcR and USP isoforms both within species and across insect species.
References


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Publications and Manuscript

The results from Project A (Functional analysis of the ligand binding domain of USP from *Drosophila melanogaster*) have been published in the following peer reviewed journal article:


The results from Project B (Characterization of the EcR isoforms from *Drosophila melanogaster*) result in the following publication:


The results from Project C (Characterization of ecdysteroid receptors from diverse insect species) resulted in the following publication and manuscript:


(V) Beatty JM, Spindler-Barth M, Henrich VC. A cross species comparison of EcR and USP in the functional ecdysone receptor...52

Project D (Utility of cell culture systems in the characterizing the heterodimeric partnership of EcR and USP) resulted in the following book chapter:

Functional studies on the ligand-binding domain of Ultraspiracle from Drosophila melanogaster

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Abstract
The functional insect ecdysteroid receptor is comprised of the ecdysone receptor (EcR) and Ultraspiracle (USP). The ligand-binding domain (LBD) of USP was fused to the GAL4 DNA-binding domain (GAL4-DBD) and characterized by analyzing the effect of site-directed mutations in the LBD. Normal and mutant proteins were tested for ligand and DNA binding, dimerization, and their ability to induce gene expression. The presence of helix 12 proved to be essential for DNA binding and was necessary to confer efficient ecdysteroid binding to the heterodimer with the EcR (LBD), but did not influence dimerization. The antagonistic position of helix 12 is indispensible for interaction between the fusion protein and DNA, whereas hormone binding to the EcR (LBD) was only partially reduced if fixation of helix 12 was disturbed. The mutation of amino acids, which presumably bind to a fatty acid, evoked a profound negative influence on transactivation ability, although enhanced transactivation potency and ligand binding to the ecdysteroid receptor was impaired to varying degrees by mutation of these residues. Mutations of one fatty acid-binding residue within the ligand-binding pocket, I323, however, evoked enhanced transactivation. The results confirmed that the LBD of Ultraspiracle modifies ecdysteroid receptor function through intermolecular interactions and demonstrated that the ligand-binding pocket of USP modifies the DNA-binding and transactivation activities of the fusion protein.

Keywords: dimerization; DNA binding; ecdysteroid; nuclear receptor; transactivation; two-hybrid assay.

Introduction
The ligand-binding domain (LBD) of nuclear receptors participates in several functions: ligand binding, dimerization, hormonal regulation of transactivation and interaction with comodulators. Among insects, two nuclear receptors, the ecdysone receptor (EcR) and Ultraspiracle (USP), an ortholog of the vertebrate RXR, form the functional ecdysteroid receptor. The interaction of 20-hydroxy ecdysone with this heterodimer sets off the transcriptional changes associated with insect larval and metamorphic development. Previous studies have conclusively demonstrated that ecdysteroids bind only to EcR, and there is less certainty about what ligand, if any, interacts with the LBD of USP. Other studies have shown that USP physically interacts with juvenile hormone III (JHIII) and can be induced by JHIII with specific promoters (Xu et al., 2002). Moreover, protein models suggest that JHIII can bind via specific residues in the USP (LBD) (Gesorith et al., 2003).

The combination of LBDs including the C-terminal part of the hinge region of EcR and USP show the same ligand-binding properties as reported for full-length receptors of Drosophila melanogaster (Grebe et al., unpublished; Yao et al., 1993) and tags used for purification of receptor proteins or other fused protein moieties have no effect (Grebe and Spindler-Barth, 2002). For the ecdysteroid receptor from Chironomus tentans it has been shown that purified receptor proteins and crude extracts possess the same Kᵢ values (Grebe and Spindler-Barth, 2002). Therefore we conclude that hormone-binding capability is an autonomous function of the LBD and is influenced only by the dimerization partner.

In previous papers we characterized dimerization and hormone-binding properties of the LBD of the ecdysone receptor EcR (Lezzi et al., 2002; Grebe et al., 2003) using mutated receptor LBDs created by site-directed mutagenesis. We now investigate the impact of USP LBD mutations on ecdysteroid receptor function.

Although Drosophila EcR is able to bind hormone in the absence of USP to a small but significant degree, a tenfold increase in ecdysteroid binding is observed after addition of USP (Lezzi et al., 2002; Grebe et al., 2003) which is accompanied by an allosteric change of the ligand-binding pocket of EcR (Grebe et al., 2003). Whereas the three-dimensional structure of the EcR LBD is unknown, some relevant insights have been obtained from the structure of the USP(LBD) of D. melanogaster (Clayton et al., 2001) and Heliothis virescens (Billas et al., 2001). Their structures show that helix 12 of the USP LBD is fixed in an antagonistic position even in the absence of a specific ligand because of a hydrophobic interaction between helix 12 residues and other amino acids located in the loop between helix 1 and helix 3. The loop is highly conserved in Diptera and Lepidoptera but not in other arthropods. According to Billas et al. (2001), the non-specific binding of a phospholipid further stabilizes this apo-position of helix 12, involving a different subset of amino acid residues than those associated with possible JHIII binding.

Using yeast two-hybrid analysis, previous studies have demonstrated that the LBDs of EcR and USP, fused to...
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**Results and discussion**

Fusion proteins of USP(LBD) with Gal4(DBD) and Gal4(AD)-EcR(LBD) were used to study the influence of mutations in the LBD of USP on EcR(LBD)/USP(LBD) heterodimer function. First the induction of reporter gene activity by the heterodimer was measured by two-hybrid assay and the influence of hormone determined. DNA binding, a prerequisite for reporter gene induction, was tested by gel mobility shift assays and ligand-binding studies were performed to determine the influence of the USP(LBD) on the binding of ecdysteroid with USP’s dimerization partner, EcR. The results are summarized in Table 1.

Most of the mutated USP fusion proteins were highly reduced in their ability to induce reporter gene activity, although it was unclear from this result alone whether the effect reflected a loss of expression, a destruction of all activity, or a specific loss of transactivation capability. By contrast, ligand binding among EcR-mutated proteins ranges from nearly normal levels to no binding at all, thus demonstrating that general receptor function was not destroyed, but that specific functions were affected by each mutation (Grebe et al., 2003). Indeed, the mutated proteins included in this study were expressed and formed heterodimer complexes that showed at least some ligand-binding and DNA-binding activity. In other words, these USP mutations affected the ability of the GAL4 fusion protein to induce transcription, but did not eliminate other LBD functions.

DNA-binding properties were characterized by gel mobility shift assays using Gal4-specific UAS. The identity of the band representing Gal4(DBD)-USP(LBD) complex with DNA was verified by a supershift in the presence of a c-Myc specific antibody recognizing the Gal4(DBD) fusion protein (Figure 1). This confirms that the retarded band was in fact due to USP(LBD) and not another transcription factor present in the yeast extract also interacting with the radiolabeled oligonucleotide. No corresponding band was observed in non-transformed yeast. No heterodimeriza-
Figure 2 Western blots of wild-type and mutated Gal4(DBD)-USP(LBD): the expression level of individual USP fusion proteins varies considerably. The intensities of the Western blot signals were quantified and compared with the signal of wild-type Gal4 (DBD)-USP(LBD) as described in the material and methods section.

The expression rates of various mutated USP fusion proteins varied approximately 6 fold for individual mutations (Figure 2). Therefore the receptor concentrations of the mutated USPs were determined by quantification of the Western blot signals using a standard curve derived from wild-type USP fusion protein to ensure that the same receptor concentration was used for subsequent biochemical studies.

Functional role of helix 12

Helix 12 is essential for hormone-dependent transactivation in most nuclear receptors and is also required for ligand binding in some receptors like EcR (Lezzi et al., 2002; Grebe et al., 2003). Helix 12 of USP is unique compared to all other nuclear receptors and is fixed to an antagonistic position even in the absence of a ligand (Billas et al., 2001; Clayton et al., 2001). Therefore we were especially interested in the functional role of helix 12. Truncation of USP’s helix 12 abolished reporter gene induction on the two-hybrid assay (Table 1), interaction with DNA, and the hormone-binding capability of USP’s dimerization partner, EcR (Figure 3). All of these receptor functions depend on the ability of the receptors to dimerize with each other. To measure dimerization directly, Gal4(DBD) fusion proteins with wild-type USP(LBD) and truncated USP(LBD) were further examined by gel filtration (Figure 4). Gal4 (DBD) eluted at a much lower apparent molecular weight (M₀) than calculated due to interactions with the gel in the absence of a Gal4 specific UAS. Dimerization mediated by the DBD was only observed in the presence of DNA. The elution pattern of the fusion protein with wild-type USP(LBD) showed a main peak even in the absence of DNA that corresponded to the predicted M₀ of the homodimer. Since dimerization via the DBD is not possible without an UAS, dimerization in this case must be mediated by the USP(LBD). The truncated receptor protein eluted at an

Figure 3 Adjusted hormone- and DNA-binding properties of EcR/USP complexes with mutations in helix 12 (H12) of USP. (A) Specific binding of ponasterone A to Gal4(AD)-EcR(LBD)/Gal4(DBD)-USP(LBD). Non-specific binding (<5% of total binding) and hormone binding to EcR(LBD) in the absence of USP (9%) were subtracted. (B) Specific binding to Gal4 specific UAS compared to wild-type Gal4(DBD)-USP(LBD). No interaction with the Gal4 specific UAS was seen if helix 12 of USP was deleted. Point mutations in helix 12 show either weak, diffuse (L490R), or normal (E493K) DNA binding and a hormone-dependent shift as typical for wild-type receptor complexes is visible for both mutations. +/− with or without 10⁻⁶ M muristerone A. \(\text{Gal4(DBD)-USP(LBD)}\), \(\text{Gal4(DBD)-USP(LBD)/Gal4(AD)-EcR(LBD) heterodimer}\).
Figure 4  Gel filtration of Gal4(DBD) and fusions with wild-type and ΔH12-USP(LBD) based on protocols described in the materials and methods section. (A) A 0.4 M NaCl extract of yeast cell expressing Gal4(DBD) was treated with DNase to prevent DNA-mediated dimerization. Gal4(DBD) eluted at an apparent $M_t$ below 10000, which is considerably lower than the calculated $M_t$ of 21500. (B) In the presence of Gal4-specific UAS GAL4(DBD) dimerizes and elutes at an apparent $M_t$ of 55000. (Calculated $M_t$ of the receptor dimer/DNA-complex: 69600.) (C) Gal4(DBD)-USP(LBD) wild type elutes at an apparent $M_t$ of 155000, which is consistent with the formation of dimers bound to UAS (calculated $M_t$ of 142000). (D) Deletion of helix 12 causes a shift to an apparent $M_t$ of 115000, which corresponds to the calculated $M_t$ of 113000 of the dimer of Gal4(DBD)-USP(LBD)ΔH12 in the absence of DNA binding.

apparent molecular weight $M_t$, that corresponds fairly well to the calculated value of the homodimer without UAS. This is in agreement with the EMSA which showed no DNA binding if helix 12 of USP(LBD) was deleted (Figure 3B).

X-Ray studies revealed that helix 12 in USP is fixed in an antagonistic position even in the absence of a specific ligand (Billas et al., 2001; Clayton et al., 2001). Two amino acids were tested: L490 which interacts with the L1-L3 loop and stabilizes the antagonistic position of helix 12 and E493 which is not engaged in fixation of helix 12. The results show that reporter gene induction was severely affected by both mutations, but that hormone binding, DNA binding and hormone-dependent heterodimerization still occurred at an observable level (Figure 3). Comparison of USP<sub>L490R</sub> and USP<sub>E493K</sub> showed that the antagonistic position of helix 12 was indispensable for reporter gene induction, but did not disrupt ligand binding. Since dimerization is a prerequisite for the observed level of ligand binding (Grebe et al., 2003), it was inferred that dimerization is nearly normal in USP<sub>E493K</sub> and only moderately reduced in USP<sub>L490R</sub>. DNA binding as shown by EMSA was reduced for L490R but was normal for E493K. The data obtained with E493K demonstrate clearly that, besides dimerization and DNA binding, an additional factor is important for reporter gene induction.

A functional role of helix 12 according to the model proposed by Westin et al. (1998) requiring a flexible helix seems questionable. Based on homology models (Sasorith et al., 2003), an agonistic position preferred after docking of a putative ligand such as juvenile hormone seems possible, although this ligand does not contact amino acid residues of helix 12 directly (Sasorith et al., 2003).

Helix 12 of USP is centrally important not only for DNA binding and transactivation but also for hormone binding with the heterodimerization partner, EcR. The retained ability for dimerization in the absence of helix 12 in USP rules out the possibility that the observed effects are caused by a generally altered three-dimensional structure of the ligand-binding pocket that affects all receptor functions simultaneously. Rather, the influence of helix 12 in USP(LBD) on ligand binding of the heterodimerization partner EcR and on DNA-binding ability of the Gal4 (DBD) demonstrates both intra- and intermolecular allosteric effects of the USP(LBD).
Function of Ultraspiracle ligand-binding domain

Mutations of amino acids that contact the non-specifically bound phospholipid

Another set of mutations is characterized by substituted residues that form a salt bridge with the hydrophilic end of a phospholipid such as the one that copurified with USP extracted from *E. coli* (Clayton et al., 2001). While the phospholipid has not generally been viewed as a natural ligand for USP, it apparently is important for stabilizing USP structure during purification. Among the putative phospholipid-binding sites, S376 is universally conserved among known USP proteins, but K379 is not. In both cases, mutational changes severely reduced the activity in two-hybrid experiments, and hormone inductibility was also eliminated at a hormone concentration of 25 μM (Table 1). The reduced hormone binding (Figure 5A) in combination with the weak DNA binding (Figure 5B) may be partially responsible for this effect, although weak heterodimerization in the presence of hormone was still observed in EMSAs. However, S376A did not completely eliminate hormone-induced two-hybrid activity. In fact, USP$^{S376A}$ evoked a higher level of induction at an elevated dosage of muristerone A (80 μM = 13.94-fold induction, 150 μM = 51.60-fold induction) further revealing a residual level of ligand-binding activity in the heterodimer.

A third set of mutations was created for hydrophobic residues that lie within the ligand-binding pocket of USP (L281, W318, L322, I323, V326) and apparently contact the phospholipid via hydrophobic interactions. Each of these residues is partially to highly conserved among USP proteins in insects. Four of these mutations eliminated both basal and ligand-induced activity on the two-hybrid assay, whereas two different substitutions of a residue that lies at the base of the USP ligand-binding pocket, I323A and I323V, behaved as superinducers on the two-hybrid assay (Table 1, Henrich et al., 2000). Position 323 is occupied by an amino acid that encodes a leucine or isoleucine in all known insect USPs. Ligand-induced activity is much higher at both low (Table 1) and high dosages of muristerone A (unpublished results). This is especially remarkable because ligand binding was slightly reduced by these same mutations. The opposite effects of these mutations compared to the other amino acids involved in phospholipid binding suggests an additional or different role for I323. In fact, both the alanine and valine substitutions of I323 remove the large side chains that protrude into the ligand-binding pocket. Other substitutions, notably I323D, which introduces a negative charge, and I323F (data not shown) with a rather bulky side chain are deleterious and eliminate yeast two-hybrid activity. Hormone binding is reduced (Table 1, Figure 5A) in heterodimers carrying a mutation of amino acids engaged in phospholipid binding (Clayton et al., 2001; Billas et al., 2001).

The size of the side chain seems to be very critical in L322, because reduction in L322G caused a complete loss of receptor activities. The length of the side chain
was sufficient to allow DNA binding in USP<sup>335AR</sup>, and seems to be more important than the introduction of an additional charge.

**Amino acids of the ligand-binding pocket of USP with no contact to a putative ligand**

In USP<sup>324DS</sup>, hormone binding to EcR was reduced indicating that hormone-induced dimerization is still possible. DNA binding was reduced only partially, but two-hybrid activity was abolished completely. This example revealed that an additional condition for the receptor heterodimer is necessary for transactivation of reporter gene activity. Its failure also shows that specific functions are selectively destroyed by individual mutations.

The E392 residue is highly conserved among all USP receptors and we, therefore expected that this amino acid plays an essential role for USP function. Ligand binding of the heterodimer carrying the USP<sup>E392A</sup> mutation was almost destroyed and two-hybrid activity was severely reduced, but not completely abolished (Figure 6A).

For L410F, I414A, L415F, a hormone-dependent supershift indicating heterodimerization was still observed although the DNA-binding capacity was reduced in the absence of hormone. This was further confirmed by the observation that ponasterone A binding to EcR was reduced in the presence of these mutated USPs. Two-hybrid activity was completely abolished, thus underlining the general importance of amino acids in the ligand-binding pocket of USP for reporter gene induction and indicating once more that ligand and DNA binding alone is not sufficient for two-hybrid activity.

P463 is located in helix 10, which lies along a dimerization interface. It is reasonable to assume that exchange of proline by asparagine destroys the dimerization capability of USP<sup>LBD</sup>. All receptor functions were abolished except DNA binding, demonstrating that dimerization mediated by the LBD of USP is not required for DNA binding of the fusion protein, but that dimerization of the Gal4 moieties is sufficient.

**Two-hybrid assay**

The behavior of the USP mutations on the yeast two-hybrid assay revealed a different pattern of effects than previously noted for analogous EcR mutations (Lezzi et al., 2002; Grebe et al., 2003). In the former case, mutations of the EcR LBD evoked a range of effects. Many of the mutations impaired both basal and ligand-induced functions indicating that a basal process is disturbed.

Two-hybrid assays are generally considered to be dimerization assays. This may be justified, when the bait is the same and the interaction with different preys is examined as was shown with the EcR mutations which were coupled to the activation domain of Gal4 and probed with the wild-type Gal4(DBD)-USP<sup>LBD</sup>. In experiments presented in this paper the bait itself was modified, meaning that mutated USP<sup>LBD</sup>s fused to the DNA-binding domain of Gal4 were used. Consequently, the Gal4(DBD) is not considered as a constitutive functional unit for monitoring reporter gene activity. Instead,

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**Table 1** Effect of mutations in the ligand-binding domain of USP on reporter gene activity (two-hybrid assay), ecdysteroid- and DNA-binding properties of Gal4(AD)-EcR(LBD) and Gal4(DBD)-USP<sup>LBD</sup> fusion proteins.

<table>
<thead>
<tr>
<th>Mutational site</th>
<th>Position</th>
<th>% wild type no hormone</th>
<th>% wild type 25 µm murA</th>
<th>Fold induction</th>
<th>Ligand binding&lt;sup&gt;a&lt;/sup&gt; (% EcR/USP wild type±SD)</th>
<th>EMSA</th>
</tr>
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<tbody>
<tr>
<td>Wild type</td>
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<td>100</td>
<td>8–25</td>
<td>100</td>
<td>(+/+/+/+)</td>
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<tr>
<td>L281Y</td>
<td>H3</td>
<td>52</td>
<td>32.2</td>
<td>2.2</td>
<td>43 ± 6</td>
<td>(+/+/+/+)</td>
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<tr>
<td>W318F</td>
<td>H5</td>
<td>1.2</td>
<td>0.1</td>
<td>1</td>
<td>28 ± 2</td>
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<td>L322G</td>
<td>H5</td>
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<td>0 ± 2</td>
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<td>L322R</td>
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<td>0.1</td>
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<td>I323A</td>
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<td>70.5</td>
<td>354.4</td>
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<tr>
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<td>14.66</td>
<td>36 ± 10</td>
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<td>53 ± 4</td>
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<td>1</td>
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<td>(+/+)</td>
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<tr>
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<td>H7</td>
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<td>1</td>
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<td>65 ± 8</td>
<td>(+/+)</td>
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<td>64 ± 10</td>
<td>(+/+)</td>
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<tr>
<td>E493K</td>
<td>H12</td>
<td>36.0</td>
<td>4.0</td>
<td>6.36</td>
<td>89 ± 16</td>
<td>(+/+/+)</td>
</tr>
<tr>
<td>ΔH12</td>
<td>H12</td>
<td>1.34</td>
<td>0</td>
<td>1</td>
<td>0 ± 2</td>
<td>(–/–)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Ligand binding to EcR in the absence of USP is 9 ± 3% and is subtracted.

<sup>b</sup>DNA binding in the absence of hormone/supershift in the presence of hormone (+, strong signal; +, weak signal; –, no signal; n.d., not determined).

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genes, is impaired or even abolished in several mutated USP fusion proteins. As shown by EMSA (Figure 6B), the positive impact of wild-type USP(LBD) on DNA binding of the Gal4 moiety depends not only on the presence but also on the unique antagonistic position of helix 12 in Ultraspiracle. These results may explain the failure to observe any effects of helix 12 USP mutations used in transfection studies with insect cells (Hu et al., 2003). Because the mutated USP proteins may have lost their ability to interact with DNA, only the positive DNA binding of endogenously expressed wild-type USP would be measured in the insect cells, thus masking the effect of the mutated protein.

**Dimerization, ligand and DNA binding are not sufficient for transactivation**

A comparison of the two-hybrid data and the results of DNA- and ligand-binding tests of some mutations (USP3276A, USP323V and USP323A) are not always in parallel. The superinduced reporter gene levels evoked by USP323V and USP323A may be interpreted as the consequence of enhanced dimerization that offsets a reduction in ligand-binding capability by the EcR/USP dimer, although direct experimental proof is missing. Alternatively, the results may indicate an additional regulatory step necessary for transactivation besides ligand and DNA binding or dimerization partners such as a co-modulator. This is illustrated by USP3493K which clearly demonstrates that despite retaining its dimerization, hormone- and DNA-binding abilities, an additional factor or co-modulator is required for transactivation as proposed already by Tran et al. (2001) and VomBaur et al. (1998) for vertebrate receptors. Mutations that allow a normal fold-induction but at a highly reduced quantitative level and the superinducer mutations indicate that USP plays an active role in transactivation of the reporter gene and that its function is not restricted to dimerization only.

Dimerization in nuclear receptors is mediated by several dimerization interfaces. Our aim is to study each dimerization site and its regulation separately to evaluate the impact of each site. In the present study we concentrated on the dimerization properties of the ligand-binding domains of EcR and USP. In the Gal4 fusion proteins the A/B domains of EcR and USP were absent. Therefore the dimerization properties of this receptor domain (Rymarczyk et al., 2003) did not influence our results. We are currently determining the influence of the C and D domain on dimerization properties.

**Materials and methods**

**Yeast strain**

Saccharomyces cerevisiae strain Y190 was cultured according to manufacturer instructions (Clontech Laboratories, Palo Alto, CA, USA). Cells were transformed with lithium acetate (Guthrie
and Fink, 1991) and selected by auxotrophy for tryptophan (pAS2-1) and leucine (pACT2), respectively.

**Yeast expression plasmids**

DNA encoding the C-terminal part of the D domain and the E domain of the Drosophila ecdysone receptor EcR (aa 375–652) was cloned into the expression vector pACT2 (Li et al., 1994; Lezzi et al., 2002) resulting in a Gal4(AD)-EcR(LBD) fusion. For expression of Gal4(DBD)-USP(LBD) the corresponding domain of Ultraspireacle (aa 172–508) was cloned either into the vector pGBKT7 (Louvet et al., 1997) or into the vector pAS2-1 (Harper et al., 1993; Lezzi et al., 2002).

**Construction of site-directed mutations**

All site-directed mutations in Gal4(DBD)-USP(LBD) were carried out with a site-directed mutagenesis kit (Stratagene, Amsterdam, Netherlands) following the manufacturer’s instructions. Briefly, the plasmid vector is amplified by polymerase chain reaction with a pair of oligonucleotide primers in which the site of mutagenesis is included, so that during replication cycles, the mutated plasmid is the predominant PCR product. The original template strands are methylated and destroyed by the restriction enzyme, DpnI, and the new plasmids were transformed into E. coli and recovered. All mutated products were verified by sequencing and also checked for possible second site mutations in both the USP(LBD) and the GAL4(DBD) portion of the plasmid. In cH12 aa 490-508 were deleted. The oligonucleotides used to produce the mutations described are available upon request.

**Yeast two-hybrid conditions**

All wild-type and mutated plasmids were tested according to the procedures described by Lezzi et al. (2002), except that yeast transformations were carried out with the Frozen EZ Transformation II kit (ZymoResearch; Orange, CA, USA) following manufacturer’s protocols. In all cases, three colonies were tested for each replicate, and three replicates were performed for each mutation. Additionally, all mutational results were compared with simultaneously run wild type controls, since the absolute level of lacZ activity reported on the yeast two-hybrid assay varies even under controlled conditions, though general levels of relative performance were stable.

**Preparation of yeast extracts**

Single colonies (not older than 4 days) of yeast transformants carrying the expression plasmids were picked and cultured at 30°C overnight in 5 ml selective medium containing 2% glucose with vigorous shaking (150-200 rev/min) to disperse the cells thoroughly. They were then diluted in 50 ml YPD medium (20 g/l peptone, 10 g/l yeast extract, 2% glucose) and grown under the same conditions until the OD$_{600}$ reached 0.6–0.8. Cells were harvested by centrifugation (1500 g, 5 min, 4°C) in precooled tubes and washed with 50 ml ice-cold binding buffer (20 mM HEPES, 20 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.9). The pellets were frozen in liquid nitrogen for approximately 20 s and disrupted for 2 min at 2000 rev/min using a Micro-dismembrator S (B. Braun Biotech International, Melsungen, Germany). After thawing, homogenates were diluted with binding buffer and supplemented with a mixture of protease inhibitors (aprotinin, leupeptin, pepstatin, benzamidine, antipain, chymostatin; final concentration 2 µg/ml each and 1 mM phenylmethyl-sulfonyl fluoride) immediately before use. After short treatment with ultrasonic power (microtip 2×2 s, 90 Watt, Branson Sonifier, B-12; Branson, Danbury, CT, USA) the samples were centrifuged (100 000 g, 1 h, 4°C) and frozen in aliquots at –80°C until use.

**Western blot and quantitative determination of fusion proteins**

Yeast extracts were diluted with sample buffer (final concentration: 100 mM Tris, 3%, SDS, 2% 2-mercaptoethanol, 10% glycerol, 0.05% bromphenol blue, pH 8.8) and boiled for 3 min (Laemmli, 1970). 10–20 µg protein (Bradford, 1976) were applied on each lane of an acrylamide gel and subjected to electrophoresis using a Hoefer mini-V, (300 V, 15 mA; Amersham Biosciences, Freiburg, Germany). Gels were electroblotted on nitrocellulose membranes (BA 85, 45 µm pore size, Schleicher and Schuell, Dassel, Germany) according to Khyse-Andersen (1984). The membranes were soaked in blocking buffer (5% milk powder, 1% fat in 20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween 20, pH 7.6, 0.02% Thimerosal). EcR(LBD) fusion protein was probed with a Gal4(AD)-specific antibody (S398-1, Clontech Laboratories) diluted in blocking buffer 1:5000. USP fusion proteins were probed either with Gal4(DBD) specific antibody (# sc-577, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:100 or with c-Myc specific antibody (3800-1, Clontech Laboratories) diluted 1:1000. Specific signals were detected with horseradish peroxidase conjugated secondary antibodies diluted 1:10000 (anti-mouse IgG, Sigma A-9001, Sigma-Aldrich, Taufkirchen, Germany) or 1:500 (anti-rabbit IgG, Sigma A-6667), in TBS-T (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween 20, pH 7.6). Detection and quantification was done as described in detail by Rauch et al. (1998). Specific signals were imaged with Fluor-S Multimager (Bio-Rad Laboratories, Hercules, CA, USA) and evaluated with the software Multi-Analyset/PC (Version 1.1, Bio-Rad Laboratories).

**Ligand-binding assays**

Yeast extracts were diluted with binding buffer and supplemented with protease inhibitors as described above immediately before use. Ligand binding was determined with [3H]-ponasterone A (specific activity 2.5 TBq/mmol; kind gift of Dr H. Kayser, Syngenta, Basel, Switzerland) using a filter assay described in detail previously (Turberg and Spindler, 1992). Radiolabeled ponasterone A was used, because the affinity to EcR is higher compared to 20-OH-ecdysone. Fusion proteins were quantified by Western blots as described above and normalized based on wild-type expression levels. Receptor proteins were mixed with 4-5 nM [3H]-ponasterone A and incubated for 1 h at room temperature. Non-specific binding determined in the presence of 0.1 mM non-labeled 20-OH-ecdysone was subtracted. Purity of [3H]-ponasterone A was checked routinely by HPLC analysis. Ligand-binding data of mutated receptors were expressed as % of wild-type hormone binding (~100%).

**Electrophoretic mobility shift assay (EMSA)**

The oligonucleotides dgal 1: 5'-GATGCGCACAGTGGCCGGAG-GACAGCTCCCGGTTGACAT-3' and dgal 2: 5'-GATCATCGA-ACCCGAGGACTGTGGCCTGGACGTG-3' were formed by
annealing 5' extensions using the sequence GATC labeled with [α-32P]-dCTP by fill-in reaction with Klenow polymerase.

The reaction mix contained EMSA buffer [20 mM HEPES, pH 7.4, 100 mM KCl, 5% (v/v) glycerol; 2 mM dithiothreitol, 0.1% NP-40]. yeast cell extracts with EcR or USP fusion proteins, 1 μg non-specific competitor poly[d(dC)] and approximately 10 fmol labeled oligonucleotide. 10−11 μg muristereone a (final concentration) was used where indicated. Muristereone a was used instead of 20-OH-ecdysone, because the affinity to EcR is higher. After incubation for 30 min at room temperature, the samples were loaded on a 5% nondenaturing polyacrylamide gel in 0.5× TBE (45 mM Tris, 45 mM boric acid, 0.5 mM EDTA pH 8.0) and separated at 10 V/cm for 2 h. Gels were dried, scanned with a phosphorimager (Fluorescent Image Analyzer FLA-3000 series, FUJIFILM, Düsseldorf, Germany) and evaluated with software Aida Image Analyzer 3.25 (Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany).

Chromatography

Samples were prepared exactly as described for gel shift experiments. 500 μg protein (1 μg/μl) were incubated for 30 min at room temperature and subjected to size exclusion chromatography (Superdex 200 HR 10/30, Amersham Pharmacia Biotech). After equilibration of the column with elution buffer (20 mM K-phosphate, pH 7.4, 50 mM KCl, 1 mM EDTA, 10% glycerol), the sample was loaded on the column and separated (flow rate of 0.25 ml/min, 4°C). Fractions were collected (500 μl) and proteins precipitated with 7.5% TCA (final concentration). A molecular weight marker kit (Sigma-Aldrich) was used to calibrate the column. Fractions were subjected to Western blotting with Gal4(DBD) or c-Myc specific antibodies and quantified as described above.

Acknowledgments

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References


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Analysis of transcriptional activity mediated by *Drosophila melanogaster* ecdysone receptor isoforms in a heterologous cell culture system

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Abstract

Ecdysteroid regulation of gene transcription in *Drosophila melanogaster* and other insects is mediated by a heterodimer comprised of Ultraspiracle (USP) and one of three ecdysone receptor (EcR) isoforms (A, B1 and B2). This study revealed that the EcR/USP heterodimer displays isoform-specific capabilities. EcRB1 is normally induced with a form of USP that is missing its DNA-binding domain (DBD), although potentiation by juvenile hormone (JH) III is reduced. The EcRA and B2 isoforms, however, display almost no response to ecdysteroids with the DBD-USP. A mutation, K497E, in the shared ligand-binding domain of the EcR isoforms caused elevated EcRB2-specific affinity for a canonical ecdysone response element. The effects of directed modification and mutagenesis offer a strategy for developing hypotheses and considerations for studying in vivo function.

Keywords: Juvenile hormone, mutagenesis, Ultraspiracle, retinoid X receptor, repression.

Introduction

The insect ecdysteroid receptor is a heterodimer comprised of two nuclear receptors, the ecdysone receptor (EcR; Koelle et al., 1991) and Ultraspiracle (USP; Henrich et al., 1990; Oro et al., 1990; Shea et al., 1990), which are stabilized by the presence of the insect moulting hormone, 20-hydroxyecdysone (20E; Yao et al., 1992, 1993; Thomas et al., 1993). The heterodimer interacts with several defined DNA sequence elements to regulate the transcription of target genes (Antoniewski et al., 1993; Vogtli et al., 1998; Devarakonda et al., 2003). Both EcR and USP are required for the normal progression of premetamorphic development in *Drosophila melanogaster* (Henrich et al., 1994, 2000; Hall & Thummel, 1998; Bender et al., 1997; Li & Bender, 2000). Ecdysteroids are the only endogenous class of steroid hormones in *D. melanogaster* and other insects, and these trigger both general and cell-specific transcriptional responses (Riddiford et al., 2000; Thummel, 2002).

The diversity of ecdysteroid responses found among developing *Drosophila* tissues involve three natural isoforms of EcR (A, B1 and B2), which differ only in their N-terminal trans-activation domain (Talbot et al., 1993) and exhibit different transcriptional capabilities (Hu et al., 2003). Isoform-specific mutations lying within this domain disrupt distinct and essential processes during metamorphic development. The B isoforms have generally been associated with larval processes (Bender et al., 1997; Schubiger et al., 1998, 2003), and the B2 isoform most efficiently rescues larval development in EcR mutants (Li & Bender, 2001). EcRB2 is also required for proper development of the larval epidermis and the border cells of the developing egg chamber (Cherbas et al., 2003). The A isoform has been implicated in the remodelling of neurons during metamorphosis (Robinow et al., 1993; Truman et al., 1994; Davis et al., 2005), is required for normal development of wing disc margins (Cherbas et al., 2003) and is essential for normal metamorphosis (Davis et al., 2005). Mutations of EcR coding regions shared by the three isoforms arrest development at allele-specific times during the interval from embryogenesis to the onset of metamorphosis (Bender et al., 1997) and disrupt normal oogenesis (Carney & Bender, 2000). However, the in vivo distribution of isoform expression is not clearly correlated with developmental
requirements. Several tissue-specific processes do not require a specific isoform, and in some instances do not even require the EcR N-terminal domain (Cherbas et al., 2003). This complex relationship between EcR disruption and resultant phenotype belies the fact that each EcR isoform has both redundant and isoform-specific properties.

Drosophila USP also contributes to the complexity of ecdysoid receptor function. USP is essential for normal metamorphosis (Hall & Thummel, 1998) although usp mutations generate phenotypes that are distinct from those caused by EcR mutations (Li & Bender, 2001). USP also fulfills distinct larval and metamorphic functions (Henrich et al., 2000) and possesses both inducible and repressive transcriptional properties (Schubiger & Truman, 2000; Ghbeish et al., 2001; Ghbeish & McKeown, 2002). Experimental studies with mammalian and insect cultures have further raised the possibility that USP's cognate ligand is juvenile hormone (Jones et al., 2001; Sasorith et al., 2002), although this possibility has not been reconciled with in vivo responses to exogenous application of juvenoids in Drosophila (Zhou & Riddiford, 2002; Dubrovsky et al., 2004; Wilson et al., 2006).

Heterologous mammalian cell cultures have been utilized frequently to assess the function of the EcR/USP dimer because such cells have no endogenous response to ecdysteroids, and become responsive to ecdysteroids only when cotransfected with EcR and either USP or its mammalian homologue, the retinoid X receptor (RXR; Palli et al., 2003). Such studies have already demonstrated that the individual Drosophila EcR isoforms are not equivalent in their performance, although all can mediate transcriptional activity in response to ecdysteroids (Mouillet et al., 2001). In cell cultures, ecdysteroid response can further be potentiated by the simultaneous presence of juvenile hormone (JH) III, thus reducing the ecdysteroid dosage necessary for maximum receptor induction by about 10-fold (Henrich et al., 2003). Synergistic ecdysteroid/JH effects have also been observed in insect cells (Fang et al., 2005) and in vivo (Dubrovsky et al., 2004). The effects of structurally altered receptors, individual ligands and different DNA response elements have also been tested and compared in heterologous cell culture systems (Vogtli et al., 1998; Henrich et al., 2003).

This study examines the relative performance of the three Drosophila EcR isoforms in terms of their activation by ecdysteroids via a canonical 27 kDa heat shock protein (hs27) ecdysone response element (EcRE; Riddihough & Pelham, 1986), the ability of juvenile hormone to potentiate transcriptional activity of the receptor complex and EcR interaction with Drosophila USP in Chinese hamster ovary (CHO) cells. We also examined specific site-directed mutations in the EcR ligand-binding domain (LBD) that were previously shown to perturb the normal function of EcR in a yeast two-hybrid system (Lezzi et al., 2002; Grebe et al., 2003; Bergman et al., 2004; Przibilla et al., 2004). In the present study, the three EcR isoforms displayed properties in the cell culture system that have implications for in vivo analysis.

**Results**

Functional differences among the three *D. melanogaster* EcR isoforms were inferred by previous studies in both Drosophila cell cultures (Hu et al., 2003) and heterologous cell culture systems (e.g. Mouillet et al., 2001; Henrich et al., 2003). These studies have already demonstrated isoform-specific capabilities for JH III-mediated potentiation of transcriptional activity, although JH III alone exerts no effect on receptor activity. Further, EcR/mammalian fusion proteins are responsive to ecdysteroids, but only the *Drosophila* EcR isoform induces ecdysteroid-dependent transcription when partnered with RXR, the mammalian orthologue of USP (Henrich et al., 2003).

**Effect of DmUSP on EcR isoform activity**

Three fusion proteins derived from DmUSP (VP16-USPII, VP16-USPI and VP16-USPIII) were used to investigate the properties of the natural Drosophila EcR isoforms with DmUSP. DmUSP forms a heterodimer with EcR in mammalian cell cultures, but is not capable of inducing ecdysteroid-dependent transcription. When the N-terminal domain of DmUSP was replaced with the VP16 activation domain, ecdysteroid-responsive transcriptional activity was observed. To measure induction, cells were incubated with 1 µM muristerone A (murA) for 24 h, a regimen that evoked a maximal transcriptional response from all three EcR isoforms in preliminary ligand dosage studies.

The VP16-USPI fusion protein includes six amino acids in the amino-terminal domain (aa 98–103) that are conserved among all insect USP sequences along with the DNA-binding domain (DBD), hinge region and LBD of DmUSP (aa 104–507). The VP16-USPII construct is identical to VP16-USPI, except that these six conserved amino acids are not included. In preliminary studies, the VP16-USPI and VP16-USPIII exhibited only minor differences in transcriptional activity when partnered with EcR (data not shown), indicating that the conserved portion of the N-terminal region has no measurable effect upon hsp27-EcRE-mediated gene expression. Therefore, VP16-USPII was used for other experiments reported here, except as noted.

When tested with the VP16-USPII fusion protein, the EcRBI isoform showed basal and murA-induced levels of transcription that were about four-fold higher than those of the other two isoforms (Fig. 1), even though EcRB1 generated a much weaker immunostaining signal than EcRB2 on Western blots (Fig. 2a). Basal transcriptional levels of EcRA were comparable to those generated by EcRB2 but the fold-induction induced by murA was relatively

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low with the EcRA isoform. The A isoform also generated the weakest immunostaining signal on Western blots, raising the possibility that the weak inducibility could be related to low levels of EcRA expression. When this hypothesis was tested, 250 ng of EcRA-encoding plasmid transfected into cells evoked a maximal level of basal and induced levels of EcRA-induced transcription (Fig. 3). In other words, the relatively weak inductive response of EcRA expression was not caused by a rate-limiting level of available EcRA in the cell, because fold-induction reached a maximal response when as little as 10 ng of EcRA-encoding plasmid was transfected into cells. As in a previous study with a different USP construct, EcRB2 showed the strongest potentiation response to JH III when measured as fold-induction with a 0.1 µM concentration of murA (Henrich et al., 2003).

The VP16-USPIII fusion protein encodes the VP16 AD attached directly to the hinge region and LBD of DmUSP and is analogous to the Choristoneura fumiferana (Cf) USP fusion protein utilized for a previous study (Henrich et al., 2003). The dispensibility of a functional DmUSP DBD for EcRB1-mediated, ecdysteroid-inducible transcriptional activity via an hsp27 EcRE has been demonstrated previously (Ghbeish et al., 2001). Because the CfUSP construct had evoked a relatively robust response with all of the three EcR isoforms, it was hypothesized that the equivalent VP16-USPIII construct would behave similarly.

For the A and B2 isoforms, however, VP16-USPIII produced a reduced basal transcriptional level, a severe reduction in responsiveness to murA and the elimination of any potentiation effect of JH III. Only the B1 isoform conformed to predictions by showing an elevated responsiveness to murA when paired with either VP16-USPII or VP16-USPIII, although the potentiating effect of JH III with the EcRB1/VP16-USPIII dimer was reduced significantly (Fig 1, t-test, P < 0.05). A reduction of potentiation by about
one-half relative to induction by 1 µM murA alone was also observed in two controlled replicates of this experiment.

The activity, as measured by fold-induction, seen with the previously employed VP16-USP III resembled the effects of VP16-USP II in this study, although only the CF form lacks the USP DBD. Western immunostaining of the VP16 domain in cell extracts transfected with the USP constructs generated bands of predicted size and similar density, thus confirming the integrity and expression of the USP constructs used (Fig. 2b).

Effects of site directed mutations on isoform function
Mutant EcR proteins produced by site-directed mutagenesis offer the opportunity to assess the effect of specific structural changes upon receptor function, and potentially can locate specific functional attributes based on the defects caused by the mutation. A fraction of EcR LBD mutations previously tested in a yeast two-hybrid system impair specific receptor characteristics (Lezzi et al., 2002; Grebe et al., 2003; Bergman et al., 2004). Two site-directed mutations described in these studies were tested in each of the three EcR isoforms to determine whether the substitutions evoked the same effect in whole receptors as they did in analogous yeast two-hybrid fusion proteins. Further, these common region mutations were tested in each isoform to explore the possibility that a common region mutation differentially disrupts isoform function.

The K497E mutation. The K497 residue lies in helix 4 and aligns with a consensus cofactor binding site in nuclear receptors. The site has also been implicated in the formation of a salt-bridge with helix 12 to mediate ligand-dependent transcriptional activity (Wurtz et al., 1995a,b). Two different substitutions of this residue, K497A and K497E, result in a dramatically elevated level of ligand-independent transcriptional activity in the yeast two-hybrid system (Bergman et al., 2004). The similar effects caused by two different mutations of K497 strongly suggest that the elevation of basal transcription results from a loss of function normally associated with the K497 residue, although ecdysteroid binding affinity in the mutant fusion protein is actually reduced (Grebe et al., 2003).

When the DmEcR isoforms carrying the K497E mutation were tested with VP16-USP II, the basal rate of transcription was substantially elevated in the EcRB2(K497E) compared to wild-type EcRB2 (t-test, P < 0.001), as predicted from the yeast two-hybrid study, but no significant effect on basal transcription was observed in either EcRA(K497E) or EcRB1(K497E). MurA-induced levels, however, were similar to wild-type levels in all three isoforms (Fig. 4).

In order to test whether this heightened activity resulted from an abnormally high affinity for DmUSP, a competition experiment was devised by which increasing amounts (0–500 ng) of the plasmid encoding EcRB2(K497E) were transfected along with a fixed amount of the plasmid containing the nonmutated EcB2 isoform (100 ng) and VP16-USP II (100 ng). Transcription was then observed in the absence of murA (Fig. 5). When equimolar amounts of EcR-B2 (K497E) and wild-type EcR-B2 were mixed

Figure 4. Effects of three site-directed mutations on basal and muristerone A (murA)-induced levels of transcription on three ecdysone receptor (EcR) isoforms of Drosophila melanogaster. All relative luciferase unit values were normalized according to β-galactosidase activities and were transformed for all data points relative to mean EcRB2 activity in the absence of hormone. Therefore, fold-induction levels for all three graphs are comparable to a common reference level (basal EcRB2 = 1, indicated by *). EcRB1 scaling is reduced to account for higher quantitative levels of transcription. Each data point shows the standard deviation based on a sample size of 3 or more. Unshaded boxes indicate transcriptional levels in the absence of murA, and darkened boxes indicate transcriptional activity in the presence of 1 µM murA.
Figure 5. Basal transcription levels resulting from transfection with 100 ng of test ecdysone receptor B2 (EcRB2) and varying levels (100–500 ng) of mutant EcRB2 isoforms. Levels of M504R and K497E mutants are shown in leftmost bars with no test EcR. EcRB2(K497E) was used as a proxy for wild-type EcRB2 to compete with EcRB2(M504R) because the K497E mutation confers a high basal level of activity allowing for competition analysis to be performed. All data points based upon three replicates plotted with standard deviation.

The substantially elevated transcription associated with B2 (K497E) is not found in any of the isoforms carrying the A483T mutation, which in turn, corresponds to an in vivo larval lethal mutation that disrupts a site required for physical interaction between EcR and the corepressor, SMRTER (Tsi et al., 1999; Carney & Bender, 2000; Fig. 4). Interestingly, A483T did not discernibly affect basal transcriptional activity of the B1 isoform, but significantly reduced its ligand-induced transcriptional activity (t-test, P < 0.01).

The three K497E isoforms were also tested by electrophoretic mobility shift assay (EMSA) to determine whether the elevation of transcription in the mutated B2 is associated with a higher affinity for the hsp27 response element. The shift revealed that the B2(K497E) isoform has a stronger affinity for the hsp27 EcRE in the absence of hormone than any of the wild-type isoforms or the mutant A or B1 isoforms (Fig. 6). Unexpectedly, none of the K497E mutated isoforms showed a high level of affinity for the hsp27 EcRE in the presence of murA, even though wild-type EcR isoforms showed this elevated affinity and transcriptional activity in the mutants approximated the levels found in the equivalent wild-type EcR. This paradoxical result is currently unexplained. Reduced ligand affinity caused by the K497E substitution (Grebe et al., 2003) and/or the absence of a contextual sequence surrounding the EcRE element may have obliterated the shift of the mutant EcR complex.

Figure 6. Electrophoretic mobility shift assay using 27 KDa heat shock protein ecdysone response element (EcRE) tested with extracts from Chinese hamster ovary cells transfected with VP16-USPII and wild-type or EcR (K497E) vectors encoding DmEcRA, B1 and B2 isoforms.

substitution (Grebe et al., 2003) and/or the absence of a contextual sequence surrounding the EcRE element may have obliterated the shift of the mutant EcR complex.

The M504R mutation. Ecdysteroids stabilize the EcR/USP heterodimer which in turn, recognizes promoter elements to regulate transcription. It follows that a receptor unable to bind to a hormone is also unable to be stabilized by the hormone, and ultimately, fails to elevate transcription in the presence of the hormone. Based on alignments of the nuclear receptor superfamily, a mutation was made at a consensus site for ligand binding (M504) in helix 5 of DmEcR (Wurtz et al., 1995a,b). This site corresponds with a predicted ligand-binding site in the retinoic acid receptor (RAR; Bourguet et al., 2000), although this site does not correspond to any that are predicted to contact ecdysteroids in the Heliothis EcR model (Billas et al., 2003). When tested for heterodimerization using a yeast two-hybrid assay, the mutant EcR showed normal dimerization affinity for USP in the absence of hormone, but the elevated rate of heterodimerization associated with ligand binding is abolished, and in fact, ligand binding is also eliminated in this mutant (Grebe et al., 2003).

Consistent with those two-hybrid results, basal levels of transcription in this study were unaffected by the mutation in any of the three natural EcR isoforms whereas murA-induced rates of transcription were almost completely eliminated (Fig. 4). In the absence of hormone, EcRB2(M504R) competed with EcRB2(K497E), which in turn, competed with wild-type EcR and served as a proxy for wild-type dimerization. In the absence of hormone, transfection with equal amounts of plasmid encoding K497E and M504R mutant EcRB2 (100 ng of each) reduced the abnormally
high level of transcriptional activity caused by the K497E mutant (Fig. 5). In other words, the EcRB2(M504R) mutant dimerized normally with DmUSP in the absence of hormone. On the other hand, the M504R mutant receptor failed to displace wild-type receptor in the presence of murA. Fold induction was 9.50 ± 1.90 in cells transfected with 100 ng of wild-type EcRB2-expressing plasmid, and did not change significantly even when 500 ng of EcRB2 (M504R)-expressing plasmid was also added (fold induction: 7.35 ± 0.61). This strongly suggests that the ligand-bound, wild-type EcRB2 has an intrinsically higher affinity for USP than the EcRB2 (M504R) receptor (Fig. 5), which is incapable of ligand-binding (Grebe et al., 2003). This result is also consistent with the supposition that when an ecdysteroid binds to EcR, the affinity of EcR and USP is increased.

Discussion

The in vitro analysis described here is intended to establish a basis for assessing the in vivo function of D. melanogaster EcR and USP, with the ultimate aim of understanding how the heterodimer governs individual transcriptional processes underlying development. These studies, along with those reported earlier, have shown that the native EcR isoforms possess both shared and unique characteristics. Further, an analysis of common region EcR mutations led to the identification and characterization of two site-directed mutations in EcR that affected specific molecular functions, one of which also specifically disrupted EcRB2.

The interaction with USP is not equivalent for the three EcR isoforms

These experiments showed that the USP DBD is essential for both ecdysteroid-inducible transcription and JH III potentiation mediated by EcRA and EcRB2 via an hsp27 EcRE. By contrast, the absence of USP DBD did not impair EcRB1 responsiveness to murA. EcR/USP interactions with the hsp27 EcRE have shown that the USP DBD binds to the 5′ half-site and recruits EcR to the 3′ half-site (Grad et al., 2001). The ability of EcRB1 to maintain its transcriptional inducibility when USP lacks its DBD is consistent with previously reported results (Schubiger & Truman, 2000; Ghbeish et al., 2001) and suggests that this isoform can interact with an hsp27 EcRE differently from the A and B2 isoforms. These results further suggest that EcRB1 is specifically responsible for the in vivo appearance of ecdysteroid-inducible gene expression in usp mutant tissues whose alleles impair USP DBD function (Henrich et al., 1994; Schubiger & Truman, 2000). These same usp mutations destroy the normal in vivo repression of Broad-Complex and IFTZF1 gene expression (Schubiger & Truman, 2000; Ghbeish et al., 2001; Ghbeish & McKeown, 2002). There is evidence that the EcR/USP dimer acts in a ligand-independent fashion to block specific developmental pathways (Schubiger et al., 2005) and the results reported here further indicate that these interactions with mutant USP products vary among the three isoforms.

While the ability of EcRB1 to respond to murA did not require USP to have a DBD, JH potentiation of EcRB1 was reduced by about half when the DBD was removed from USP. This observation argues that ecdysteroid inducibility and JH potentiation of the EcR/USP complex are functionally separable. Finally, the disparity between equivalent USP constructs from different insect species (C. furnieriana and D. melanogaster) highlights the possibility that species-specific aspects of the USP LBD are important for ecdysteroid receptor function, and by corollary, that the CHO cell culture system can delineate functional differences in ecdysteroid receptor activity for different insect species.

The three isoforms display differences in transcriptional capabilities

The differences observed in the reported characteristics of the three EcR isoforms with a canonical hsp27 EcRE imply that the three isoforms show discernibly different in vivo capabilities. By extension, it is conceivable that the three isoforms also vary in their affinity for other ecdysone response elements and natural ecdysone-responsive promoters. The dimerization interface of EcR and USP likely depends on the nature of the response element, because direct repeat elements require a different relative orientation than the hsp27 element that was used in this study (Perera et al., 2005). In turn, this may involve important functional differences that can be genetically dissected.

An unexpected indication of isoform-specific function is revealed by the effects of the K497E mutation. The most notable molecular effect of the mutation is that affinity of the B2(K497E) mutant receptor for the hsp27 EcRE is strongly and specifically elevated in the absence of hormone. While the possibility that this mutation has destroyed a cofactor binding site cannot be formally ruled out, there is no difference in the size of the mutant and wild-type B2 complex assessed by EMSA, as might be expected if a cofactor interaction were involved. Moreover, if a cofactor is involved in this mutational effect, it must occur in mammalian and yeast cells, because the mutation evokes similar effects in both systems. The failure to detect a shift in any of the K497E-mutant isoforms in the presence of hormone, however, leaves open the possibility that other proteins and promoter elements influence transcriptional activity, and that they are unable to interact with the mutant receptor.

Cell culture competition experiments reported here have also shown that K497E has roughly normal dimerization capabilities. Therefore, the high basal transcriptional activity caused by the K497E mutation in a yeast two-hybrid system likely results from a high affinity of the EcR/USP dimer for a response element, rather than a high affinity between the two LBDs. The K497E mutant EcR retained
responsiveness to murA although ligand-binding is impaired (Grebe et al., 2003), which may be the basis for the reduced fold-induction in K497E mutant receptors.

Several possible explanations, which are not mutually exclusive, may account for the isoform-specific effects of the K497E mutation. Most plausibly, the mutation affects the ligand-dependent transcriptional function (AF2) associated with helix 12, which normally interacts with the K497 region in the presence of an activating ligand. The exact nature of this effect is unknown, although the removal of the basic lysine charge caused by either an alanine or glutamic acid substitution conceivably promotes a continuous holo-conformation in helix 12 and quasi-AF2 transcriptional activity. A related possibility is that the mutation disrupts an interaction normally occurring between K497 and the B2 domain. By corollary, the isoform specificity could arise from a steric hindrance that prevents a similar interaction between K497 and the larger N-terminal domains of A and B1.

From a regulatory standpoint, the effect of K497E clearly illustrates that normal transcriptional activity is not synonymous with maximal transcriptional activity for EcR. Whatever the mechanistic basis for the high basal activity, the effects of K497E further indicate that basal transcriptional activity normally includes a repressive process, which is mutationally subverted. The K497E receptor activity profile contrasts with the one previously described for the A483T mutation associated with larval lethality (Carney & Bender, 2000) and in vitro corepressor binding (Tsai et al., 1999). Given these distinctions between the two mutational effects, it is conceivable that different molecular mechanisms confer a repression of receptor activity, and that these are genetically separable.

**Mutational effects and phenotypic analysis**

The mutations described in this study were selected to investigate specific attributes as a basis for subsequent mutational analysis in *Drosophila*. Receptor functionality is actually a composite of numerous subfunctions including interactions with DNA, receptor partners, ligand, transcriptional cofactors and chaperones (Arbeitman & Hogness, 2000), as well as interdomain interactions within each receptor. Therefore, mutations that selectively disrupt specific EcR functions can be employed to assess their effects upon individual developmental processes. By contrast, in vitro studies have led to the implication that in vivo missense mutations of the EcR LBD (Bender et al., 1997) are hypomorphic, causing partial and nonspecific impairments of dimerization, ligand-binding capability and transcriptional response (Bergman et al., 2004). If so, larval lethality induced by such EcR mutations apparently results from chronically deficient ecysteroid receptor function.

While the validity of the in vitro capabilities reported here for in vivo events will depend upon specific experiments, some consistencies in performance have been noted. For instance, EcRB1 not only mediates the highest level of transcriptional activity in cell cultures, it is also the only isoform that completely restores polytene puffing in the *Drosophila* larval salivary gland of EcR- mutants (Bender et al., 1997). Alternatively or additionally, the unique EcRB1/USP interaction seen in cell culture may reveal a transcriptional response in salivary glands that the other isoforms are unable to perform as efficiently. Similarly, EcRB2 is the only isoform potentiation by JH using 20E as an agonist as well as the most effective isoform for rescuing embryonic lethality in EcR-null mutations through the larval stages, during which both juvenile hormone and ecysteroid titres are periodically elevated (Henrich et al., 2003). Taken together, the ability of B2 activation to be maximized by the presence of JH may be particularly important for larval–larval transitions, because ecysteroid peaks during this developmental period could be too low by themselves to trigger some ecysteroid-inducible responses. JH itself is derived from the insect mevalonate pathway (Belles et al., 2005), which in turn is driven by the intake of dietary nutrients. The ability of JH to potentiate ecysteroid activity suggests that this is a functional interface between the nutritional state of feeding larvae and the hormonal processes that trigger moulting processes. When the A and B1 Mandra EcR isoforms are expressed together in a Mandra cell line, ecysteroid-responsive gene expression is reduced, suggesting a difference in transcriptional capabilities consistent with those observed for the *Drosophila* EcRA and B1 isoforms in this study (Hiruma & Riddiford, 2004).

In summary, the characterization of the ecysteroid receptor complex noted here represents a systematic approach for classifying the effects of EcR and USP mutations on specific subfunctions. Transgenic flies expressing these mutant EcR isoforms can now be tested for their ability to rescue essential functions destroyed by endogenous EcR mutations and also, for their effects upon ecysteroid-dependent transcriptional activity.

**Experimental procedures**

This study employed Chinese hamster ovary (CHO) cells to evaluate the transcriptional activity mediated by *Drosophila melanogaster* EcR and USP using methods described previously (Tsai et al., 1999; Henrich et al., 2003). Unless noted otherwise, 250 ng of each EcR and USP-encoding plasmid was transfected into 2 ml of cell culture medium for these experiments. As described in Henrich et al. (2003), a reporter gene carrying five tandem copies of the hsp70 EcRE (Riddiford & Pelham, 1986) is attached to a constitutive thymidine kinase promoter and a luciferase reporter gene (pEcREli-LUC). Vectors expressing EcR, USP, the reporter gene and a constitutively active CMV promoter fused to a β-galactosidase gene were co-transfected by lipofection (Gene Therapy Systems Inc, San Diego, CA) into the CHO cells following...
the manufacturer's protocols. Transcriptional activity mediated by the hsp27 EcRe was measured by detecting the luminescence produced by luciferase in extracts from transfected cells and cell mass was determined by measuring β-galactosidase activity in these extracts. The EcR isoform vectors (A, B1 and B2) used for this study have been described previously (Fig. 7; Mouillet et al., 2001; NT033778). Additionally, three different VP16-USP vectors were utilized in this study and their construction is described below.

After a 4 h transfection period, the hormone treatment was added by diluting murA (Alexis Corporation, San Diego, CA), JH I (Scitech, Prague, Czech Republic), and/or JH III (Sigma, St Louis, MO) dissolved in dimethylsulphoxide (DMSO) to a final culture medium concentration of 0.1% DMSO. The cells were harvested 24 h after treatment and the contents of the cells extracted for measuring the luciferase and β-galactosidase activities.

Normalization of data points
Luciferase activity was normalized by weighting its activity relative to the constitutive expression of the β-galactosidase gene, whose enzymatic activity is proportional to cellular mass. These values were expressed as relative luciferase units (RLUs). These data were further transformed so that the mean value of a designated control group for each experiment was assigned a value of 1. Using these normalized values, standard deviations were calculated based on at least three independent samples for all data points. Fold-induction is therefore the ratio of mean normalized RLU values of an experimental group relative to the mean normalized RLU value of a designated control group. Individual values were tested to determine whether significant differences existed between specific comparable groups (t test, P < 0.05).

Construction of USP vectors
USP constructs were made by subcloning three different D. melanogaster USP inserts into the pVP16 vector (Clontech, Mountain View, CA). The resulting vector encodes a fusion protein consisting of the viral protein 16 (VP16) activation domain attached to various carboxy-terminal fragments of the USP open reading frame. The USP portion of each fusion gene was isolated by PCR from a plasmid, pZ7-1 (Henrich et al., 1990; AY069393). Each forward primer was tagged with an EcoRI restriction site on the 5’ end and the reverse primer (dUSPR) was tagged with a HindIII site on its 5’ end. The VP16-USPI vector includes the last six amino acids of the N-terminal domain and the remaining carboxy-terminal portion of the DmUSP open reading frame (amino acids 98–507; Henrich et al., 1990). This portion also encodes the two cysteine–cysteine zinc fingers of the DBD, the hinge region, and the LBD. The fragment was isolated by PCR using a forward primer, 5′-TTTGAATTCTAGGGAGAAGGACCTTGTCG-3′ and the reverse primer (dUSPR): 5′-TTTAAAGTCTAGTCGACCCTTACCC-3′ (underlining designate EcoRI and HindIII restriction sites, respectively). The slightly shorter VP16-USPII vector does not include the N-terminal amino acids, starts at the USP DBD and codes for amino acids 104–507. The USPII insert was isolated using the forward primer, 5′-TTTGAATTCTGTCCTATTCCGGGAGATCGG-3′ with the aforementioned dUSPR reverse primer. A third vector, VP16-USPIII, codes for amino acids 170–507 and resembles the VP16-USPIII except that it lacks the USP DBD. It was generated using a forward primer with the sequence 5′-TTTGAATTCTAGCGGCAAGCGTCCAGGAG-3′ and the dUSPR primer. After an initial 5 min melting step at 94 °C, PCR was used to amplify the inserts under the following cycling conditions: 94 °C melting for 1 min, 58 °C annealing for 1 min, 68 °C extension for 2 min, over 30 cycles.

The PCR products and the pVP16 vector were double digested with the EcoRI and the HindIII restriction enzymes, the names were electrophoresed in a 1% agarose gel and the appropriate bands were excised from the gel (Qiagen, Valencia, CA). The gel-extracted PCR products were ligated into the pVP16 vector using T4 ligase (New England Biolabs, Beverly, MA) and the ligation mixture was added to 45 µl Ultracompetent XL10-Gold E. coli (Stratagene, La Jolla, CA). The cells were then streaked on to Luria-Bertani (LB) agarose plates and transformants selected with ampicillin and grown with selection in liquid culture. Plasmid DNA was extracted and the vectors verified by restriction analysis and DNA sequencing.

Site-directed mutagenesis
Site-directed mutations were produced in each of the three EcR isoform vectors by changing one or two nucleotides in the codon corresponding to a specific amino acid position using a Quik-Change II site-directed mutagenesis kit (Stratagene). The presence of the mutations and the integrity of the remaining coding region of the protein were verified by sequencing. The mutations K497E, M504R and A483T were made in each of the three EcR isoforms using the following primers and their reverse complements: K497E: 5′-CAG ATC ACG TTA CTA GAG GCC TGC TCG TCG G-3′ M504R: 5′-CTC GTC GGA GGT GAG GAT GCT GGG TAT G-3′ A483T: 5′-G TT TTT CCT AAA GGT CTA CCA ACA ACG TTT ACA AGA ATA CCC CAG G-3′. After subsequent transformation of the mutagenesis reaction mixture in the Ultracompetent XL10-Gold E. coli, the transformants were cultured as described previously and plasmid DNA extracted using a Qiagen midiprep kit.
Electrophoretic mobility shift assays

Extracts transfected with the appropriate EcR and USP vectors were prepared according to the method described in Kitareewan et al. (1996). A double stranded hap27 response element probe was constructed using the forward primer 5'-ACCGACAAAGGGTT- TCATGACACTTGT-3' and the complementary reverse primer. The probe was end-labelled by fill-in reaction with the Klenow fragment and [α-32P]dCTP according to published protocols (Mouillet et al., 2001). After labelling, the probe was purified by centrifugation, applied to an affinity column and purified following the manufacturer's instructions (Mini Quick Spin Columns, Roche Applied Sciences, Indianapolis, IN).

The binding reactions were prepared as reported previously (Mouillet et al., 2001). Protein extracts were added to the binding reactions after normalization by β-galactosidase reporter gene activity. A Bradford assay measured the total protein content for these extracts between 100 and 150 μg (Bradford, 1976). An 11 μl volume of cell extract with the lowest-β-galactosidase reporter gene activity was added to the binding reaction. All other extracts were added proportionally to the reaction based on β-galactosidase activity relative to the one with the lowest activity. The reaction mixture. Finally, approximately 30 000 cpm of the radio-labelled hap27 element was added to the binding reaction and incubated with the extract for 20 min at room temperature. A 5% native polyacrylamide gel was used to separate the complexes.

Western blots

Cellular extracts with equal amounts of β-galactosidase activity were loaded onto 1 lane of a 15% polyacrylamide gel and subjected to electrophoresis (Biometra, Gottingen, Germany) at 15 mA. The gel was then electroblotted (MiniVE Blotter Module, Amersham Pharmacia Biotech, Piscataway, NJ) onto a nitrocellulose membrane (NC 45, 0.45 μm, Serva, Heidelberg, Germany) at 300 mA and 20 V. The membrane was then soaked in blocking buffer (3% (w/v) milk powder, 1% (v/v) BSA, 20 mM Tris/HCl, 137 mM NaCl, 0.1% (v/v) Tween 20, pH 7.6). ECR was probed with the monoclonal IgG mouse antibody DDA2.7 diluted 1 : 1000 in blocking buffer; this antibody recognizes the α-domain shared by all three DmEcR isoforms (Koelle et al., 1991). A peroxidase-conjugated secondary antibody (anti-mouse IgG, Sigma-Aldrich, Taufkirchen, Germany) was diluted 1 : 1000 (20 μg/ml Tris/HCl, 137 mM NaCl, 0.1% (v/v) Tween 20, pH 7.6) to detect specific signals. The membrane was exposed to X-ray film (Hyperfilm, Amersham) and the image developed.

For USP Western blots, similar protocols were followed and a monoclonal mouse IgG antibody that detects the N-terminal VP16 domain was utilized (Santa Cruz Biotechnology, Inc; Santa Cruz, CA) after a 1 : 200 dilution in blocking buffer.

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References


Drosophila ecdysteroid receptor isoform activity


Properties of ecdysteroid receptors from diverse insect species in a heterologous cell culture system – a basis for screening novel insecticidal candidates

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Insect development is driven by the action of ecdysteroids on morphogenetic processes. The classic ecdysteroid receptor is a protein heterodimer composed of two nuclear receptors, the ecdysone receptor (EcR) and Ultraspiracle (USP), the insect ortholog of retinoid X receptor. The functional properties of EcR and USP vary among insect species, and provide a basis for identifying novel and species-specific insecticidal candidates that disrupt this receptor’s normal activity. A heterologous mammalian cell culture assay was used to assess the transcriptional activity of the heterodimeric ecdysteroid receptor from species representing two major insect orders: the fruit fly, Drosophila melanogaster (Diptera), and the Colorado potato beetle, Leptinotarsa decemlineata (Coleoptera). Several nonsteroidal agonists evoked a strong response with the L. decemlineata heterodimer that was consistent with biochemical and in vivo evidence, whereas the D. melanogaster receptor’s response was comparatively modest. Conversely, the phytoecdysteroid muristerone A was more potent with the D. melanogaster receptor than with the L. decemlineata receptor. The additional presence of juvenile hormone III potentiated the inductive activity of muristerone A in the receptors from both species, but juvenile hormone III was unable to potentiate the inductive activity of the diacylhydrazine methoxyfenozide (RH2485) in the receptor of either species. The effects of USP on ecdysteroid-regulated transcriptional activity also varied between the two species. When it was tested with D. melanogaster EcR isoforms, basal activity was lower and ligand-dependent activity was higher with L. decemlineata USP than with D. melanogaster USP. Generally, the species-based differences validate the use of the cell culture assay screen for novel agonists and potentiators as species-targeted insecticidal candidates.

Insect development is largely driven by the action of ecdysteroids and its modulation by juvenoids. For all insects and many other arthropods, ecdysteroid action is mediated by the heterodimerization of two nuclear receptors, the ecdysone receptor (EcR) and its partner, ultraspiracle (USP), the insect ortholog of the
vertebrate retinoid X receptor (RXR). Many essential characteristics of ecdysteroid action are well described in Drosophila melanogaster [1,2], and have since been confirmed and further investigated in other insect species [3,4]. Generally, one or more isoforms of EcR and USP in a given species trigger an orchestrated and multilayered hierarchy of transcriptional changes in target cells that ultimately mediate the morphogenetic changes associated with molting, metamorphosis, and reproductive physiology [5].

Although the basic molting mechanism is highly conserved, it is apparent that the characteristics of the EcR–USP heterodimer vary among species. This is readily seen in the species-specific effects of the diacylhydrazines, nonsteroidal agonists that show order-specific differences in receptor affinity and in vivo toxicity [6]. Biochemical and cell culture studies of EcR and USP have also revealed species-specific functional characteristics that presumably underlie differences in ecdysteroid-driven developmental events [7–11]. Steroids and nonsteroidal agonists bind exclusively to the EcR ligand-binding domain (LBD), although the presence of USP increases ligand-binding affinity [12–15].

The diversity of ligand-responsive characteristics seen among ecdysteroid receptors from various insect species suggests a basis for identifying and screening for compounds that perturb normal receptor function [12,13,15,16]. Ecdysteroid receptor-mediated transcriptional activity has been measured in mammalian cells, which have no endogenous response to insect ecdysteroids, by transfecting them with the genes encoding EcR and USP, along with an ecdysteroid-inducible reporter [17–19]. An analysis of species-specific versions of EcR and USP and site-directed mutations in this heterologous cell system has generally established that the effects of ecdysteroids and other diacylhydrazine-based agonists can be measured by reporter gene activity [8,19,20]. Furthermore, the Drosophila EcR–USP heterodimer is potentiated by the presence of juvenile hormone (JH) in mammalian cells; that is, JH dramatically reduces the ecdysteroid concentration necessary to attain maximal induction from an ecdysteroid-inducible reporter gene [9,21]. The mechanism for potentiation has not been elucidated, although it reveals a modulatory action that may be useful for identifying novel insecticides acting as disruptors of normal ecdysteroid action. This possibility increases the importance of evaluating the heterologous cell culture assay as a valid tool for the assessment of ecdysteroid receptor capabilities from specific species.

Hundreds of phytocompounds that act as nonsteroidal and steroidal agonists of the insect ecdysteroid receptor have been identified [22,23], and a large number of JH analogs and mimics have also been isolated from plants [24]. If the cell culture assay has utility as a method for detecting novel inducers and/or JH potentiators of EcR–USP, then receptors from an insect species such as the Colorado potato beetle, Leptinotarsa decemlineata, are expected to evoke a profile of response that varies considerably from those previously reported for D. melanogaster. Furthermore, these characteristics are expected to be consistent with in vivo measurements of ecdysteroid activity in L. decemlineata [16,20,25–27]. L. decemlineata belongs to a relatively primitive insect order, the Coleoptera. Owing to its worldwide importance as a pest insect and its well-established ability to develop resistance to insecticides, the species has been well studied for its susceptibility to a variety of agonists [28,29].

The L. decemlineata ecdysteroid receptor shows the general structural features shared by all EcR and USP sequences characterized among insects and other arthropods [5,30,31]. Two EcR isoforms (A and B) have been identified so far in the L. decemlineata genome. L. decemlineata USP (LdUSP) carries an LBD that is remarkably similar to the vertebrate RXR, and lacks many of the features found in D. melanogaster USP (DmUSP), such as glycine-rich regions and a B-loop between helices 2 and 3 [30–32]. This divergence between the Coleopteran USP LBD (often referred to as RXR in this order) with those of the Lepidoptera and Diptera has been noted, suggesting a concomitant functional divergence [32]. Whereas the cell culture assay has been employed to survey the responses of ecdysteroid receptors from several species, this work focuses on a direct and thorough comparison of several attributes associated with well-described ecdysteroid receptors from two insect species for which relevant biochemical and in vivo information exists. The comparative profiles demonstrate an approach for developing a screening system to identify and characterize candidate insecticidal compounds showing both inductive and potentiative activity.

**Results**

The DNA-binding domains (DBDs) of Leptinotarsa and Drosophila EcR and USP are identical at every amino acid position that is conserved among all EcR and USP DBD sequences, respectively, and share an overall identity of over 90% in both cases [31]. Therefore, it was expected that the canonical hsp27 ecdysone response element (EcRE) would allow direct comparisons of agonist inducibility when tested with EcR–USP from each of the two species. Sequence conservation is
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not as extensively shared in the LBD, where the identity between *D. melanogaster* EcR (DmEcR) and *L. decemlineata* EcR (LdEcR) is about 67% [21] (Fig. S1). USP LBD conservation is < 39% between the two species [21] (Fig. S2).

The N-terminal (A/B) domains of EcR are also divergent in the two insect species [31] (Fig. 1), although all of the isoforms from both species share almost complete identity over a stretch of 35–37 amino acids that lie just to the N-terminal side of the DBD (Fig. 1C). The EcRA isoforms from the two species share a few similar motifs in the middle region of the A/B domain (Fig. 1A), whereas LdEcRB shares some identity with DmEcRB1 only in the most N-terminal region (Fig. 1B).

Effects of selected agonists on EcR–USP transcriptional activity in the two species

In an initial series of experiments, the basal and ligand-induced properties of the three *D. melanogaster* isoforms (DmEcRA, DmEcRB1, and DmEcRB2) with the VP16-USP heterodimer used in earlier studies were compared with those of the *L. decemlineata* isoforms (EcRA and EcRB) paired with the equivalent VP16-LdUSP construct [18]. Activity was determined by measuring reporter gene (luciferase) activity mediated by the hsp27 EcR after normalization for cell mass using β-galactosidase activity registered via a constitutive promoter.

In order to compare the efficacy of agonists, maximally inducing doses of several ecdysteroids and the most inductive nonsteroidal agonist, methoxyfenozide (RH2485), based on preliminary experiments, were tested.

The pattern of response was similar for each of the three *D. melanogaster* isoforms (Fig. 2A). In all cases, muristerone A (MurA) (2.5 μM) evoked the strongest fold induction, and the greatest absolute level of transcriptional activity. RH2485 also evoked a response from all three DmEcR isoforms, with lesser responses from the natural molting hormone, 20-hydroxyecdysone (20E), and makisterone A (MakA), the latter being the most abundant ecdysteroid in late third instar whole body titers of *D. melanogaster* [33]. The relatively modest response to natural ecdysteroids such as 20E has been noted in previous cell culture studies. Also, differences in the quantitative levels of transcription were previously reported, with DmEcRB1 showing the highest levels of basal and induced activity, and EcRA displaying the lowest levels of activity [9].
The response profile observed for each of the two LdEcR–LdUSP heterodimers varied considerably from those seen with the DmEcR–DmUSP heterodimers (Fig. 2B). RH2485 evoked a much higher fold induction (up to 25-fold) from the L. decemlineata heterodimers. By contrast, the response of LdEcR–LdUSP to MurA and 20E was relatively modest as compared with that of DmEcR–DmUSP. Minimal induction was seen with MakA with receptors from either species. Differences in normalized induction in this experiment and others are not attributable to differences in cell growth caused by the effects of the individual ligands. The β-galactosidase reporter gene measurements used to normalize transcriptional activity (by providing an estimate of cell mass) varied by < 20% for all the ligand regimens applied. Also, the absolute β-galactosidase values varied by < 20% between experiments; that is, cell growth rates were relatively constant (data not shown).

Immunoblots were also performed with cell extracts expressing the EcR isoforms employed in this study, to determine whether transcriptional activity levels are related to expression levels. Although the signal evoked from individual isoforms varied to some degree, as noted in previous work [9], the strength of signal did not correlate with differences in transcriptional activity. In summary, each of the isoforms within a species generated a similar responsiveness to maximal dosages of individual agonists. Whereas the EcR N-terminal domain influences the quantitative level of transcription for a given isoform, it had no effect on relative ligand responsiveness. Importantly, the relative induction by individual agonists was species-specific for all of the tested ligands, and the responsiveness to RH2485 was much higher in Leptinotarsa than in Drosophila, whereas DmEcR–DmUSP was more responsive to MurA than to any other agonist.
Effects of selected ecdysteroids and nonsteroidal ecdysteroid agonists on transcriptional activity in the two species

The potency of natural and nonsteroidal agonists was further evaluated by comparing the dose response of DmEcRB2–DmUSP with those of the two LdEcR–LdUSP complexes. Three natural ecdysteroids, MurA, ponasterone A (PonA), and MakA, were tested in receptors from both species (Fig. 3A–C). MurA was significantly more potent with receptors of D. melanogaster than with those of L. decemlineata. Whereas DmEcR–DmUSP showed a maximal response in the range of 1–10 µM MurA, LdEcR–LcUSP required about 50 µM MurA to show a maximal response. Nevertheless, the maximal induction evoked by MurA at 50 µM was over 30-fold with L. decemlineata. Receptors from both species were maximally induced by 1 µM PonA, and neither species responded strongly to MakA, even at 50 µM.

Four nonsteroidal ecdysteroid agonists, halofenozide (RH0345), methoxyfenozide (RH2485), RH5849, and tebufenozide (RH5992), were also tested over a range of dosages with receptors from both species (Fig. 4A–C). The maximal fold induction evoked by nonsteroidal compounds was considerably higher among the LdEcR dimers than it was for the compared DmEcRB2–DmUSP heterodimer. Except for RH5849, each of the RH compounds evoked a maximal induction at 10 µM with the LdEcR–LdUSP dimers that was > 10-fold. The order of fold induction obtained for the pooled results (i.e. LdEcRα and LdEcRβ) was RH2485 > RH5992 > RH0345 > RH5849; one-way ANOVA, P ≤ 0.01). By contrast, the Drosophila receptor showed a more modest induction with all of the nonsteroidal ecdysteroid agonists, never exceeding 10-fold (Fig. 4A).

An electrophoretic mobility shift assay (EMSA) was also performed using cell culture extracts expressing DmEcRB1–DmUSP and DmEcRB2–DmUSP or the LdEcR–LdUSP combinations to verify their interaction with the hsp27 EcRE. The observed shifts associated with the hsp27 EcRE revealed that DmEcRB1–VP16–DmUSP showed an increased shift intensity in the presence of agonist, and that that of DmEcRB2–VP16–DmUSP was modestly increased by the presence of agonist (Fig. 5) [9]. Under identical experimental conditions, the two LdEcR–LdUSP complexes showed little change in shift intensity when an agonist was present. The variability among the individual EcR–USP pairings could be attributed to the selected conditions, which had been optimized for testing DmEcR–DmUSP.
Effect of JH on EcR–USP transcriptional activity in the two species

When Chinese hamster ovary (CHO) cells expressing DmEcR–DmUSP are challenged with JHIII alone, no effect on transcriptional activity is observed [9]. However, the simultaneous presence of JHIII in a cell culture medium that already contains ecdysteroids reduces the concentration of ecdysteroids necessary for maximal transcriptional activity by about 10-fold. In other words, JHIII potentiates the responsiveness of EcR–USP to ecdysteroids [9,14,21]. Using the same paradigm employed for measuring potentiation in the Drosophila system, a submaximal dosage of MurA together with JHIII was simultaneously tested with cells expressing LdEcR–LdUSP. Under these conditions, partial and significant potentiation by JHIII was observed in the L. decemlineata receptor (Fig. 6A; \( P < 0.01 \), t-test).

The potentiation testing paradigm was then modified by testing the nonsteroidal agonist RH2485 instead of MurA. No potentiation by JHIII was seen in either D. melanogaster or L. decemlineata, using RH2485 as an agonist (Fig. 6B). This result indicates that potentiation by JHIII is not a general cellular effect, but depends upon the specific agonist–EcR interaction.

Effects of L. decemlineata and D. melanogaster USP constructs on ecdysteroid-inducible transcriptional activity

As noted, when VP16-DmUSP/ΔDBD is tested with the three D. melanogaster EcR isoforms, EcRA and EcRB2 heterodimers form a relatively inactive dimer [9] (Fig. 7A). However, DmUSP/ΔDBD retains nearly normal activity when paired with EcR-B1, indicating that the nature of the EcR–USP interaction is isoform-specific [9,34] (Fig. 7A). The analogous VP16-LdUSP/ΔDBD was tested with LdEcRA and LdEcRB. In both cases, the expression of VP16-LdUSP/ΔDBD, as verified by immunoblots (data not shown), resulted in a heterodimer with severely reduced transcriptional activity (Fig. 7B).

In order to compare the capabilities of DmUSP and LdUSP further, cross-species heterodimers were tested for transcriptional activity (Fig. 7C). At least four functional differences were observed: (a) the DmEcRB1 and DmEcRB2 isoforms display a higher level of ligand-dependent (induced) transcriptional activity with VP16-DmUSP than with the equivalent VP16-DmUSP; (b) the same EcRB1 and EcRB2 isoforms display a lower level of ligand-independent (basal) transcriptional activity with VP16-DmUSP than with VP16-DmUSP; (c) VP16-LdUSP/ΔDBD forms a
relatively inactive dimer with DmEcRB1, unlike VP16-DmUSP/DBD; and (d) VP16-DmUSP consistently evokes a lower quantitative level of transcriptional activity, with both its own EcR isoforms, and with the two L. decemlineata EcR isoforms.

Discussion

A controlled assessment and comparison of the Leptinotarsa and Drosophila EcR–USP heterodimers in this study reveals a variety of distinctions between them in terms of quantitative level of transcriptional activity, ligand responsiveness, and capability for potentiation by JHIII. These findings are generally consistent with expectations from other in vivo and biochemical work with the two species’ receptors, and indicate that the CHO cell culture assay system can be validly employed to characterize individual insect EcR–USP heterodimers for their responsiveness to agonists and potentiators.

Utility of the cell culture as a screening assay for novel agonists

The differences in characteristics of the ecdysteroid receptors from the two species studied here, and the general consistency with previously published results [25–27], suggest a basis for screening plant extracts and candidate insecticides affecting EcR–USP-mediated induction or potentiation in either or both species.

The fold induction evoked by the tested RH compounds on transcriptional activity of LdEcR approximately corresponded with their ligand affinity

Fig. 5. EMSA using CHO cell extracts following transfection and incubation in the absence and presence of MurA, RH5849, and RH5992, using the hsp27 EcRE as a labeled probe. Asterisk designates shift band. All extracts were equilibrated by β-galactosidase activity prior to loading. Densitometry readings corresponding to designated shift bands are indicated below the image and adjusted relative to the signal generated by LdEcRB (equals 1.0).

Fig. 6. Effects of JHIII on transcriptional activity induced by (A) MurA and (B) RH2485 of DmEcRB2–VP16-DmUSP and analogous LdEcR–VP16-LdUSP complexes. Parentheses in (A) indicate a potentiation effect, and arrows in (B) indicate an absence of potentiation when RH2485 is the agonist. All transcriptional activity levels are adjusted to DmEcRB2–VP16-DmUSP in the absence of ligand (assigned a value of 1.0). No effect upon transcriptional activity was observed when JHIII was tested with RH2485.
Nevertheless, although RH0345 is not the most efficacious of the RH compounds in the cell culture assay, it is actually the most toxic of these compounds in *L. decemlineata*, owing to its relative persistence in target tissues [35]. This observation highlights the reality that a robust fold induction in the assay is not necessarily the best indication of toxicity. The study alternatively suggests that ligand potency may be the best primary criterion for isolating insecticidal candidates within a given species, even if fold induction is modest. The potency of RH0345 with the LdEcR isoforms was similar to those of RH2485 and RH5992, and all three of these RH compounds showed greater potency and efficacy than RH5849, which is weakly toxic in *L. decemlineata*. Finally, all of the RH compounds yielded a higher fold induction with the *L. decemlineata* receptor than with the receptor of *D. melanogaster*, which is relatively unresponsive to the effects of RH compounds [36], thus suggesting that fold induction can serve as a basis for predicting differences in the toxicity of a compound between species. The weak inductive effects of the natural ecdysteroids (MurA, PonA, MakA, and 20E) further show a lack of correspondence between fold induction and ligand affinity, as the affinities of the natural ecdysteroids for EcR are higher than the affinities of the diacylhydrazines [12].

The differences in fold induction observed between the natural steroids and the nonsteroidal agonists is predictable, as these agonist classes involve different amino acid interactions in the ligand-binding pocket. Nevertheless, both DmEcR and LdEcR carry the same residue at each of the putative binding sites ascribed to the RH compounds [8], consistent with the suggestion that other features of the ligand-binding pocket account for species differences in responsiveness to RH compounds [13].

**EcR and USP**

Transcriptional activity levels varied widely among the three *Drosophila* isoforms and two *Leptinotarsa* isoforms. Such quantitative differences may prove important for *in vivo* functions. In *Manduca*, the presence of a B-isoform increases transcriptional activity normally mediated by the A-isoform alone, heightening the possible relevance of these differences for *in vivo* regulation [37].

There is growing evidence that changes in net activity induced by ecdysteroids and nonsteroidal agonists in the cell culture system involve not only allosteric changes in the receptor itself, but also factors such as the effect of DNA and ligand on receptor stability and the regulation of nuclear receptor transport in the cell [38–41].
Therefore, differences between basal and induced transcriptional activity must be viewed as a net effect resulting not only from changes in the level of receptor molecule activity, but also from changes in stability and intracellular localization. Possible differences in these parameters among EcR–USP dimers from different species have not been explored extensively, although the relationship between protein stability and ligand interactions has been noted for *Drosophila* E75 and its interaction with heme [42]. Degradation of DmEcR is seen at specific developmental periods [43].

The studies also demonstrated that DmUSP and LdUSP are not interchangeable in terms of transcriptional activity, although USP does not affect ligand affinity when tested in cross-species dimers [12]. Species-specific differences in USP structure have already been implicated in the regulation of developmental events associated with larval growth and subsequent metamorphosis [44]. The effects observed in cross-species EcR–USP dimers further suggest that USP plays a role in determining the quantitative level of transcriptional activity.

**Implications for a mechanism of potentiation**

As noted earlier, the effects of potentiation suggest a low-affinity interaction between EcR–USP and JHIII. A similar effect for DmEcR–DmUSP has been observed for methyl farnesoate and other substrates within the mevalonate pathway [14]. The mechanism for this effect upon EcR–USP activity remains unknown, although the ability of JHIII to potentiate ecdysteroid inducibility has also been observed with polychlorinated biphenyls, whose activity is associated with members of the basic helix–loop–helix Per-Arnt-Sim (bHLH-PAS) transcription factor family [45]. Members of this family, in turn, include the *Drosophila* methoprene-tolerant (MET) gene product [46], and MET is known to bind to JHIII [47]. Mutations of the MET gene in *Drosophila* block the normally lethal effects of methoprene application [46]. Mammalian bHLH-PAS transcription factors bind to nuclear receptors, leaving the possibility for a MET–EcR–USP interaction. A physical interaction between MET and both EcR and USP has been reported [48], although its relevance for the functional effects of JHIII remains to be explored. The homolog of MET in *Tribolium castaneum* mediates JH action, further raising the possibility of a similar role in modulating ecdysteroid receptor action [49]. Nonsteroidal ecdysteroid agonists are known to confer a markedly different shape upon the ligand-binding pocket of EcR than natural ecdysteroids [8] that could prevent interactions with regulatory cofactors such as MET via the LBD.

It is important to recognize that USP itself binds to JH and methyl farnesoate under certain experimental conditions [50]. Alternatively, the effect of RH2485 on EcR is to alter the shape of its ligand-binding pocket, thus blocking potentiation mediated by USP binding to JHIII. Finally, although MET explains some JH-mediated activities in *T. castaneum*, it does not account for all of them [49], leaving open the possibility that JH acts via multiple modes of action. The inability to see potentiation with nonsteroidal compounds at least demonstrates that the effects of JHIII cannot be attributed to a generalized cellular action upon the transcriptional complex that includes EcR and USP. Rather, the occurrence of potentiation depends upon the specific agonist.

**Summary**

The comparative study of the *Leptinotarsa* and *Drosophila* EcR–USP complexes further establishes the utility of the heterologous CHO cell culture system for assessing the effects of agonists/antagonists and other modulators on EcR–USP-mediated transcriptional activity. The insect ecdysteroid receptor is a commercially proven target for insecticidal action, and the assay provides a conceptual basis for high-throughput screening and identifying compounds that perturb receptor function, not only in terms of classic ecdysteroid agonist functions, but also for those compounds that are capable of mimicking or evoking the potentiation effect induced by JHIII in this assay.

**Experimental procedures**

**Cell culture, EMSA, and western immunoblotting**

All aspects of cell culture methodology, ligand application, transfection, reporter gene measurement, western immunoblotting and EMSAs have been previously reported [9,21]. Briefly, CHO cells were grown to confluence and transfected (250 ng each) with: (a) a plasmid vector containing the luciferase gene controlled by a constitutively active promoter, (c) one of the EcR-encoding vectors described below; and (d) one of the USP-encoding vectors described below. After transfection for 6 h, cells were incubated with or without agonists and/or JHIII for 24 h, cells were harvested, and extracts were processed for the studies. The reagents tested included: MurA (Alexis Biochemical, San Diego, CA, USA), PonA, MakA (AG Scientific, San Diego, CA, USA), and JHIII (Sigma Chemical, St Louis, MO, USA). The diacylhydrazine-based agonists that were tested included RH0345, RH2485, RH5849, and RH5992, all...
> 95% pure, and kindly provided by Rohn and Haas Co. (Spring House, PA, USA). Western immunoblots of LdEcR and DmEcR were performed with the 9B9 and DDA 2.7 monoclonal antibodies, respectively, obtained from the Developmental Studies Hybridoma Bank at the University of Iowa.

Band densities were measured, using BioRad (Hercules, CA, USA) QUANTITY ONE software from the EMSA and western immunoblot images. The pixel intensity of the band was determined for the defined band area and adjusted relative to one of the signals, as designated, to calculate the relative band density.

Vector description and construction

All DmEcR and DmUSP expression vectors and the luciferase (and β-galactosidase) reporter gene vectors have been described previously [9,21]. The expression vectors encoding the natural isoforms of DmEcR are denoted DmEcRA, DmEcRB1, and DmEcRB2.

The following protocols were used to construct the LdEcR cell culture vectors encoding its two natural isoforms (LdEcRA and LdEcRB). The LdEcRA ORF was isolated by PCR from pBluescriptKS + LdEcRA [31], using the forward primer 5'-TTTT GAG GCG GTT CAA GAA-3' and the reverse primer 5'-TTTT TCTAGA CTA TGT CTT CAT GTC GAC GTC-3'. The underlined portions of the primers represent the inserted BamHI and XbaI restriction sites, respectively. The vector pcDNA3.1+ + and the LdEcRA amplicon were digested with the restriction endonucleases BamHI and XbaI. The digestion products were purified from an agarose gel excision, and then ligated to create the vector pcDNA3.1+-LdEcRA. The LdEcRB fragment was removed from pBluescriptKS + LdEcRB [31], and the vector pcDNA3.1+- (Invitrogen, Carlsbad, CA, USA) was linearized by restriction digestion with BamHI and XhoI. Both restriction products were purified by excision from an agarose gel and then ligated to produce the vector pcDNA3.1+-LdEcRB.

The vectors encoding DmUSP have also been described previously [9]. For these vectors, the N-terminal (A/B) domain of DmUSP was replaced with the VP16 activation domain, as the DmUSP A/B domain displays minimal transcriptional activity in CHO cells [18]. Two constructs were produced: VP16-DmUSP includes the USP DBD, whereas VP16-DmUSP/ΔDBD has had the DBD deleted.

The analogous VP16-LdUSP and VP16-LdUSP/ΔDBD vectors were constructed for this study as follows. The LdUSP and LdUSP/ΔDBD fragments were isolated by PCR from pBluescriptKS + LdUSP [31], using the forward primer 5'-TTTT GAATTC TGC TCG ATTTGC GGG GAC GAC-3' and the reverse primer 5'-TTTT GAATTC AAG CTG GGT CCC GAG GGG GAC GAC-3' (which lies just to the 3'-side of the DBD-encoding sequence). Each primer was paired with the reverse primer 5'-TTTT AAGCTT CTA AGT ATC CGA CTG GTT TTC-3', which is the complement of the 3'-end of the LdUSP LBD. The ecdysteroid receptor DBD fragment was inserted into the vector pVP16-LdUSP (Clontech, Mountain View, CA, USA). Both amplicons and the pVP16 vector were digested with EcoRI and HindIII restriction endonucleases. Ligation of the products into the linearized pVP16 vector resulted in the pVP16-LdUSP and pVP16-LdUSP/ΔDBD constructs. All constructs were subsequently verified by DNA sequencing.

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Supporting information

The following supplementary material is available:

Fig. S1. Amino acid alignment of linker region and ligand-binding domain (LBD) of ecdysone receptors.

Fig. S2. Amino acid alignment of linker region and ligand-binding domain (LBD) of ultraspiracle/RXR receptors.

This supplementary material can be found in the online version of this article.

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Chapter 14
The Multidimensional Partnership of EcR and USP

Vincent C. Henrich, Joshua Beatty, Heike Ruff, Jenna Callender, Marco Grebe, and Margarethe Spindler-Barth

Abstract  Cellular signaling of the insect ecdysteroids is mediated by two nuclear receptors, the ecdysone receptor (EcR) and Ultraspiracle (USP). Considerable evidence exists that each participates in a dimerization partnership that is subject to a variety of regulatory interactions and pathways in the cell. Heterologous cell culture has been used to reconstitute and study the transcriptional activity mediated by the EcR/USP heterodimer. These studies have utilized site-directed mutagenesis and other structural modifications to dissect functional features of each dimer pair. From this work it is apparent that EcR and USP transcriptional activity depends upon the isoforms tested, the type of agonist, and the presence of juvenile hormone. USP influences the ligand affinity of EcR as revealed by the effects of mutated USP, and mutations of EcR also reveal a repressive function that apparently involves an interdomain interaction and isoform-specific. EcR retains some activity and ligand affinity in the absence of USP which may prove relevant for in vivo regulation. Cross-species comparisons and pairings of EcR and USP have shown that these dimers vary considerably in their ligand responsiveness, level of transcriptional activity, and isoform-specific effects, which in turn, is likely to be useful for developing novel insecticides that are targeted at specific receptors. The results of the cell culture work have formed a foundation for testing hypotheses about isoform-specific regulation by EcR and USP, examining the role of juvenile hormone (JH) and candidate cofactors such as MET, which itself mediates JH activity, and testing the phenotypic effects of selected mutational modifications of EcR and USP in vivo.

Keywords  Ecdysone • cell culture • juvenile hormone • nuclear receptor
14.1 Introduction

Ongoing reports of ecdysone receptor (EcR) and Ultraspiracle (USP) sequences from arthropods and insect species might inadvertently create the impression that the relationship between these two nuclear receptors is static, with little to understand about it that could influence developmental processes (Henrich, 2005, and references therein). At least two features of the relationship between EcR and USP may not have been noted explicitly from studies across various experimental systems. First, a growing body of evidence indicates that the functional relationship between isoforms of EcR and USP are not equivalent, and that the EcR/USP relationship depends on contextual factors. Moreover, the observed characteristics tend to be conserved in a variety of heterologous cell types and in vivo, indicating that they involve intrinsic features of EcR and USP. Secondly, while EcR and USP share easily discernible similarities across a wide range of species, there are readily observable structural and functional differences among them. This is expected: the evolutionary distance between the Chironomids and Drosophilids within the Diptera order is 200 million years, which approximates the evolutionary distance between humans and vertebrate poikotherms. In other words, the diversity between insect orders is sufficiently large to allow for considerable diversification of EcR and USP function, with potentially important consequences for biological functions (Laudet and Bonneton, 2005).

14.2 Rationale for Using Heterologous Cell Cultures to Test EcR and USP Function

Mammalian cells, such as the well-described Chinese hamster ovary (CHO) cells, have been utilized for functional studies of the ecdysteroid receptor. The experimental paradigm is straightforward: Genes encoding EcR and USP are introduced simultaneously by cotransfection and expressed under the control of a promoter that is constitutively active in the cells (Christopherson et al., 1992). The N-terminal (trans-activation) domains of EcR and/or USP, which interacts with other cofactors, are often replaced with the VP16 activation domain (AD) that is active in mammalian cells. The domain replacement removes the uncertainty associated with the sequential and functional variability in the N-terminal region of EcR and USP isoforms. This domain is also variable in sequence between insect species and consequently, difficult to compare among them (Mouillet et al., 2001; Palli et al., 2003).

The cotransfection also includes a transcriptional reporter carrying single or tandem copies of an ecdysone response element (EcRE), such as the canonical inverted palindrome, hsp27 EcRE (Riddihough and Pelham, 1987; Dobens et al., 1991), attached to a weak constitutive mammalian promoter (such as thymidine kinase), and a reporter gene encoding luciferase or green fluorescent protein. After transfection, the
cells are challenged with test ligands dissolved in the cell culture medium. Later, the cells are harvested, processed, and the reporter protein activity measured, thus providing an indication of transcriptional activity mediated by the EcR/USP complex.

Despite its widespread use over the years, an obvious question is: Why use a heterologous, mammalian cell culture system to study the insect ecdysteroid receptor? The answer is that mammalian cells possess no endogenous response to ecdysteroids and therefore, any response to ecdysteroids seen in these cells can be ascribed to the expression of EcR and USP. For the purposes of measuring ecdysteroid-inducible transcriptional activity via the hsp27 EcRE, for instance, both receptors are expressed in the system. Conveniently, CHO cells do not express a detectable level of the vertebrate USP homologue, RXR (Nieva et al., in press) that can form a transcriptionally active dimer with EcR in the presence of some ecdysteroids (Christopherson et al., 1992; Henrich et al., 2003). The vertebrate EcR homologue, FXR, which is highly responsive to juvenile hormone as a dimer with RXR, is also undetectable in CHO cells (Kitareewan et al., 1996). Control experiments have shown that the expression of USP alone in the cells evokes no transcriptional activity via the hsp27 EcRE. By contrast, when EcR alone is expressed in CHO cells, a basal and induced transcriptional response via the hsp27 EcRE is detectable in cell culture, albeit at a level that is comparatively low (this will be discussed later see Costantino et al., 2008).

The absence of an endogenous ecdysteroid response in mammalian cells also provides the opportunity to introduce and test structurally modified forms of EcR and USP, so that the relationship between structure and individual receptor subfunctions: dimerization, DNA affinity and binding, ligand affinity and binding, and cofactor interactions, can be dissected. By contrast, insect cells usually possess an endogenous response to ecdysteroids, and typically express some combination of EcR, USP, and unidentified cofactors, so that interpretation can be confounded by endogenous basal receptor activity and the presence of undefined insect comodulators. When comparing species specific receptor attributes, the introduction of nuclear receptors from one insect species (for instance, Leptinotarsa decemlineata) into the cell line of a different insect species (such as a Drosophila S2 cell line) could be more difficult than simply using CHO cells, which require a heterologous reconstitution of the ecdysteroid-inducible transcriptional system.

While the logic of assembling a transcriptional system in a heterologous cell type may address the conceptual concern, a second question arises: Is there any evidence that the system is valid for species-specific EcR/USP comparisons, structure-function study, and understanding biologically relevant mechanisms? For the case of species-specific attributes, differences in agonist responsiveness for several insect receptor complexes have been reported that conform to expectations based on in vivo work (Graham et al., 2007a, b). For structure-function analysis, the cell culture system has proven itself by verifying ligand-binding residues for 20-hydroxyecdysone (20E) and BY106830 in the ligand-binding domain (LBD) of Heliothis zea EcR based on computational models. Site-directed mutations of the predicted binding sites yield results that confirm the model (Billas et al., 2003).
14.3 Comparison of EcR/USP Characteristics Across Heterologous Cell Systems

Comparison of receptor ligand binding properties when expressed in different cell types (bacteria, yeast, mammalian and insect cells) reveal that EcR and USP properties tend to be independent of the cellular context (Lezzi et al., 2002; Grebe et al., 2003, 2004; Bergman et al., 2004; Przibilla et al., 2004; Beatty et al., 2006). In other words, the effects of mutagenesis and receptor alteration relate to features of the receptor partners, rather than idiosyncracies of the experimental systems. Of course, cell culture insights cannot supplant in vivo experimentation, but the system has produced several testable hypotheses for subsequent in vivo study.

In the yeast system, the GAL4 activation domain (AD) has been attached to the EcR LBD, and the GAL4 DNA-binding domain (DBD) is attached to the USP LBD (Lezzi et al., 2002). The interaction of these GAL4 fusion proteins is tested by measuring lacZ reporter activity through the UAS promoter element where the GAL4 domains are brought together by the dimerization of the EcR and USP LBDs. The paradigm allows for testing the interaction of the EcR and USP LBDs under a variety of experimental conditions, without the possible complications posed by interdomain interactions with other portions of EcR and USP.

Site-directed mutations have been introduced into both of the two-hybrid fusion proteins and tested for their effects (Grebe et al., 2003, 2004; Bergman et al., 2004; Przibilla et al., 2004). These included point mutations which correspond with larval lethal in vivo mutations of EcR (Bender et al., 1997). In yeast, the mutant proteins show modestly reduced basal and inducible transcriptional activity, that is, hypomorphic activity (Bergman et al., 2004) and reduced ligand affinity (Grebe et al., 2003). The partial activity seen in the two-hybrid system may explain the ability of mutants carrying these hypomorphic alleles to survive to the larval stages, since null EcR mutations cause embryonic lethality (Bender et al., 1997). Many other EcR mutations differentially affect ligand association and dissociation, revealing that these processes involve distinct mechanisms that together determine ligand affinity. As a partner for EcR, USP primarily reduces ligand dissociation and thereby increases ligand affinity of the EcR/USP complex (Grebe et al., 2004).

The possibility of interdomain signalling has been inferred in the two-hybrid system from the effect of several mutations in the USP LBD which were tested in GAL4 fusion proteins for their effects on basal and induced transcriptional activity, ponasterone A binding, and DNA binding. Some of the USP LBD substitutions result in an EcR/USP two-hybrid dimer displaying reduced ponasterone A binding (Fig. 14.1) and a severe loss of transcriptional activity, which ultimately is traced to a loss of affinity for the UAS element (Przibilla et al., 2004). The effects of these mutations belie a modulatory effect of USP upon EcR ligand-binding affinity. Paradoxically, the USP mutations exert negligible effects on transcriptional activity when introduced into whole receptors and tested in the CHO cell culture system (data not shown). In other words, the relationship between the natural DBD of USP and its LBD is apparently different than the same USP LBD’s interaction with the GAL4 DBD in the yeast two-hybrid system.
Several EcR mutations from the yeast two-hybrid system have also been tested in full length receptors using the CHO cell system. As will be noted, these mutations bring about specific impairments of normal function which are not easily predicted or described \textit{in vivo} but which are potentially important for ascertaining receptor functions. Moreover, the cross-system consistency of mutational effects indicates intrinsic receptor functions are affected.

The M504A and M504R substitutions involve a residue associated with ligand-binding in the crystal structure of the EcR from \textit{Heliothis zea} (Wurtz et al., 2000; Billas et al., 2003) and shared by all reported EcRs in helix 5 of the LBD. The mutation virtually abolishes ligand-binding and ligand-dependent transcription in yeast and in cell cultures, but has no discernible effect on basal transcriptional levels in yeast or mammalian cells (Grebe et al., 2003; Bergman et al., 2004; Beatty et al., 2006). EcR (M504A) retains the ability to translocate into the CHO cell nucleus in the absence of ecdysteroids at the same rate as wild-type EcR, though M504R destroys this capability. Neither M504A or M504R translocate into the nucleus at the highly elevated rate that wild-type EcR displays in the presence of ecdysteroids. The failure reveals that the elevated rate of nuclear translocation is functionally dependent on the ligand-bound EcR, and this transport is facilitated by heterodimerization with USP (Nieva et al., 2005).

Two different substitutions, K497A and K497E, of a lysine residue in helix 4 of the LBD shared by all known insect EcRs. Its effects on EcR function illustrate the multifunctional properties of individual residues within the ecdysteroid receptor sequence and also reveal unexpected insights concerning receptor function. This site was originally selected because it has been associated with cofactor interactions among several
nuclear receptors (Wurtz et al., 1995a, b). Both K497A and K497E evoke the same mutant effect in the yeast two-hybrid system: a strongly elevated level of reporter activity from the EcR/USP dimer through the UAS promoter in the absence of ligand, and a modest elevation of ligand-induced reporter gene levels (Bergman et al., 2004).

The GAL4 fusion proteins encoding K497A and K497E also impose a similar effect on two distinct properties of EcR: disruption of a salt bridge between helix 4 and helix 12 and a reduction in ligand binding that is primarily associated with a severe impairment of ligand association (Grebe et al., 2004) that in turn, modestly reduces ligand-dependent dimerization. Across the salt bridge, a counterpart mutation of a residue in helix 12 only slightly decreases ligand-binding. Thus, the reduction in ligand-binding caused by K497E is not solely caused by impairment of the salt bridge. The similar effects caused by two different substitutions of K497 (A and E) further indicates that the functional change results from a loss of a function normally associated with lysine (K), rather than an unpredicted gain or change of function caused by the residue that replaces lysine.

The two-hybrid results led to a series of experiments in mammalian cell cultures to examine the properties of K497E on each of the three isoforms as partners with USP. The mutant protein has a slightly reduced dimerization capability with USP, as evidenced by the effects of competition experiments with wild-type EcR for limited amounts of USP (Beatty et al., 2006). In the CHO cells, only the EcRB2 isoform shows an increase of basal activity like the K497E mutational effects seen in the yeast two-hybrid system. Basal transcriptional activity is not significantly affected by the mutation in EcRA or EcRB1. The isoform specificity reveals an interdomain interaction that resembles isoform-specific properties seen in the vertebrate androgen receptor (Berrevoets et al., 1998). It further suggests the possibility that an EcRB2-specific corepressor interaction is normally mediated at the K497 residue.

The K497E mutation has further confirmed an EcR-mediated transcriptional function that occurs in the absence of USP, though basal and induced activity is reduced by about 3–4-fold of the level seen when USP is also present (Fig. 14.2). Consistent with this transcriptional effect, EcR by itself shows the capability

![Fig. 14.2](image-url)  
**Fig. 14.2** Induction of luciferase reporter activity induced by YFP- *D. melanogaster* EcR-B1 expressed in CHO cells in the absence of *D. melanogaster* USP according to methods of Henrich et al. (2003). Luciferase activity is normalized on receptor concentration determined by Western blot. Black bars: No hormone. White bars: 1 µM murA. SD < 15% based on n = 3
of binding to ecdysteroids, though ligand affinity is much higher when USP is co-expressed (Bergman et al., 2004; Grebe et al., 2004). Importantly, the direct involvement of EcR, even without USP, is illustrated by the effect of the K497E mutation, which elevates ligand-dependent activity in these experimental conditions via the hsp27 EcRE. The effect further implicates the K497 residue as a player in the interaction between the ligand-binding pocket and helix 12, since it apparently affects ligand-dependent transcriptional activity that is known to be an AF2 (ligand-dependent) function (Hu et al., 2003).

In summary, both EcR and USP carry out a range of subfunctions within the context of their partnership that are altered by individual mutations. Some involve not only intermolecular signalling but also interdomain signalling. Further, there is evidence that EcR is also capable of mediating transcriptional activity in the absence of USP, though it is relatively low.

14.4 EcR, USP and Juvenile Hormone Action

Juvenile hormone (JH) molecular actions were summarized recently by Berger and Dubrovsky (2005). Despite the different modes of actions for JH proposed at the molecular level, the outcome seems to be that JH-dependent transcription could be mediated by EcR and USP (Wu et al., 2006) as well as E75 (Dubrovskaya et al., 2004), whose own expression is regulated by ecdysteroids. Together, the varied modes of action and the variety of gene targets which have been studied can be interpreted as evidence for multiple modes of JH action, particularly since seemingly contradictory conclusions do not involve the same reagents, promoters, and cell types. The recent characterization of transcriptional effects of JH incubated with dissected D. melanogaster salivary glands offers an entry point for examining gene targets of JH, and further exploring the possible role of EcR and USP for evoking changes associated with juvenile hormone (Beckstead et al., 2007).

The cell culture system has also shown that insect USP and vertebrate RXR are not functionally equivalent (Billas et al., 2001; Clayton et al., 2001). RXR does not form a transcriptionally active dimer with D. melanogaster EcR in the presence of 20E (Henrich et al., 2003). Exogenously introduced USP, but not the mammalian RXR, facilitates nuclear transport of EcR in mammalian cells (Nieva et al., 2005).

RXR is activated by the JH analogue, methoprene, and physically interacts with methoprene acid (Harmon et al., 1995), thus forming a basis for examining the ligand status of JH and JH analogues on RXR’s insect orthologue, USP. Based on the RXR analogy, an obvious possibility for JH action is that USP is acting as a JH receptor via its ligand binding pocket, and a computational model has been reported that allows for JH binding inside the USP ligand-binding pocket (Sasorith et al., 2002). Using a DR12 response element in insect cells, Jones et al. (2001) showed that a USP-mediated transcriptional response is detected via homodimerization, and that high affinity binding with the JH precursor, methyl farnesoate, is abolished by mutating the C472 residue in the USP LBD (Jones et al., 2006).
CHO cells transfected with EcR and USP possess no capability for mediating a response to juvenile hormone or analogues such as methoprene and pyriproxfen via an hsp27 EcRE, and similar conclusions have been drawn from insect cells (Henrich et al., 2003; Fang et al., 2005). These experiments suggest that JH employs different mechanisms in insect cells, depending in part upon the presence of EcR and the type of promoter element involved. In CHO cells, JH exerts no effect on EcR/USP transcriptional activity but reduces the dosage of ecdysteroids necessary to achieve maximal induction by about tenfold (though it cannot act additively with ecdysteroids to evoke a supramaximal response; Beatty et al., 2006). The ability of JH and analogues to potentiate a maximal induction at otherwise submaximal ecdysteroid dosages has established a paradigm for testing known and novel compounds for their ability to affect EcR/USP activity.

The analysis has been extended to the components of the mevalonate pathway, whose starting substrate is acetyl coA, which is derived from glycolysis and the Krebs cycle and which ultimately depends on the availability of nutrients (Belles et al., 2005). Several substrates along the insect mevalonate pathway ending with JH have shown the ability to potenti ate the ecdysteroid response in CHO cells over the 20–100µM range, including farnesol, farnesoic acid, farnesyl diphosphate, and methyl farnesoate (Fig. 14.3). The broad spectrum-low affinity interaction suggests a “sensing” mechanism resembling those described for the nuclear receptor PPAR,
which regulates metabolism in vertebrates (Auwerx et al., 2003), the effect of glucose on LXR (Mitro et al., 2007), and the low affinity ligand interactions involving intracellular metabolites seen in other nuclear receptors such as PXR and FXR (Goodwin et al., 2003; Desvergne, 2007).

Conceivably, a cellular milieu laden with mevalonate pathway substrates reduces the actual level of ecdysteroids necessary to evoke a maximal inductive response. Low ecdysteroid titer peaks generally trigger the larval-larval molts during feeding larval stages in holometabolous insects, and therefore, cross talk between low ecdysteroid titers and nutritional state would ensure an appropriate trigger for larval development. The mechanism also could account for the absence of any obvious detrimental effect when JH is absent in *Drosophila*, since JH may represent just one of the sensors read by EcR and USP.

The mechanism for JH potentiation apparently is distinct from the high affinity interaction with methyl farnesoate. Alanine substitutions were made for each JH-binding site proposed in the Sasorith model and tested for their effects on ecdysteroid induction and JH potentiation by the EcR/USP complex in the CHO cell system. Basal and induced transcriptional activity was relatively unaffected in full-length receptors, and potentiation by JH was normal. Notably, the C472 residue associated with a high affinity interaction with methyl farnesoate affected neither JH-mediated nor methyl farnesoate-mediated potentiation, nor did the mutation affect ecdysteroid inducibility (data not shown).

Given the differences in behavior of USP in the presence and absence of EcR, it is conceivable that USP retains a high affinity interaction with JH as a homodimer, or in conjunction with other cofactors, whereas the EcR/USP heterodimer involves a low affinity/low specificity interaction with JH or with other cofactors that interact with JH. Differences in ligand affinity have been reported for USP’s vertebrate RXR homologue in the presence and absence of heterodimeric partners (Desvergne, 2007).

The distinct mechanisms described so far may simply reveal that the functional effects of JH depend not only on USP, but also on EcR. For instance, it is plausible that JH modulates the ability of EcR and USP to recruit comodulators, such as the enhanced corepressor recruitment seen in the presence of JH (Maki et al., 2004). When viewed individually and compared together, these results further imply that JH utilizes more than one mode of action to affect transcriptional activity in the cell (Feyereisen, 1998).

Another indication that JH plays multiple cellular roles is based on the gene encoding the Met-Tolerant (MET) protein. Drosophila MET protein belongs to the bHLH-PAS family and binds to JH and methoprene, though a functional or physical connection between MET and specific gene targets remains to be identified. In the absence of JH, MET dimerizes with a second bHLH-PAS factor, and is displaced by the introduction of JHIII or methoprene (Godlewski et al., 2006). Other members of the bHLH-PAS family are known to interact directly with nuclear receptors via an LXXLL motif in the PAS domain (Okino and Whitlock, 2000); MET possesses several of these motifs, though no interaction with either EcR or USP has yet been described.
A few salient observations imply that a bHLH-PAS transcription factor may play(s) a role in mediating the low affinity potentiation described in CHO cells. The bHLH-PAS family includes the dioxin receptor (aka, aryl hydrocarbon receptor, ArHR), which is activated by polychlorinated biphenyls (PCBs). Remarkably, PCBs have been shown to potentiate the ecdysteroid response in CHO cells with about the same potency as JH, further implicating a common mode of action (Oberdorster et al., 1999).

Interestingly, JH potentiation only occurs with the use of natural ecdysteroid agonists. Nonsteroidal agonists, which confer a different shape to the EcR ligand-binding pocket than natural ecdysteroids, are incapable of evoking potentiation (Beatty, unpublished, 2006). Presumably the pocket shape assumed by interaction with diacylhydrazines and other nonsteroidal agonists of EcR precludes interactions with cofactors. Whether an interaction with MET is tied to the distinct effects of ecdysteroids and nonsteroidal agonists on JH potentiation in the CHO cell culture system is an active subject of investigation.

14.5 Species Specific Aspects of EcR and USP Revealed in Cell Cultures

Species specific variation of the amino acid sequences of EcR and USP and the expression of different isoforms leads to physiological consequences like altered ligand specificity in *Leptinotarsa decemlineata* (Colorado potato beetle; Ogura et al., 2005) and other insects (Graham et al., 2007a, b) and changes in the dimerization efficiency (Suhr et al., 1998) and DNA binding of *Bombyx mori* receptors (Shirai et al., 2007). Chimeric complexes of EcR and USP derived from different species may elucidate the molecular basis for these differences, and this approach has already been applied for cell culture studies of EcR function.

The evidence for interspecial differences in the USP LBD is apparent by the effects of substituting the LBD of the *D. melanogaster* USP with the equivalent domain of *Chironomus tentans*. The resulting chimeric USP rescues larval development in usp mutant flies, but suddenly fails at the onset of pupariation, meaning that the *C. tentans* USP LBD is unable to perform a function that is essential for metamorphosis (Henrich et al., 2000).

For the three ecdysteroid receptor isoforms of *D. melanogaster*, deletion of the DNA-binding domain of *D. melanogaster* USP evokes different effects. This construct, (USP-ΔDBD) forms an active dimer with EcRB1 that wholly retains its ability to mediate ecdysteroid-inducible transcriptional activity. However, while USP-ΔDBD dimerizes with all three EcR isoforms, the resulting EcRA and EcRB2 complexes are only weakly active (Beatty et al., 2006). In these cases, the USP-ΔDBD is dominant negative, that is, it forms a transcriptionally impaired complex with EcRA and EcRB2. USP *in vivo* point mutations affecting conserved residues in the DBD do not inhibit the expression of early-inducible genes, though a null mutation of USP eliminates induction. All of the USP mutations lead to premature expression of other
genes, indicating that the USP-ΔDBD normally participates in a repressive function (Schubiger and Truman, 2000; Ghbeish et al., 2001). The relationship of the cell culture results and the in vivo observations have yet to be reconciled.

By contrast, the equivalent USP-ΔDBD construct from *Choristoneura fumiferana*, forms a high activity dimer with all three *D. melanogaster* EcR isoforms (Henrich et al., 2003), and in fact, this heterospecies complex displays a distinctively higher activity level than the native *D. melanogaster* complexes using an intact USP (Fig. 14.4). The structural difference(s) that are responsible for the functional differences between the two USP sequences remains to be tested more fully. In any case, it is increasingly apparent that there is considerable divergence among the structures of insect USPs that is not obvious in sequence alignments, and that the USP proteins diverge considerably from the vertebrate RXR (Iwema et al., 2007).

**Fig. 14.4** Effects of USP-ΔDBD from two insect species on basal and induced transcriptional activity of *D. melanogaster* EcRB1 based on methods of Henrich et al. (2003). Fold-induction compared to transcriptional activity of EcRB2/DmUSPII in the absence of murA (equals 1). DmUSPII refers to the *D. melanogaster* USP in which the N-terminal domain has been replaced by VP16 AD (Beatty et al., 2006). USPIII (*D. melanogaster*) and CfUSP (*Choristoneura fumiferana*) refer to a VP16-USP (ΔDBD), from which the DBD has been removed (Henrich et al., 2003 and refs. therein)
14.6 Summary

The EcR/USP heterodimer possesses a variety of subfunctions and features which are difficult to assess in vivo and may not be apparent unless observed in isolation or through careful manipulation. The heterologous mammalian cell culture system has emerged as an effective tool to elucidate these issues. So far, the system has yielded observations that generally confirm those obtained biochemically and from yeast two-hybrid systems, indicating that these systems are dealing with actual receptor attributes. More importantly, the system has proven useful for testing computational models, structure-function studies, EcR and USP isoform comparisons within species, and interspecies receptor comparisons. New insights leading to testable hypotheses in vivo have been generated through the process about the relationship of EcR and USP isoforms, as well as the role of JH and possible cofactors. The next phase of studies will likely include many modifications and comparisons between species, with the aim of elucidating not only the evolutionarily conserved features of EcR/USP activity, but also the many nuances and variations associated with insect adaptation to specific environmental niches.

References


A cross species comparison of EcR and USP
in the functional ecdysteroid receptor

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Abstract

The functional ecdysteroid receptor is a heterodimeric complex consisting of the ecdysone receptor (EcR) and ultrapsiracle (USP). Ecdysteroids mediate the activity of the heterodimer to regulate transcription of target genes involved in insect development. In most insects, an additional level of transcriptional control is achieved by the expression of multiple EcR isoforms. Single species heterodimers of the EcR isoforms and USP from *Drosophila melanogaster* and *Leptinotarsa decemlineata* display unique functional characteristics both in the absence of ligand and in response to a range of ecdysteroids and nonsteroidal ecdysone agonists. A heterologous mammalian cell culture assay was used to analyze the roles *D. melanogaster* and *L. decemlineata* EcR and USP in the functional ecdysteroid receptor by comparing the transcriptional capabilities of cross species heterodimers. When the *D. melanogaster* EcR isoforms were tested with *L. decemlineata* USP, basal transcriptional activity was lower while ligand induced transcriptional activity in response to muristerone A or RH2485 was higher than with *D. melanogaster* USP. Heterodimers of *L. decemlineata* EcR and *D. melanogaster* USP maintained basal transcriptional activity although ligand induced activity was lower than with *L. decemlineata* USP. The presence of juvenile hormone III in addition to muristerone A potentiated maximum transcriptional activity with *D. melanogaster* EcR and *L. decemlineata* USP combinations. In contrast, the *L. decemlineata* EcR and *D. melanogaster* USP receptors exhibited weak potentiation capability similar to that observed with *L. decemlineata* USP in response juvenile hormone III and muristerone A. Receptor combinations from both species demonstrated low-level potentiation with juvenile hormone III and 20-hydroxyecdysone. Potentiation of ligand induced transcriptional activity with RH2485 and juvenile hormone III occurred exclusively in receptors with *D. melanogaster* EcRB1. The cross species heterodimers provide an indication that USP plays an important role in modifying the transcriptional activity of the functional ecdysteroid receptor.

**Keywords:** cell culture, *Drosophila*, *Leptinotarsa*, juvenile hormone, nonsteroidal agonist

**Abbreviations used**

Introduction

The physiological processes underlying insect development are mediated by ecdysteroids and further modulated by juvenile hormone. In most insects and arthropods the molting process is the outcome of a series of transcriptional events organized by the natural insect molting hormone 20-hydroxyecdysone (20E). The simultaneous presence of juvenile hormone determines the nature of the molt and sustains larval development. These developmental events occur when 20E binds to the ecdysone receptor (EcR) thereby stabilizing the formation of a functional ecdysteroid receptor with its heterodimeric partner Ultraspiracle (USP), the insect ortholog of the vertebrate retinoid X receptor (RXR). EcR and USP are members of a larger superfamily of nuclear receptors having a characteristic DNA binding domain (DBD) and ligand binding domain (LBD, Henrich et al., 1990; Oro et al., 1990; Shea et al., 1990; Koelle et al., 1991). Members of this receptor superfamily share extensive sequence homology in their DNA binding domain (DBD) characterized by two cysteine-cysteine zinc fingers that enable interaction with certain DNA response elements to regulate transcriptional events. The LBDs of both EcR and USP are structural motifs consisting of 10-12 alpha helices that form the dimerization interface for the EcR/USP heterodimer and the ligand binding pocket of EcR (Perlmann et al., 1996). Drosophila melanogaster USP (DmUSP) has generally been characterized as an orphan receptor because it does not mediate transcriptional activity through a known ligand although mammalian and insect cell culture studies have indicated that USP physically interacts with juvenile hormone III (JHIII; Jones et al., 2001, 2010; Xu et al., 2002). Other studies have demonstrated that USP binds methylfarnesoate, a precursor of JHIII, with nanomolar affinity, an interaction that can be reduced by mutation of putative docking sites (Jones et al., 2006). The D. melanogaster USP gene has no introns and encodes a single isoform of the protein that is expressed broadly in the developing tissues. In contrast, other insects such as Chironomus tentans express multiple isoforms of USP with different N-terminal domains. These USP isoforms differ in their expression profiles and ability mediate transcriptional events (Vöglti et al., 1999). Leptinotarsa decemlineata USP (LdUSP) shares more sequence homology with human and mouse RXR than with USP from Dipteran and Lepidopteran insects (Ogura et al., 2005). Recent studies indicate that USP has evolved a substantially larger heterodimerization interface in Dipteran and Lepidopteran insects (Iwema et al., 2009) a feature that has not evolved in more primitive insect orders such as Coleoptera. Structural studies have shown that Dipteran and Lepidopteran insects also share a unique antagonistic position of the USP helix 12 (Billas et al., 2001; Clayton et al., 2001; Sasorith et al., 2002; Billas and Moras, 2005). This antagonistic conformation significantly modifies the stability of the ecdysteroid receptor complex thereby affecting DNA binding, intracellular localization, and
transcriptional activity indicating that USP is not simply a passive dimerization partner for EcR (Tremmel et al., 2011).

In most insect species multiple EcR isoforms mediate the diverse range of tissue specific transcriptional responses that occur during development. Three natural isoforms of EcR (A, B1, B2) orchestrate this cascade of ecdysteroid mediated transcriptional events in D. melanogaster. The EcRA and EcRB isoforms are expressed via alternate promoter elements in the Drosophila genome. Differential splicing of the EcRB pre-mRNA gives rise to the EcRB1 and EcRB2 isoforms. Two EcR isoforms (A and B) have been identified in the Colorado potato beetle Leptinotarsa decemlineata (Ogura et al., 2005). Despite similarities in patterns of development among insects, the characteristics of the functional ecdysteroid receptor vary broadly across insect species. A good example is the species specific toxicity of the synthetic nonsteroidal ecdysone agonists, diacylhydrazines, against Lepidopteran and Dipteran targets and to a lesser extent against Coleopteran insects (Carlson et al., 2001). These species specific functional attributes of EcR and USP have been characterized by using ecdysteroid inducible reporters in a heterologous mammalian cell culture system in response to a range of steroidal and nonsteroidal ecdysone agonists (Mouillet et al., 2001; Beatty et al., 2006; 2009). The distinct responses of EcR and USP to such ecdysteroids and agonists are thought to underlie important functional roles of the ecdysteroid receptor for orchestrating developmental events in vivo. Recent studies have utilized Coleopteran and Lepidopteran cell culture based reporter assays with the intent of screening agonists based on their activation of ecdysone-inducible gene expression (Dinan et al., 2001; Swevers et al., 2004; Soin et al., 2010). The utility of an insect cell culture assay is that it allows order specific characterization of candidate agonists in cells that endogenously express adequate levels of EcR and USP (Soin et al., 2010); only an ecdysone responsive reporter construct must be transfected into the cells. In contrast, mammalian cell cultures have no endogenous ecdysteroid response; the cells must be transfected with EcR and USP genes in addition to an ecdysone responsive reporter gene construct. The insect cell culture based assay provides a high throughput method by which to screen candidate agonists in an order specific receptor background. Because the heterologous mammalian cell culture assays have no endogenous ecdysteroid response, they provide a screening method that allows the user to select which ecdysteroid inducible components are ectopically expressed in the system. Therefore, a much different utility is achieved because the system can be used to elucidate functional characteristics of EcR and USP from one species or in a cross species fashion. As an extension, the heterologous system provides a platform to analyze the effect of site directed mutations on receptor function. In order to correctly define the effect of cross species comparisons it is necessary to consider the transcriptional capabilities of the receptors of both species with their natural dimerization partners. Earlier work in the heterologous mammalian cell culture system demonstrated the basal and ligand induced transcriptional characteristics of the DmEcR isoforms paired with DmUSP (Beatty et al., 2006). These studies were expanded to compare the transcriptional capabilities of the LdEcR isoforms with LdUSP in response to a range of ecdysteroids and diacylhydrazine agonists to those of the DmEcR/DmUSP heterodimers (Beatty et al., 2009). The intent of this work was to compare the
basal and ligand induced transcriptional profiles of these nuclear receptors in order to examine how these proteins mediate transcriptional responses in a species specific fashion.

This study focuses on the roles of *D. melanogaster* and *L. decemlineata* EcR and USP by making cross species comparisons of transcriptional activity in a heterologous mammalian cell culture assay with three aims in mind: a) to characterize the transcriptional activity of cross species (DmEcR/LdUSP and LdEcR/DmUSP) pairings in response to natural ecdysteroids and diacylhydrazine agonists b) to better define species specific functional aspects of these receptors by comparing their transcriptional activities and DNA binding characteristics to those of already established same species pairings and c) to examine the species specific role of USP in the potentiation effect with JHIII.

**Experimental procedures**

**Cell Culture, EMSA, and Western Immunoblotting**

The methodologies used in this study for cell culture, reporter gene assays, vector construction, western immunoblotting and the electrophoretic mobility shift assays (EMSAs) have been previously described (Henrich et al., 2003; Beatty et al., 2006, 2009). CHO cells were seeded at a density of 3.5x10^5 cells per well in a 6-well tissue culture plate and allowed to grow for 24 hours. Near confluence, the cells were transfected with 250ng each of four different plasmid vectors; a luciferase reporter gene construct under the control of an hsp27 EcRE; a construct expressing a β-galactosidase reporter via a constitutive mammalian promoter; an EcR coding plasmid under the control of a constitutive promoter, and a plasmid vector that constitutively expresses a fusion of the herpes simplex virus viral protein 16 (VP16) activation domain attached to either DmUSP amino acids 104-508 (DmUSPII) or DmUSP amino acids 170-508 (DmUSPIII). The transfections proceeded for 4 hours after which time the ligand treatments were applied. Ligands tested in this study include the ecdysteroids murA (Alexis Biochemicals, SanDiego, CA, USA) and 20E (Sigma Chemical, St Louis, MO, USA), the nonsteroidal diacylhydrazine ecdysone agonist RH2485 (methoxyfenozide, Rohm and Haas Co., Spring House, PA, USA), and JHIII (Sigma Chemical, St Louis, MO, USA). After a 24 hour incubation, the cells were harvested and the cell extracts processed for subsequent applications. Western immunoblots of DmUSP and LdUSP constructs were probed with a VP16 monoclonal antibody (Santa Cruz).

All vectors encoding luciferase and β-galactosidase reporter genes as well as *D. melanogaster* and *L. decemlineata* EcRs and USPs have been described previously (Henrich et al., 2003; Beatty et al., 2006, 2009).
Publications and manuscript

Results

Previous studies utilizing the heterologous mammalian cell culture assay have characterized the transcriptional capabilities of EcR and USP from both *D. melanogaster* and *L. decemlineata* in response to a range of steroidal and nonsteroidal (diacylhydrazine; RH2485) ecdysone agonists (Beatty et al., 2006, 2009). These studies have established the transcriptional capabilities of receptors from both species utilizing same species heterodimers. The EcR isoforms of both species produced different transcriptional responses when challenged with various ligands in a manner dependent on the specific USP present (Beatty et al., 2009). Cross species heterodimers demonstrate that the DmEcR isoforms, when paired with LdUSPII, achieve a level of ligand induced transcriptional activity in response to murA (Figure 1A) or RH2485 (Figure 2) that is at least as great as the same species DmEcR/DmUSPII heterodimers. However, the cross species DmEcR/LdUSPII heterodimers are unable to evoke the same level of transcriptional activity as the same species heterodimers in response to 20E (Figure 3). In contrast, the LdEcR/LdUSPII pairings were unable to evoke the same level of transcriptional activity as the corresponding same species LdEcR/LdUSPII receptors for any of the ligands tested (Figure 4). Additionally, the receptors of both species exhibit distinct DNA binding properties both in the absence and presence of ligand. DmEcR isoforms generally demonstrate a ligand induced increase in DNA binding in both same species (Figure 5C) and cross species heterodimers (Figure 5A, 5B). The LdEcR isoforms demonstrate DNA binding capabilities in the presence and absence of ligand with all USP constructs (Figure 5B). This study utilized cross species receptor pairings to identify unique functional attributes of the individual receptor proteins.

Effect of steroidal and nonsteroidal ecdysone agonists on ecdysteroid receptor function:

Comparison of basal transcriptional activity of EcR isoforms from *D. melanogaster* and *L. decemlineata* (same species pairings)

The *D. melanogaster* and *L. decemlineata* EcR isoforms, when paired with their respective USPs, are able to mediate basal transcriptional responses in the absence of ligand. The basal transcriptional activity mediated by same species EcR/USP heterodimers occurs in an isoform specific pattern. In a heterodimer with DmUSPII, DmEcRB1 produces the highest level of basal transcriptional activity while the DmEcRA/DmUSPII heterodimer evokes the lowest basal activity of the *D. melanogaster* isoforms (Figure 1A; Beatty et al., 2006). An intermediate level of basal transcriptional activity is mediated by the DmEcRB2/DmUSPII heterodimer. A similar isoform specific pattern of basal transcriptional activity is observed with the *L. decemlineata* isoforms. The LdEcRA/LdUSPII heterodimers exhibit greater levels of basal transcriptional activity than the equivalent LdEcRB complex (Figure 1B; Beatty et al., 2009). In general, the basal transcriptional activity associated with the LdEcRA isoform is slightly higher than that of the DmEcRB2 complex while the basal transcriptional activity of the LdEcRB heterodimer is slightly lower (Figure 1A-B).
**Effect of steroidal and nonsteroidal ecdysone agonists on ecdysteroid receptor function:**

Ligand induced transcriptional activity of EcR isoforms from *D. melanogaster* and *L. decemlineata* (same species pairings)

Both DmEcR and LdEcR isoforms in a heterodimer with their respective USPs are capable of mediating transcriptional activity in response to murA, 20E, and RH2485 (Beatty et al., 2009). Similar to basal transcriptional activity, the observed levels of ligand induced transcriptional activity vary in a manner dependent on the EcR isoform specific N-terminal sequence of the receptor. Generally, the DmEcR/DmUSPII heterodimers were more responsive to murA (Figure 1A) while the *L. decemlineata* receptors achieved significantly higher levels of transcriptional activity in the presence of a maximal dosage of the synthetic nonsteroidal ecdysone agonist RH2485 (Figure 4). Receptors of both species produced only a modest, and isoform specific, response to 20E (Figure 3).

The pattern of transcriptional response with the individual EcR isoforms was similar for each of the ligands tested. Specifically, when the transcriptional activity of *D. melanogaster* isoforms is compared relative to DmEcRB2 basal activity, DmEcRB1 exhibits the highest absolute levels of basal and induced transcriptional activity, with DmEcRA producing the lowest levels of activity. DmEcRB2 displayed an intermediate level of basal and ligand induced transcriptional activity (Figure 1A; Beatty et al., 2006, 2009). It is interesting to note that the fold induction achieved by both DmEcRB2 and DmEcRB1, compared to their respective basal transcriptional activities, is roughly equivalent. In other words, DmEcRB2 is just as inducible as DmEcRB1 although its absolute level of transcriptional activity is lower. In every case murA evoked the highest level of transcriptional activity among the *D. melanogaster* isoforms. The nonsteroidal ecdysone agonist RH2485 also precipitated a strong response in these isoforms, producing transcriptional activity nearly equivalent to that evoked by murA (Figure 2A-B; Beatty et al., 2009). The natural insect molting hormone 20E produced the lowest level of transcriptional activity in all three *D. melanogaster* EcR isoforms with DmUSP. Overall, the efficacy of 20E was much lower than that of murA for all receptor combinations tested; maximum transcriptional induction of DmEcRB isoforms with DmUSPII treated with 10µM 20E is about half that of murA treatments (Figure 3; Beatty et al., 2009).

The *L. decemlineata* receptors demonstrated a similar isoform specific pattern of response when challenged with various ligands. Generally, LdEcRA/LdUSPII heterodimers achieved greater levels of ligand induced transcriptional activity than the equivalent LdEcRB complex. MurA demonstrated a much lower potency with *L. decemlineata* heterodimers than with those of *D. melanogaster* (Beatty et al., 2009). In fact, 20E evoked a transcriptional response nearly equivalent to that of murA with both LdEcR/LdUSPII heterodimers (Figure 4; Beatty et al., 2009). Both LdEcR isoforms with LdUSPII exhibited greater absolute transcriptional activity in response to 10µM RH2485 than the corresponding DmEcR/DmUSPII heterodimers (Figure 4; Beatty et al., 2009).

The DmEcR/DmUSPII pairings are generally more sensitive to steroidal ligands, whereas the LdEcR/LdUSPII heterodimers evoke a greater transcriptional response in the presence of the nonsteroidal diacylhydrazine ecdysone agonist RH2485.
Effect of steroidal and nonsteroidal ecdysone agonists on ecdysteroid receptor function:
Comparison of basal transcriptional activity of EcR isoforms from D. melanogaster and L. decemlineata (cross species pairings)

Cross species DmEcR/LdUSP II heterodimers demonstrate lower basal transcriptional levels than same species D. melanogaster pairings. In every case where a DmEcR isoform is coexpressed with LdUSP II, the basal transcriptional activity mediated by the receptor is about half that observed from the same species heterodimers (Figure 1A). The DmEcR isoforms continue to exhibit an isoform specific pattern of basal transcriptional activity in which DmEcRB1/LdUSP II produces the greatest basal transcriptional activity and DmEcRA/LdUSP II generates the lowest. An intermediate basal transcriptional response is observed with the DmEcRB2/LdUSP II heterodimer (Figure 1A). In contrast, both LdEcR isoforms when coexpressed with DmUSP II display similar levels of basal transcriptional activity to those associated with the same species L. decemlineata heterodimer (Figure 4). These results suggest that the basal transcriptional capabilities of the DmEcR isoforms are contingent on the USP heterodimeric partner while the basal response mediated by the LdEcR isoforms is largely unaffected by the specific USP present.

Effect of steroidal and nonsteroidal ecdysone agonists on ecdysteroid receptor function:
Ligand induced transcriptional activity of EcR isoforms from D. melanogaster and L. decemlineata (cross species pairings)

In every case where a DmEcR isoform was coexpressed with LdUSP II the transcriptional activity evoked by the complex with murA (Figure 1A) or RH2485 (Figure 2A-B) was at least equivalent to that of a same species DmEcR/DmUSP II response, although induction with the natural molting hormone 20E was significantly reduced (Figure 3). Each LdEcR isoform paired with DmUSP II was unable to achieve the level of ligand induced transcriptional activity produced with LdUSP II (Figure 4). Inductive transcriptional responses are mostly maintained, and in some cases increased, when cross species DmEcR/LdUSP II heterodimers are challenged with various ligands. The DmEcRB1/LdUSP II heterodimer evokes significantly higher maximal transcriptional activity in response to 1µM murA than the DmEcRB1/DmUSP II heterodimer (Figure 1A; Beatty et al., 2009). Transcriptional activity of the DmEcRB2/ LdUSP II heterodimer can be induced at least as well as the DmEcRB2/DmUSP II pairing when treated with 1µM murA (Figure 1A). The 20E mediated transcriptional response is significantly lower when DmEcRB1 is paired with LdUSP II (Figure 3). The nonsteroidal ecdysone agonist RH2485 is able to evoke a significantly higher level of transcriptional activity with the DmEcRB1/LdUSP II heterodimer than with DmEcRB1/DmUSP II (Figure 2A). The maximal transcriptional activity induced by RH 2485 with DmEcRB2 is equivalent when this receptor is paired with either DmUSP II or LdUSP II (Figure 2B).

Cross species LdEcR/DmUSP II heterodimers exhibit a weaker transcriptional response to maximal dosages of all ligands tested when compared with the transcriptional activity of the same species.
LdEcR/LdUSPII heterodimers (Figure 4). When LdEcRA is coexpressed with DmUSPII the maximum level of induced transcriptional activity in response to 50µM RH2485 is about 25% lower, from 22 fold induction with LdEcRA/LdUSPII to nearly 17 fold with LdEcRA/DmUSPII (Figure 4). RH2485 mediated transcriptional activity is greater for the LdEcRB/LdUSPII complex than the LdEcRB/DmUSPII cross species heterodimer. Although basal transcriptional levels were largely unaffected, the LdEcRB1/LdUSPII heterodimer produced a nearly 2.5 fold greater level of reporter gene activity than the cross species LdEcRB1/DmUSPII complex in the presence of 50µM RH2485 (Figure 4).

The inducibility of cross species DmEcR/LdUSPII and LdEcR/DmUSPII heterodimers is largely modulated by the interaction with the USP present. Induced transcriptional activity is at least maintained with the DmEcR/LdUSPII receptors in response to murA and RH2485 (Figure 1A; Figure 2), whereas the level of induced transcriptional activity is diminished for the LdEcR/DmUSPII pairings in response all ligands tested (Figure 4).

**Effect of JHIII on transcriptional activity of the EcR isoforms from* D. melanogaster* and* L. decemlineata* (same species pairings)**

Previous studies have shown that JHIII is capable of mediating a maximal transcriptional response with the functional ecdysteroid receptor in the presence of a submaximal dosage of murA, although JHIII alone has no effect (Henrich et al., 2003; Beatty et al., 2006). This effect, termed potentiation, reduces the concentration of ecdysteroid by about tenfold that is necessary to achieve a maximal transcriptional response. *D. melanogaster* receptor combinations are capable of potentiation by JHIII when also treated with either murA (Figure 1A) or the natural molting hormone 20E (Figure 3), but only DmEcRB1 demonstrates potentiation with the nonsteroidal agonist RH2485 (Figure 2A). A submaximal dosage (1µM) of 20E in combination with JHIII is able to evoke a slight but significant potentiation effect when DmEcRB1 is paired with DmUSPII (Figure 3). Low level potentiation also occurs with *D. melanogaster* heterodimers in response to the diacylhydrazine RH2485 and JHIII (Figure 2A-B). Under these circumstances, only the DmEcRB1/DmUSPII heterodimer demonstrates any increase in induced transcriptional activity that may be attributed to the presence of JHIII (Figure 2A).

In contrast, LdEcR/LdUSPII receptors only demonstrate a weak ability to potentiate transcriptional activity in the presence of murA (Figure 1B) or 20E in combination with JHIII (Figure 3). This low-level potentiation effect disappears completely when the LdEcRB/LdUSPII heterodimer is exposed to RH2485 and JHIII (Figure 2C). Generally speaking, potentiation in same species heterodimers is largely affected by the interaction of ligand with the particular EcR present, an effect that is modulated by USP.
Effect of JHIII on transcriptional activity of the EcR isoforms from *D. melanogaster* and *L. decemlineata* (cross species pairings)

Cross species DmEcR/LdUSPII heterodimers can be potentiated with various ligands and JHIII. Potentiation, as a proportion of absolute transcriptional activity, is diminished in LdEcR/DmUSPII receptors. All three DmEcR isoforms paired with LdUSPII in the presence of a submaximal dosage of murA (0.1 µM) can be potentiated to maximum transcriptional activity in response to 80 µM JHIII (Figure 1A). Potentiation occurs to a much lesser extent with the DmEcRB1/LdUSPII receptor in response to 20E and JHIII (Figure 3).

The ability of RH2485 and JHIII to potentiate transcriptional activity in the DmEcRB1/LdUSPII cross species heterodimer is considerably lower when compared to the maximum transcriptional response evoked by 10 µM RH2485 (Figure 2A). DmEcRB1 is capable of low level potentiation with RH2485 when paired with the USP of either species. In contrast, DmEcRB2 paired with either DmUSPII or LdUSPII exhibits no potentiation response when treated with RH2485 and JHIII (Figure 2B).

The potentiation response evoked with the DmEcR/LdUSPII heterodimers is diminished when the LdEcR isoforms are paired with DmUSPII. Because the LdEcR/DmUSPII heterodimers are not as sensitive to murA and 20E, the potentiation effect evoked by submaximal dosages of these ligands with 80 µM JHIII is more difficult to ascertain than with the DmEcR/LdUSPII heterodimers. Although murA induced transcriptional activity of the cross species LdEcRA/DmUSPII heterodimer is lower, treatment with 0.1 µM murA and JHIII evokes transcriptional activity at least as great as with 1 µM murA (Figure 1B). The LdEcRB/DmUSPII heterodimer retains only partial ability to be potentiated in response to murA in combination with JHIII (Figure 1B). The potentiation response is nearly eliminated when LdEcRB/DmUSPII heterodimer is challenged with 1 µM 20E and 80 µM JHIII (Figure 3). The nonsteroidal ecdysone agonist RH2485 is unable to potentiate a transcriptional response with JHIII via LdEcRB1 when paired with the USP construct of either species (Figure 2C).

Generally, cross species DmEcR/LdUSPII and LdEcR/DmUSPII heterodimers both retain some amount of potentiation capability with ecdysteroids or steroidal ecdysone agonists, while both receptors demonstrate little or diminished potentiation with the nonsteroidal agonist RH2485.

Basal DNA binding characteristics of the ecdysteroid receptor complex (same species, cross species pairings)

EMSAs were performed with CHO cell extracts to examine the DNA binding properties of the ecdysteroid receptors with a radiolabeled hsp27 EcRE. EMSA analysis demonstrates that the DmEcRB2/DmUSPII complex binds the hsp27 EcRE in the absence of hormone, an interaction that increases with the addition of 2.5 µM murA (Figure 5A, DB2 C1). The signal intensity of the basal and induced DmEcRB2/DmUSPII complex is greater than that of the corresponding DmEcRB1/DmUSPII complex (Figure 5A, DB1 C1 and C2; Figure 5C). When either DmEcRB2 or DmEcRB1 is paired with
LdUSPII the resulting complex is capable of interacting with the hsp27 EcRE in the absence of ligand (Figure 5A, DB2 C2, DB1 C3).

When LdEcR isoforms are paired with LdUSPII basal DNA binding is observed with the hsp27 EcRE. The LdEcRA/LdUSPII heterodimer forms two distinct complexes with the DNA (Figure 5B, LA C1, C2; Figure 5C) while only one complex is observed with the LdEcRB/LdUSPII complex in the absence of ligand (Figure 5B LB C1; Figure 5C). A higher basal affinity for the hsp27 EcRE is observed for both LdEcRA and LdEcRB in a cross species complex with DmUSPII than with LdUSPII (Figure 5B, LA C3, LB C2, C3). DNA binding is also observed with both LdEcR isoforms and DmUSPIII in the absence of ligand (Figure 5B, LA C2, LB C1), although the signal intensity of these complexes is lower than that of the same species LdEcR/LdUSPII receptor complexes.

These results indicate that the basal interactions of the DmEcR receptors with the hsp27 EcRE are dependent on the USP present, while basal DNA binding of LdEcR receptors is mostly independent of the modulatory effect of a specific USP.

**Induced DNA binding characteristics of the ecdysteroid receptor complex (same species, cross species pairings)**

EMSA analysis reveals that the formation of heterodimeric complexes capable of interaction with the hsp27 EcRE is mediated by ligand and associated with the specific EcR present. These DNA binding studies further demonstrate that LdUSPII exerts a species specific effect on the DNA binding ability DmEcRB isoforms in the presence and absence of ligand.

Same species DmEcRB1/DmUSPII heterodimers form a faintly visible complex in the absence of hormone (Figure 5A, DB1 C2). The addition of murA causes a significant increase in the intensity of the DmEcRB1/DmUSPII complex on the EMSA gel (Figure 5A, DB1 C1, C2). An indication of the species specific effect of USP on DNA binding is revealed when DmEcRB1 is paired with LdUSPII. The DmEcRB1/LdUSPII complex (Figure 5A, DB1 C3, C4) exhibits increased band intensity on the EMSA gel over that of the corresponding DmEcRB2/LdUSPII complex (Figure 5A, DB2 C2) in response to murA. In the presence of 2.5µM murA, the relative mobility of the DmEcRB2/LdUSPII complex (Figure 5A, DB2 C2) was lower (the band migrated a shorter distance through the polyacrylamide gel) than that of the same species DmEcRB2/DmUSPII heterodimer complex (Figure1A, DB2 C1).

The LdEcR isoforms paired with LdUSPII exhibit similar DNA binding characteristics in the presence and absence of ligand. The LdEcRA/LdUSPII heterodimer interacts with the hsp27 EcRE resulting in two distinct complexes observed in the EMSA gel image (Figure 5B, LA C1, C2; Beatty et al., 2009). The addition of 2.5µM murA slightly increased the band intensity but not the relative mobility of the LdEcRA/LdUSPII complexes (Figure 5B, LA C1, C2; Figure 5C). Treatment of the LdEcRB/LdUSPII heterodimer with 2.5µM murA resulted in a band with somewhat greater intensity than the vehicle treatment (Figure 5B, LB C1, Figure 5C).
The DNA binding affinity of both LdEcR isoforms increases when these receptors are coexpressed with DmUSPII. A cross species LdEcRA/DmUSPII heterodimer generated a band (Figure 5B, LA C3) of greater intensity that migrated further into the EMSA gel than the upper LdEcRA/LdUSPII complex (Figure 5B, LA C2). The addition of 2.5µM murA had no effect on the band intensity or the relative mobility of this cross species LdEcRA/DmUSPII complex. The corresponding LdEcRB/DmUSPII heterodimer formed two complexes (Figure 5B, LB C2, C3) that migrated further into the polyacrylamide gel than any other complex containing LdEcRB. This complex appeared unaffected by the addition of hormone, having a similar affinity for the hsp27 EcRE in the presence and absence of murA.

The nature of the DNA interaction is primarily species specific in that it is a product of the EcR present in the receptor complex. Both same species and cross species complexes containing DmEcR exhibit increased DNA binding in response to ligand. As an extension, DmEcRB1 is capable of a ligand induced shift. In contrast, the complexes containing LdEcR isoforms demonstrate no appreciable shift of DNA interacting complexes and exhibit only subtle increases in DNA binding in response to ligand.

Influence of USP DBD on DNA binding characteristics of the ecdysteroid receptor complex (results with USPIII)

A heterodimer containing DmUSPIII with DmEcRB1 demonstrates greater DNA binding and complex formation than the corresponding DmEcRB2/DmUSPIII receptor. The DmEcRB1/DmUSPIII complex demonstrated the ability to bind to the hsp27 EcRE at a low level in the absence of ligand (Figure 5A, DB1 C3). A very faint complex is also observed with the DmEcRB2/DmUSPIII complex in the absence of ligand (Figure 5A, DB2 C2). There is very little increase in DNA binding of the DmEcRB2/DmUSPIII complex in the presence of ligand. In contrast, the addition of 2.5µM murA produces both an increase in signal intensity and a ligand induced shift characterized by the formation of a second complex with the DmEcRB1/DmUSPIII heterodimer (Figure 5A, DB1 C4).

In contrast to the DmEcR/DmUSPIII heterodimers, both LdEcR isoforms evoke DNA binding to the hsp27 EcRE in both the presence and absence of murA when paired with DmUSPIII (Figure 5B, LA C2, LB C1; Beatty et al., 2009). LdEcRA combined with DmUSPIII interacts with the hsp27 EcRE resulting in the two distinct complexes observed in the EMSA gel image (Figure 5B, LA C1, C2; Beatty et al., 2009). The complexes formed by the LdEcRA/DmUSPIII heterodimer exhibit most of their mass in the band of lower electrophoretic mobility, which migrated a shorter distance through the polyacrylamide gel (Figure 5B, LA C2).

DNA binding studies with DmUSPIII demonstrate that of the DmEcRB isoforms only DmEcRB1 is able to compensate for the lack of the USP DBD to interact with the hsp27 EcRE in both the presence and absence of ligand. In contrast, both LdEcR/DmUSPIII pairings demonstrate equivalent basal and ligand induced DNA binding.
Transcriptional activity and DNA binding of the receptor complex is not correlated with USP concentration

Western immunoblots indicate that the relative protein concentration of LdUSP II is much lower than DmUSP II. Despite the relatively weak immunoblot signal (Figure 6), a high level of LdUSP II mediated transcriptional activity is observed in both cross species and same species heterodimers with this receptor (Figure 1A; Figure 4). Conversely, DmUSP III exhibits a greater western immunoblot signal than DmUSP II or either of the LdUSP constructs (Figure 6), although this DBD deficient construct has demonstrated reduced transcriptional capabilities in previous studies (Beatty et al., 2006; 2009). Despite the much lower concentration of LdUSP II in cell culture extracts, the DmEcR isoforms paired with LdUSP II mediate a transcriptional response at least as strong as the corresponding DmEcR/DmUSP II heterodimers when exposed to 2.5 µM murA (Figure 1A). DNA binding studies also demonstrate that complexes containing DmUSP II are capable of a much higher affinity interaction with the hsp27 EcRE in the presence of murA (Figure 5A, DB2 C2, DB1 C1, C2; Figure 5B, LA C3, LB C2, C3) when compared to complexes containing DmUSP III (Figure 5A, DB2 C2, DB1 C3, C4; Figure 5B, LA C2, LB C1). These findings indicate that the modulatory role of USP in the functional ecdysteroid receptor is such that the transcriptional activity and the DNA binding capabilities of the receptor complex are not directly correlated with the concentration of this heterodimeric partner.

Discussion

Earlier studies have utilized the heterologous mammalian cell culture system to assess the influence of the unique N-terminal domains of the D. melanogaster EcR isoforms on the transcriptional capabilities of the functional ecdysteroid receptor and characterized the effects of site directed mutations in the common region of these proteins (Beatty et al., 2006). As an extension, the heterologous cell culture assay has been developed as a tool for assessing the effect of ecdysteroids and agonists on the activity of ecdysteroid receptors from diverse species (Beatty et al., 2009). The in vitro analysis of this study is meant to assess species specific functional attributes of the EcR isoforms and USP from D. melanogaster and L. decemlineata. Cross species comparisons have the potential to reveal unique properties of the proteins that may be important for in vivo function but remain undetected or ambiguous in same species pairings. Such studies may help to interpret the mechanisms by which steroidal and nonsteroidal agonists mediate transcriptional regulation in a species specific fashion.
USP plays an active role in mediating the effects of EcR in the functional ecdysteroid receptor: the functional heterodimer and modes of DNA binding mediate transactivation

The *L. decemlineata* LBD shares all of the structural motifs associated with the superfamily of nuclear receptors although when compared to other insect species only shares about 67% sequence identity in this domain (Ogura et al., 2005). The LBD represents an important juncture in the functional divergence of EcR and USP across insect orders (Bonneton et al., 2003; Iwema et al., 2009). Despite having divergent N-terminal domains that share only a few structural motifs, the *D. melanogaster* EcR DBD shares greater than 90% amino acid identity with the DBD of *L. decemlineata*. Crystallographic studies of the DBD from EcR and USP indicate that the C-terminal extension of the DBD, the T-box, forms an α-helix that allows EcR to interact in a unique fashion with the hsp27 EcRE. The T-box is highly conserved among EcR, RXR, and USP proteins having the amino acid sequence KREAVQEER in DmUSP (Figure 7). This feature conducts the EcR/USP heterodimer with the appropriate orientation to the EcRE, and in the process creates a substantial bend in the helical axis of the DNA molecule (Jakób et al., 2007). Similarly, the RAR/RXR heterodimer can bind a DNA response element via the T-box of RXR and the zinc finger DNA binding motif of RAR. Both of these moieties undergo an extensive conformational change upon dimerization yielding a complex that binds with a defined polarity to the cognate retinoic acid response element (RARE; Rastinejad et al., 2000). The structure of the heterodimeric EcR-USP complex is stabilized in a progressive fashion by sequence specific contacts with the DNA response element allowing cooperative binding of the complex through a series of structural transitions mediated by the T-box (Jakób et al., 2007). These findings are consistent with biochemical analysis of the interaction of the EcR-USP complex with an hsp27 EcRE (Grebe et al., 2003; Azoitei and Spindler-Barth, 2009). The T-box can also act in an inhibitory manner by interfering with contacts on the DNA strand and overlapping portions of the zinc finger binding domain. This feature of the D domain is also thought to perform other regulatory functions in nuclear receptors (Rastinejad et al., 2000). It is interesting to note that *in vivo* studies of a chimeric USP construct with a duplication of the conserved T-box motif, KREAVQEER, caused sudden arrest of development at the prepupal stage in *D. melanogaster usp* mutant larvae (Henrich et al., 2000). The chimeric construct consisted of the DmUSP amino acids 1-178 (A/B and DBD, including the T-box) fused with a three amino acid linker to the *Chironomus tentans* USP (CiUSP) amino acids 175-451 (D domain and LBD). The resulting d/cUSP transformants expressing one copy of the chimeric USP experienced developmental arrest in the late third instar. However, expression of up to four dosages of the d/cUSP construct rescued development of the mutant larvae (Henrich et al, 2000). This result implies that the duplicate T-box in the d/cUSP construct may interfere with the cooperative effect that DNA binding has on heterodimerization and subsequent regulatory functions performed by USP within the heterodimer.

Modes of regulation seem to be conserved across members of this superfamily of nuclear receptors. RXR, the vertebrate homolog of USP, is able to heterodimerize with thyroid receptor (*T3r*) and RAR and mediate transcription via a human promoter element. The nuclear receptor corepressor (NCoR) is recruited by the hinge region of *T3R* and RAR and mediates ligand independent repression of the
heterodimer complex. In the presence of ligand, NCoR remains bound to the heterodimer, indicating that the hinge region and LBD of both TR and RXR undergo substantial conformational changes to release the corepressor when the heterodimeric complex binds DNA. The specific conformational change in the protein complex is dependent on the nature of the DNA binding site (Hörlein et al., 1995). For example, RXR heterodimers selectively recognize the geometry of direct repeats of the half site 5’ AGGTCA 3’ separated by 1-5 base pairs (Umesono et al., 1991; Mangelsdorf and Evans, 1995). In the case of the TR/RXR heterodimer, structural studies of the DBDs from these receptors have revealed that cooperative asymmetric binding can only occur when the response element half sites are separated by 4bp (Rastinejad et al., 1995). Deletion of the N-terminal domain of USP exhibited no effect on transactivation of the TR/USP heterodimer via a human response element. A USP mutant with an impaired DNA binding domain was able to heterodimerize with TR but unable to interact with a promoter to mediate transcriptional activity, indicating that transactivation is a function of TR requisite on dimerization with USP (Hatzivassilou et al., 1997). Consistent with this finding, DmUSPIII is able to form a heterodimer with the three DmEcR isoforms, but is unable to mediate transcriptional activity with the DmEcRA and DmEcRB2 isoforms.

As with TR, transactivation seems to be a function mediated by interaction of the unique N-terminal domain of DmEcR with the DBD and D domain or C-terminal extension (the T-box) of USP. Both of these moieties facilitate heterodimerization and enable interaction with the hsp27 EcRE. EMSA analysis demonstrates that DmEcRB1 and DmEcRB2 retain the ability to interact with the hsp27 EcRE (Figure 5A), but only DmEcRB1 retains the ability to mediate basal and induced transcriptional responses in the presence of the DBD deficient DmUSPIII (Beatty et al., 2006). The retention of transcriptional activity mediated by the DmEcRB1/DmUSPIII heterodimer provides an indication that in the absence of the D. melanogaster DBD the DmUSP D domain performs a function necessary for the heterodimer to achieve transactivation. This result also implies that the DmUSP D domain mediates an interaction involving the isoform specific N-terminal domain of DmEcR. Conversely, DmUSPIII paired with either LdEcR isoform exhibits normal interaction with the hsp27 EcRE in EMSA analysis (Figure 5B) but demonstrates severely reduced basal and induced transcriptional capabilities (Beatty et al., 2009). An observation indicating that the D domains from USPs of different species do not perform equivalent functions in their corresponding ecdysteroid receptors.

The presence of USP plays a role in ligand affinity of EcR that affects DNA binding (Azoitei and Spindler-Barth, 2009) and subsequently stabilizes heterodimer formation (Rastinejad et al., 2000). Full length DmUSPII evokes a conformation of the functional ecdysteroid receptor that allows the complex to bind the hsp27 EcRE with a higher affinity. This result is further supported by recent studies showing nearly identical ligand binding affinity of the DmEcR isoforms in the presence of the hsp27 EcRE if a full length DmUSP is also present (Azoitei and Spindler-Barth, 2009). When DmEcR isoforms are paired with the DBD deficient DmUSPIII in the presence of the hsp27 EcRE an isoform specific effect on ligand binding is observed. Under such conditions, the ligand affinity of the DmEcRB1/DmUSPIII heterodimer is significantly higher than that of the corresponding DmEcRB2/DmUSPIII pairing (Azoitei and Spindler-Barth, 2009). This finding is confirmed by results
of the current study demonstrating the significantly greater DNA binding affinity of DmEcRB1 over that of DmEcRB2 with a DBD deficient DmUSP.

Deletion of the DmUSP DBD causes a reduction in basal and hormone induced DNA binding among all DmEcR isoforms (Braun et al., 2009). The formation of similar complexes on EMSA gels when DmEcRB1 is paired with either LdUSP II or DmUSP III suggests a species specific effect on DNA binding mediated by USP via the EcR isoform specific N-terminal region. Recent studies have associated a motif specific to the DmEcRB1 N-terminal domain with a stabilizing effect on the antagonistic position of helix 12 in the LBD (Tremmel et al., 2011). Because the EcR LBD forms the dimerization interface with USP, the conformation provided to the heterodimer via this interdomain interaction allows the T-box of USP to conduct the complex to the appropriate half site of the hsp27 EcRE with a geometry that facilitates DNA binding. Rastinejad et al. (2000) demonstrated that the T-box of RXR coordinates such a structural transition thereby modifying the position of the upstream zinc finger and resulting in a cooperative conformation change that facilitates DNA binding and dimerization. The similar electrophoretic mobility of DmEcRB1/DmUSP III and the DmEcRB1/LdUSP II complexes in the presence of murA could indicate that the DBD deficient DmUSP III is capable of performing a regulatory function similar to that of LdUSP II. The complexes formed with the DmEcRB1/DmUSP III and DmEcRB1/LdUSP II heterodimer are of equivalent relative mobility. This finding, coupled with transcriptional data demonstrating severely reduced transcriptional activity for DmUSP III with DmEcRA and DmEcRB2 but not with DmEcRB1 (Beatty et al., 2006), suggests that the DmUSP D domain may function in a repressive fashion in the absence of the DmUSP DBD.

Recent studies provide evidence that the sequence downstream of the T-box of EcR, known as the hinge region or D domain, may be involved in molecular recognition for protein modification and cofactor interactions. These regions of low structural complexity and high flexibility are thought to allow multiple binding partners and facilitate involvement in multiple signaling pathways (Jakób et al., 2007). In D. melanogaster USP, the D domain is a glycine rich region similar to that of the mammalian transcription factor Yin-yang 1 (YY1), which has been shown to interact with the histone deacetylase RPD3 to mediate repression of target genes (Yang et al., 1996). Although there is no direct evidence to support the recruitment of such repressive factors by the D domain of DmUSP, the unique sequence and transcriptional capabilities of this USP indicate a yet undetermined function within this domain. The role of this domain may be the active recruitment of such corepressors or it may be a passive role creating flexibility within the molecule to allow proper positioning of the isoform specific N-terminal domains and common DBD relative to the LBD of the protein. Such a passive role would be conceivable, as it would explain how the D domain of DmUSP provides a physical link between the ligand independent activation function (AF1) of the AB domain and the ligand dependent activation function (AF2) of the LBD. More precisely, the T-box or C-terminal extension is the only portion of the DmUSP D domain shown by structural studies to contain an α-helix followed by two amino acids (Q175 and R178) that form direct phosphate linkages with the minor groove of the DNA response element. Also, several specific amino acids in the zinc fingers of the DmUSP DBD are known to form direct contacts for the dimerization interface with DmEcR (Jakób et al., 2007). The glycine rich portion of the Drosophila D domain shares no similar residues with USP amino acid
sequences from other species (Figure 7) and according to protein prediction algorithms, is a coiled region containing no distinct structural motifs. Flexibility in this region allows the molecule to coordinate the structural transitions necessary to incorporate DNA binding and recognition of the hsp27 EcRE with ligand binding. The D domain may help to coordinate these events by facilitating changes in the three dimensional structure of the heterodimer that are promoted by the geometry of the DmUSP DBD relative to the position of its LBD. Specifically, the unique DmUSP D domain may provide flexibility in the molecule that allows multiple conformations of the ecdysteroid receptor complex. This possibility would account for multiple structural arrangements of the EcR/USP heterodimer that facilitate dimerization, interaction with ligands and cofactors or corepressors, and graded recognition of a number of DNA response element sequences. Transactivation studies with DmUSPIII agree that the USP DBD is necessary to achieve a functional conformation of the heterodimer with DmEcRA and B2 isoforms (Beatty et al., 2006, 2009). DmUSP likely requires the DBD in order to achieve a geometry in the protein that facilitates cooperative binding to DNA and supports dimerization. DmUSPIII retains some degree of transactivation with DmEcRB1 because the longer AB domain of this isoform supports a structural transition in the heterodimer that allows interaction with the hsp27 EcRE. The severe reduction in basal and murA induced transcriptional activity with DmEcRA and DmEcRB2 and the lack of DNA binding in EMSA analysis demonstrates the inability of DmUSPIII to guide the heterodimer to the 3' binding site of the EcRE. This failure of transactivation and DNA binding indicate a role of the EcR AB domain that is modulated by the DmUSP DBD. In contrast, the DmUSP DBD is unnecessary for the LdEcR/DmUSPIII heterodimers to achieve a conformation of the receptor complex that is capable of interaction with the hsp27 EcRE.

RXR and RAR are known to function in a cooperative manner to facilitate DNA binding and dimerization. The cooperative effect is evoked when one protein binds to the DNA causing a bend in the helix that allows the other protein of the transcription factor complex to bind. Such structural distortions of the DNA have been associated with recruitment of other regulatory factors to the complex (Fujii et al., 1999, Rastinejad et al., 2000). This effect may reconcile the retention of DNA binding capabilities of the LdEcR/DmUSPIII complexes exhibited by EMSA analysis (Figure 5B) with the significantly reduced basal and induced activity observed in the transcriptional assays (Beatty et al., 2009). More specifically, a change in the geometry of the complex relative to the DNA associated with the deletion of the USP DBD affects interaction of the complex with corepressors. This results in a complex that can effectively bind DNA, but fails to mediate transcriptional activity.

As mentioned previously, the differences observed with the DBD deficient USP proteins may underlie the difference between passive and active repression. Hörllein et al. (1995) demonstrated that repression by NCoR bound to the RAR/RXR heterodimer functions in a ligand independent fashion where the conformational change associated with DNA binding of the receptor complex evokes a substantial enough conformational change to release the bound corepressor. Typically, such active derepression would transpire through a conformational change, precipitated by ligand, that subsequently releases the bound corepressor.

A number of amino acid motifs have been characterized in proteins that mediate repression of gene transactivation (Chen et al., 1993; Paroush et al., 1994; Dawson et al., 1995; Smith and Jaynes,
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1996; Jimenez et al., 1997, 1999). One such moiety, the LXXLL amino acid sequence, has been associated with binding of a number of factors that modulate activation of target genes. Such factors participate in active repression where the corepressor is able to interact with the binding groove of the receptor in the absence of ligand. The binding groove is a cleft formed on the outer surface of a protein that facilitates interaction with other proteins. Such a moiety is generally hydrophobic in nature and provides an interface by which a number of nuclear receptors interact with corepressors bearing the characteristic amphipathic helix of the LXXLL motif. Interaction with ligand evokes a conformational change in the protein that disrupts the binding groove of the receptor thereby releasing the corepressor. The antagonistic position of DmUSP helix 12 obstructs the binding groove for LXXLL corepressor binding motifs (Billas et al., 2001). Another *Drosophila* nuclear protein, *Drosophila* hormone receptor 38 (DHR38; Sutherland et al., 1995), has been shown to interact strongly with USP. Structural studies of DHR38 indicate that the binding groove of this protein, normally associated with LXXLL interactions, is obstructed by hydrophilic amino acid residues. Interestingly, the DHR38/USP heterodimer is able to mediate transcriptional events in response to ecdysteroids (Baker et al., 2003). *D. melanogaster* USP is typically described as being locked in an inactive conformation, although it may be better described as being locked in a semi-active conformation in which active modes of repression are mediated by coordinated conformational changes of USP in the functional ecdysteroid receptor including changes mediated by recognition of and binding to specific response elements.

Effects of various ligands in cross species pairings (may underlie the modes of action of JHIII): Modes of action for JHIII coordinated by ecdysteroids and agonists

Despite similarities with RXR, USP demonstrates a distinct structural and functional divergence from orthologous nuclear proteins. Recent findings by Tremmel et al. (2011) indicate that USP serves more than a passive role in the functional ecdysteroid receptor by contributing to heterodimer stability. In this manner, USP influences the DNA binding properties and subsequent transcriptional capabilities of the receptor complex. Such studies paired with evolutionary and structural analysis of these proteins indicate that the unique antagonistic position of the USP helix 12 in Dipteran and Lepidopteran insects plays a large role in modulating this interaction. This position of helix 12 covers the permanently bound lipid molecule in the ligand binding pocket created by the DmUSP LBD (Billas et al., 2001). Structural studies indicate that this ligand binding pocket is large enough to accept a substantially larger ligand (Clayton et al., 2001).

Studies of the EcR/USP heterodimer have established that two distinct ligands can mediate different effects via a single response element (Forman et al., 1995) a result that has been demonstrated in a number of *in vitro* transcriptional assays and biochemical assays (Vöglti et al., 1999; Lezzi et al., 2002). The extreme flexibility of the EcR LBD allows the protein to adopt a conformation that can accept a number of ligands, an effect that is further modulated by a series of structural transitions with USP that coordinate binding of the EcR/USP complex to the hsp27 EcRE. Species specific affinity for
ligand has been established particularly for the nonsteroidal ecdysone agonist diacylhydrazines (Carlson et al., 2001). Carmichael et al. (2005) characterized a second lobe of the *Heliothis virescens* EcR LBD that extends into the ligand binding pocket. Such variation is thought to form the basis for preferential binding of the diacylhydrazines to Lepidopteran receptors. Studies of the evolutionary divergence of Lepidopteran and Dipteran EcR and USP from receptors of other species indicate that the unique torsional ability of helices within the USP LBDs of these species achieve a dimerization interface with larger surface area thereby stabilizing heterodimer formation (Iwema et al., 2009). Consistent with these findings, the potentiation effect is preserved when DmEcR isoforms are paired with either DmUSPII or LdUSPII proteins in the presence of murA and JHIII, although JHIII alone is unable to evoke an effect (Beatty et al., 2006, 2009). When challenged with ligands other than murA in the presence of JHIII potentiation is significantly reduced. A result indicating that potentiation is largely mediated by the LBD of the EcR in a species specific fashion. Potentiation is reduced when the LdEcR isoforms are paired with DmUSPII providing evidence that potentiation is largely mediated by the modulatory effect of USP on the functional ecdysteroid receptor. This finding suggests that the potentiation effect is modified through an interaction between the shared LBD and the unique AB domains of the EcR isoforms thereby accounting for the isoform specific nature of this interaction. The reduced potentiation with the LdEcR/DmUSPII heterodimers provides an indication that potentiation is facilitated by the ligand binding properties of EcR and further mediated by the modulatory effect of USP on the functional ecdysteroid receptor.

JHIII is not alone in its ability to potentiate a transcriptional response. Other precursors of JHIII and components of the mevalonate pathway such as farnesoic acid and methylfarnesoate interact with the DmEcR/DmUSP heterodimer to mediate potentiation in the presence of murA (Henrich et al., 2009), a finding that has led to the hypothesis that the combined effect of mevalonate pathway components serve as an indicator of nutritional status in developing insects. It is well known that such juvenoids interact with the EcR/USP complex, but the exact mechanisms of potentiation are largely unknown. It is likely that there are multiple modes of action for juvenoids.

In *D. melanogaster* mutations of the gene methoprene tolerant (Met) confer resistance to methoprene (Ashok et al., 1998), an analog of JHIII that would normally cause lethality in developing flies. Met is a member of the basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) transcription factor family known to interact with EcR and USP (Li et al., 2007) and mediate transcription of target genes in response the JH (Li et al., 2011). Members of the bHLH-PAS family of transcription factors are capable of protein-protein interactions via the HLH and PAS domains. The presence of a predominantly basic region preceding the HLH moiety enables a dimer of bHLH transcription factors to recognize half-sites and bind specific DNA response elements. Recent structural studies have shown that PAS domains are also capable of direct interaction with ligands to mediate transcriptional activity (Scheuermann et al., 2009). Met and FISC, another bHLH-PAS protein from the mosquito *Aedes aegypti*, form a heterodimer in the presence of JHIII that interacts with the juvenile hormone response element (JHRE) to mediate transcription of target genes in response to JHIII (Li et al., 2011). FISC, a homolog of the *D. melanogaster* gene Taiman, is a known coactivator of the functional ecdysteroid receptor complex. Flexibility within the EcR ligand binding pocket enables the receptor to adopt a
conformation capable of interaction with a variety of steroidal and nonsteroidal ecdysone agonists (Billas et al., 2003). The transcriptional capabilities of same species pairings of EcR and USP in a heterologous mammalian cell culture system indicate that these ligand induced conformational changes may affect the recruitment of Met or other modulatory cofactors to the functional ecdysteroid receptor complex in a species specific fashion (Beatty et al., 2009). This possibility can be reconciled with the outcome of the cross species comparisons of the current study. In every cross species pairing the type of EcR present mediates the transactivation capabilities of the receptor complex in response to a range of ligands. The effect is potentiating with JHIII, in a species specific fashion, to the extent that USP will allow. This observation leaves open the possibility that JHIII mediates transcription by direct interaction with USP. It is known that a USP homodimer is capable of high affinity binding with JHIII (Jones et al., 2006). Although no change in transcriptional activity is observed when either same species or cross species EcR/USP heterodimers are challenged with JHIII, cooperative structural transitions induced by ligand interactions with EcR and the presence of a DNA response element may lead to a conformation of USP capable of direct interaction with JHIII. Alternately, this conformational change may just as easily lead to an interaction with another cofactor such as MET (or a MET homolog present in the mammalian cells), which is known to evoke transcription of target genes in response to JHIII (Li et al., 2011). In this mode of action the overall conformation conferred on the ligand binding pocket of EcR would determine the extent of JHIII mediated transcriptional events. More specifically, potentiation occurs when the altered shape of the EcR ligand binding pocket leads to a stable dimerization interface causing a subsequent conformational change that increases the affinity of USP for the JHIII bound cofactor. This transition could conceivably precipitate the recruitment of other cofactors as well as dissociation from repressive factors thereby allowing the complex to bind with the appropriate orientation to the hsp27 EcRE. The geometry of this complex is critical to the establishment of sequence specific contacts with the DNA that, in turn, recruit USP to the 5’ half site of the hsp27 EcRE. The exact mechanism by which JHIII modifies the action of the ecdysteroid receptor has yet to be elucidated.
Literature


Figure 1. Effects of JHIII on murA mediated transcriptional activity with A) DmEcR with DmUSPII or LdUSPII and B) LdEcR with LdUSPII or DmUSPII. All luciferase activity is reported relative to DmEcRB2/DmUSPII basal activity =1 (not shown).
Figure 2. Effects of JHIII on RH2485 mediated transcriptional activity when tested with A) DmEcRB1 heterodimers, B) DmEcRB2 pairings and C) combinations of LdEcRB1 with LdUSP II and DmUSP II.
Figure 3. Effects of JHIII on transcriptional activity mediated by DmEcRB isoforms and LdEcRB1 paired with either DmUSPII or LdUSPII in the presence of 20E.
Figure 4. Fold induction caused by maximal dosages of murA, 20E, and RH2485 with cross species LdEcR/ LdUSPII heterodimers compared to LdEcR/ DmUSPII transcriptional responses.
Figure 5. EMSA showing the interaction of EcR and USP from CHO cell extracts with a P\textsuperscript{32} labeled hsp27 EcRE probe. A). D. melanogaster EcR isoforms with either DmUSP variants or LdUSP\text{II}. B). L. decemlineata EcR with LdUSP\text{II} or DmUSP\text{ variants} and C). same species pairings (taken from Beatty et al., 2009, Figure 2A). Complexes indicated by arrows are labeled according to the EcR they contain (DB1, DB2, LA, LB) and numerically (C1- C4) relative to the complexes formed by single species heterodimers. Protein quantities were normalized and loaded according to β-galactosidase activity.
Figure 6. Western blot of *D. melanogaster* and *L. decemlineata* USP proteins probed with anti-VP16 primary antibody. Cell extracts from vehicle treatments were normalized for loading onto the polyacrylamide gel according to β-galactosidase reporter gene activity.
Figure 7. Clustal W amino acid alignment of the DBD C-terminal extension (D domain) from human RXRα (hsRXRα), LdUSP, DmUSP, and CfUSP. Boxed regions indicate α-helices. The D domain of each sequence is shaded. Bold type indicates identity across amino acid sequences. Arrows indicate amino acid residues (R153, N154, Q157, R160, Q175 and R178 in DmUSP) that interact directly with DNA as observed by Jakób et al (2007). Amino acid residues of the zinc finger α-helix corresponding to positions R153, N154, and R155 in DmUSP have been implicated in dimerization (Jakób et al., 2007).
Summary

The studies presented here represent a progressive approach to characterize the functional attributes associated with EcR and USP from *D. melanogaster* and *L. decemlineata*. In order to first characterize *D. melanogaster* USP, the yeast two-hybrid assay was employed to assess the effect of site-directed mutation of specific amino acid residues on functions associated with the USP LBD. These results paired with earlier studies, which characterized specific amino acid residues of the EcR LBD (Grebe et al., 2003) and determined the nature of heterodimerization between the EcR and USP (Lezzi et al., 2002), established that these domains can function autonomously in the fusion proteins used for *in vitro* analysis. While this approach facilitated the characterization of proteins through mutational analysis, it is limited to a domain specific portion of the functional possibilities associated with nuclear receptor function. The heterologous mammalian cell culture system afforded the possibility to examine the effects of whole *D. melanogaster* receptors in a cellular context. This system was preferred over an insect cell culture system because all of the components necessary to evoke expression of the reporter gene are transfected exogenously into the system. This characterization of *D. melanogaster* EcR and USP in the heterologous mammalian cell culture system provided a platform for the controlled assessment of receptors from *L. decemlineata*. Once the transcriptional activity, ligand binding, and DNA binding properties of the receptor had been characterized for the EcR isoforms and USPs of both species, cross species comparisons offer an additional look at properties unique to the receptors of both species.

The yeast two-hybrid analysis demonstrated that the USP LBD affects DNA binding and transactivation capabilities of the ecdysteroid receptor. Mutants with a deletion of helix 12 were capable of dimerization but eliminate ligand binding to the EcR/USP complex and are unable to bind DNA. The antagonistic position of helix 12 also proved to be important. The helix 12 mutation L490R evoked normal DNA binding and dimerization capabilities, but severely reduced the ligand binding capability of the ecdysteroid receptor. This finding demonstrates the importance of the antagonistic position of the USP helix 12 in ligand induced transactivation. Mutations to all but one of the hydrophobic residues of the USP ligand binding pocket that interact with the bound phospholipid affected ligand binding to the heterodimer and consequently eliminated transcriptional activity of the heterodimer. The mutations I323A and I323V resulted in somewhat lower ligand binding properties, but had a superinductive effect on transcriptional activity. These results illustrate the importance of intermolecular interactions of the USP LBD in modulating transactivation of the ecdysteroid receptor.

The heterologous mammalian cell culture system was utilized to analyze the transcriptional activity of the *D. melanogaster* EcR isoforms. This study revealed that the EcR/USP heterodimer exhibits an isoform specific pattern of transcriptional activity in which EcRB1 mediated the highest absolute levels of basal and ligand induced transcriptional activity. The lowest basal and ligand induced transcriptional activity was observed with EcRA and an intermediate level of transcriptional activity was evoked with DmEcRB2. The EcR isoforms also demonstrate potentiability in the presence of a submaximal dosage of murA and JHIII to maximum transcriptional activity. The USP DBD plays an important role in transactivation that demonstrates an isoform specific function of EcR. A DBD
deficient USP, USPIII, evokes a normal basal and ligand induced transcriptional response with EcRB1, but exhibits severely reduced basal and ligand induced transcriptional capabilities with EcRA and EcRB2. An amino acid substitution in the conserved EcR LBD, K497E, elucidates an isoform specific effect. EcRB2 K497E causes a nearly four fold increase in the basal levels of transcription observed with this receptor. The mutant also demonstrates increased affinity for the hsp27 EcRE in EMSA studies. EcRA and EcRB1 K497E exhibited only a slight increase in basal transcriptional activity. All of the K497E mutants maintained normal transactivation capabilities. By using the mammalian cell culture system to analyze the characteristics of both mutant and non-mutant EcR isoforms and USP, this study formed the basis for characterizing the receptors of other species.

Based on the characterization of EcR and USP from *D. melanogaster*, the heterologous cell culture system provides a platform by which to characterize EcR and USP from diverse species. In this respect, the cell culture system was used to analyze the properties of EcR and USP from *L. decemlineata*. The system was then developed as a screening tool to examine the effect of ligands, or insecticidal candidate compounds, on ecdysteroid receptor function. Like the receptors of *D. melanogaster*, the LdEcR isoforms exhibit isoform specific capabilities in heterodimers with LdUSP. Generally, the basal and induced profiles of transcriptional activity were species specific. The LdEcR/LdUSPII receptors produced the greatest transcriptional activity in response to nonsteroidal ecdysone agonists. In contrast, the DmEcR/DmUSP were more sensitive to murA. Potentiation was observed with receptors from both species in response to murA and JHIII. However, JHIII was unable to potentiate a response with the nonsteroidal agonist RH2485 in either species. EMSA studies revealed that the LdEcR/LdUSP receptors interact with DNA in a manner independent of ligand, whereas the DmEcR/DmUSP receptors demonstrate different basal and induced affinities for the hsp27 EcRE.

Cross species receptor combinations revealed the species specific function of USP in the ecdysteroid receptor. When the DmEcR isoforms were tested with LdUSPII, the basal levels of transcriptional activity were lower and the murA induced level of transcriptional activity was higher than for the corresponding same species DmEcR/DmUSPII heterodimer. Conversely, when the LdEcR isoforms are paired with DmUSP the basal levels of transcriptional activity remain unchanged. However, the inductive response is lower than with LdEcR/LdUSPII receptor combinations. The cross species DmEcR/LdUSPII displayed a distinct ligand induced shift in the EMSA gel, but displayed diminished basal DNA binding affinity. The basal DNA binding affinity of LdEcR isoforms increased with DmUSPII but displayed no ligand induced shift or increase in DNA binding. These studies form a basis to characterize the capabilities of receptors from other insect species and to further develop the heterologous cell culture system as a tool to understand the roles of EcR and USP in the functional ecdysteroid receptor.
Publications and manuscript

Contribution to publications and manuscript

Publication I:
Designed mutagenic primers and conducted site-directed mutagenesis for all mutations presented in this study. Verified mutant clones by DNA sequencing.

Publication II:
Designed and subcloned the USP constructs utilized in this study. Verified USP constructs by DNA sequencing. Responsible for all aspects of site-directed mutagenesis including mutagenic primer design. Verified mutant clones by DNA sequencing. Conducted all cell culture experiments and analyzed transcriptional activities. Performed western blot of USP constructs. Participated in DNA binding analysis. Produced all figures used in publication. Authored the experimental procedures section.

Publication III:
Designed and subcloned the LdEcR and LdUSP constructs used in this study. Verified all L. decemlineata constructs by DNA sequencing. Designed, performed, and analyzed all cell culture experiments presented in this study. Conducted Western blots and EMSAs. Constructed all figures used in the publication. Authored the experimental procedures.

Publication IV:
Designed and conducted cell culture experiments. Performed data analysis and created figures for transcriptional activity presented in the publication.

Manuscript V:
Designed and conducted all cell culture experiments, EMSAs, and Western blots in this study. Prepared all data and figures. Authored the manuscript.
Presentation of results at meetings

47th Annual Drosophila Research Conference 2006
29. March- 2. April 2006, Houston, Texas, USA

Poster:

Beatty J, Callender J, Weinberger C, Henrich VC.
Mutational analysis of Drosophila EcR and USP in a heterologous cell culture system.

North Carolina Academy of Sciences 2008
25. March 2008, Greensboro, NC, USA

Presentation:

Characterization of the ecdysteroid receptor isoforms of Drosophila melanogaster

Fifth International Symposium on Insect Molecular Science 2006
20.-24. May 2006, Tucson, Arizona, USA

Posters:

Beatty J, Callender J, Fauth T, Weinberger C, Spindler-Barth M, Henrich VC.
Analysis of transcriptional activity mediated by Drosophila melanogaster ecdysone receptor isoforms in a heterologous cell culture system.

Weinberger C, Beatty J, Henrich VC.
Roles for EcR and its mammalian orthologue FXR as targets for natural and synthetic juvenile hormones and insecticides which disrupt a non-sterol isoprenoidal flux which controls cell growth.

16th International Ecdysone Workshop 2006

Poster:

17th International Ecdysone Workshop 2008
20.- 24. July 2008, Ulm, Germany

Poster:

Effect of juvenile hormone mimics and insect growth regulators on ecdysteroid activity in a heterologous cell culture assay.
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Erklärung

Ich versichere hiermit, dass ich die Arbeit selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt sowie die wörtlich oder inhaltlich übernommen Stellen als solche kenntlich gemacht habe.

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