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FUNCTIONAL ANALYSIS OF ECDYSONE RECEPTOR

DISSERTATION

Zur Erlangung des Doktorgrades (Dr.rer.nat.)
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Anca Azoitei

aus
Iasi, Romania

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Amtierender Dekan:

Prof. Dr. Peter Bäuerle

Erstgutachter:

Prof. Dr. Margarethe Spindler-Barth

Zweitgutachter:

Prof. Dr. Bernhard Eikmanns

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"DNA affects ligand binding of the Ecdysone receptor of Drosophila melanogaster"
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Braun S., **Azoitei A.,** Spindler-Barth M.,
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"Functional analysis of Ecdysteroid receptor from *Drosophila melanogaster* In vitro"
Azoitei A. Ruff H., Tremmel C., Braun S., Spindler-Barth M.,
Ecdysones, Structures and Functions, Smagghe G. (ed.) *Springer Verlag, Berlin (2009)*

"Influence of hormone on intracellular localization of Drosophila melanogaster ecdysteroid receptor (EcR)"
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SUMMARY

Ecdysteroids are important regulators of insect development. They perform their actions through intracellular receptors belonging to the superfamily of nuclear receptors (NRs).

In the present study, the relative performance of the three *Drosophila* ecdysone receptor (EcR) - isoforms in terms of their affinity to the ligand Ponasterone A in mammalian cell line CHO-K1 was determined.

In the absence of a heterodimerization partner hormone binding of EcR is rather weak. The presence of ecdysone response elements (EcREs) stimulates the ligand binding to different degrees depending on the EcR-isoform involved.

When Ultraspiracle (Usp), the invertebrate orthologue of mammalian RXR, is used as dimerization partner, all EcR isoforms of *Drosophila* bind the ligand Ponasterone A with the same high affinity already in the absence of EcREs.

Depending on the EcR isoform, Usp variant and EcREs, addition of DNA results in augmented ligand binding to the heterodimer EcR/Usp.

Ligand binding to heterodimers containing wild type Usp is enhanced about 5 fold with hsp27, Pal-1 and DR-1 response elements. The same results are obtained with monomeric and pentameric EcREs.

In the absence of EcREs, hormone binding is not affected if the A/B domain of wild type Usp is replaced by the activation domain (AD) of herpex simplex virus (VP16) to circumvent the inhibition of transcriptional activity of the wild type Usp. By contrast, substantial differences were observed with VP16-Usp fusion proteins in the presence of EcREs. While hsp27 monomers have no effect, the use of pentameric hsp27 enhances the ligand binding to EcRs/VP16-Usps heterodimers.

In the presence of Pal-1 and DR-1, an increase in ligand affinity is already observed with EcRE monomers and is further enhanced with 5x Pal-1 and 5x DR-1. Ligand affinity is particularly improved (about 10 fold) in the

presence of all EcREs, if UspIII - an Usp-variant lacking the C-domain (UspΔDBD) - is used as heterodimerization partner.

RXR in contrast to Usp confers ligand affinity to the receptor complex only in the presence of an EcRE.

Finally, specific mutations in the EcR ligand binding domain (LBD) showed an abolishing effect on the ligand binding function of this receptor.

ZUSAMMENFASSUNG

Ecdysteroide sind wichtige Regulatoren der Insektenentwicklung. Sie wirken über intrazelluläre Rezeptoren welche zur Superfamilie der nukleären Rezeptoren gehören.

In der vorliegenden Doktorarbeit wurde die relative Affinität der 3 Drosophila Ecdyson-Rezeptor(EcR)-Isoformen gegenüber dem Liganden Ponasteron A in der Säugerzelllinie CHO-K1 untersucht.

Ohne Heterodimerisierungspartner ist die Hormonbindung von EcR verhältnismäßig schwach. Wird jedoch Ultraspiracle (Usp), das Invertebraten Ortholog des Säuger-RXR als Dimerisierungspartner verwendet, so binden alle Drosophila EcR-Isoformen den Liganden Ponasteron A mit gleich hoher Affinität bereits in Abwesenheit von EcREs.

Die Anwesenheit von Ecdyson-responsiven-Elementen (EcREs) stimuliert die Ligandenbindung in unterschiedlichem Maße abhängig von den beteiligten EcR-Isoformen.

Abhängig von der EcR-Isoform, der Usp-Variante und der Art des EcREs, führt die Zugabe eines response Elements zu einer gesteigerten Ligandenbindung des Heterodimers EcR/Usp.

Die Ligandenbindung von Heterodimeren welche ein Wildtyp-Usp enthalten ist ca. um das 5-fache bei hsp27, Pal-1 und DR-1 Response-Elementen gesteigert. Die gleichen Ergebnisse wurden sowohl mit monomeren als auch mit pentameren EcREs erhalten.

In Abwesenheit von EcREs ist die Hormonbindung nicht beeinflusst wenn die A/B-Domäne des Wildtyp-Usp durch die Aktivierungsdomäne (AD) des Herpes Simplex Virus' (VP16) ersetzt wird um die Inhibition der transkriptionellen Aktivität des Wildtyp-Usp zu umgehen. Im Gegensatz dazu wurden in Gegenwart von EcREs mit VP16-Usp-Fusionsproteinen erhebliche Unterschiede beobachtet. Während monomere hsp27 response Elemente keinen Effekt haben, verstärkt die Verwendung von pentameren

hsp27 response Elementen die Ligandenbindung an EcRs/VP16-Usp-Heterodimere. Dahingegen wird mit monomeren Pal-1 und DR-1 response elementen ein Anstieg der Ligandenaffinität beobachtet, der mit 5x Pal-1 und 5x DR-1 weiter verstärkt wird. Die Ligandenaffinität ist vor allem dann in der Anwesenheit von EcREs gesteigert (um das 10-fache) wenn UspIII – eine Usp-Variante der die C-Domäne fehlt (UspΔDBD) - als Heterodimerisierungspartner benutzt wird.

RXR vermittelt im Gegensatz zu Usp die Ligandenaffinität des Rezeptorkomplexes nur in Anwesenheit eines EcRE.

Abschließend führten spezifische Mutationen in der EcR Ligandenbindungsdomäne (LBD) zur Aufhebung der Ligandenbindungsfunktion dieses Rezeptors.

1. Introduction

1.1 Nuclear receptors

Nuclear receptors are ancient proteins found in sponges, echinoderms, arthropods and vertebrates and shown to function as ligand-activated transcription factors which work in concert with co-activators and co-repressors to regulate gene expression (Jones and Thummel, 2005), responsible for cell growth, differentiation, homeostasis.

Phylogenetic analysis has revealed six distinct subfamilies of nuclear receptors (Laudet, 1997). One big family encloses: thyroid hormone receptors (TRs), retinoic acid receptors (RARs), vitamin D receptors (VDRs), peroxisome proliferator-activated receptors (PPARs), ecdysteroid receptors (EcRs) and “orphan receptors”, for which regulatory ligands are not known. Some of nuclear receptors act in a constitutive manner whereas others can be activated by phosphorylation. A second family is comprised of retinoid X receptors (RXRs), chicken ovalbumin upstream stimulators (COUPs), hepatocyte nuclear factor 4 (HNF4), testis receptors (TR2) and receptors involved in eye development (TLX and PNR). The third family consists of: steroid receptors and the estrogen-related receptors. To the fourth, fifth, and sixth families belong orphan receptors NGFI-B, FTZ-1/SF-1, and GCNF.

Independent of this classification, the nuclear receptors are modular in structure (Thompson et al., 1999; Klinge, 2000) and they are composed of five to six domains. The N-terminal A/B-domain is highly variable in sequence among nuclear receptors and harbours an autonomous transcription activation function, called AF-1, which can synergise with AF-2 (in the E-domain) in the presence of hormone to modulate gene expression. The C-domain or DNA-binding domain (DBD) is highly conserved and

contains two zinc-fingers, capable of recognizing specific sequences of DNA called hormone response elements (HRE). The D-domain or hinge region is responsible for intracellular trafficking and subcellular distribution. The E-domain or ligand binding domain (LBD) harbours the ligand-binding site and a ligand-dependent transcription activation function (AF-2). In addition to the DBD, the LBD is involved in dimerization of the receptor and binds coactivator and corepressor proteins. Few members of the nuclear receptor superfamily possess an F-domain, the function of which is not yet well elucidated (Figure1).

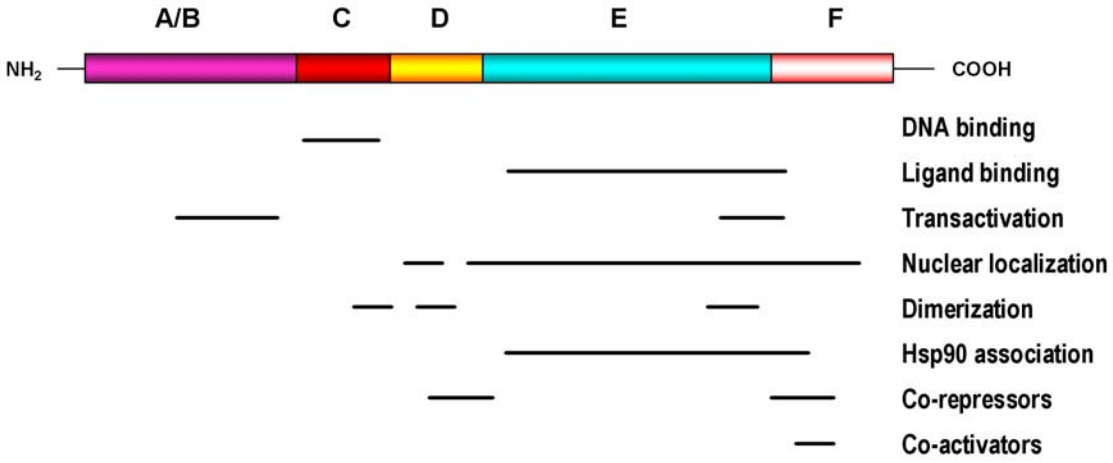


Figure 1 Schematic representation of modular nuclear receptor. A typical nuclear receptor is comprised of following domains: N-terminal A/B transactivation domain, conserved DNA-binding domain (C), hinge region or D-domain which connects the DBD and the conserved ligand binding domain (LBD or E-domain). At the C-terminus of some nuclear receptors there is F-domain (modified according to K.D.Spindler, Vergleichende Endokrinologie: Regulation und Mechanismen. Georg Thieme Verlag, Stuttgart).

1.1.1. Functional characterization of Ecdysone receptor

The ecdysteroid hormones regulate many processes in reproduction and development of insects, notably moulting and metamorphosis, by binding to the ecdysone receptor (EcR) (Riddiford et al., 1993). Titre and

spectrum of ecdysteroids change during development which may indicate that various ecdysteroids have different functions (Spindler-Barth and Spindler, 2000).

The ecdysone receptor alone may regulate some hormonal processes, since the receptor is able to bind ligand in the absence of ultraspiracle (Spindler, 2003). Furthermore, DNA binding domains of both receptors react with DNA when the heterodimerization partner is absent, EcR-DBD as a homodimer and Usp-DBD as a monomer (Niedziela-Majka et al., 2000; Grad et al., 2001).

A considerable amount of data proving that Usp alone is able to bind a ligand and exert its function independently of a heterodimerization partner was reported. Moreover, other studies claimed that Usp might be a receptor for juvenile hormone (JH), a family of farnesoate compounds (Jones and al., 2000). This suggestion is based on the chemical similarity of juvenile hormone to retinoic acid as well as binding assays. Ligand binding induces conformational changes in Usp and stabilizes its homodimerization (Jones et al., 2001). However, the reported binding affinity of Usp for JH is rather low, therefore the question of whether there is a natural ligand for Usp remains open.

EcR, which is the orthologue of the vertebrate farnesoid X receptor (FXR) or liver X receptor (LXR) forms a heterodimer with Ultraspiracle (Usp), the mammalian orthologue of retinoid-X receptor (RXRα), which at its turn is able to influence the activity of its partner receptors through the action of the ligand 9-cis retinoic acid (Antoniewski et al., 1993; Vogtli et al., 1998).
In many cases the EcR/Usp heterodimer is able to coordinate and modify the expression of more then one hundred genes in a tissue- and time-specific manner. Most studies on EcR and Usp have focussed on EcR as a ligand binding receptor, while the heterodimerization partner Usp was considered as an orphan receptor.

In *Drosophila melanogaster*, three ecdysone receptor isoforms (EcRA, EcRB1 and EcRB2) with various lengths and sequences of N-terminal A/B domain and different biological functions were described (Talbot et al., 1993). These isoforms cannot replace each other (Bender et al., 1997), arise from two RNA templates and are differently expressed.

EcRA is predominantly expressed in adult cells which proliferate and differentiate during metamorphosis, while the EcRB1 and EcRB2 are predominantly expressed in larval cells fated to die during metamorphosis. This observation led to the proposal that different EcR isoforms dictate part of the tissue specificity of the ecdysone response (Talbot et al., 1993). The existence of different isoforms of the receptor allows diversification of the hormone response. For example, 20-OH Ecdysone triggers different actions dependent on the tissue and developmental stages.

Robinow et al. (1993) showed that EcRA isoform was implicated in the remodelling of neurons during metamorphosis. By contrast, EcRB1 isoform is capable of mediating the ecdysteroid response in salivary glands cells (Bender et al.,1997), while EcRB2 seems to be the most efficient isoform for rescuing larval development in EcR mutants (Li and Bender, 2001). Phenotypic analysis of EcR isoforms mutants showed that distinct lethal phases and morphologies are associated with each type of mutant (Bender et al., 1997; Schubiger et al., 1998).

Usp is widely expressed during development and is present in all tissues analysed at the onset of metamorphosis (Henrich et al., 1994). Usp function is best understood by looking at its partners and targets. Upon partnering with EcR, Usp was shown to activate genes involved in metamorphosis (Thummel et al., 1998). Its absence during the late third instar induces rather different phenotypic effects as compared to the absence of EcR.

1.2. Receptor - Ligand interaction

Why is receptor-ligand binding important?

Any organism must posses a mechanism of interacting with its environment. Each individual cell must be able to interact with a variety of molecules, from outside and generated within the cell itself. Receptor-ligand binding has an important role in the function of living organisms and was described to be involved in many cell functions including hormone receptor, gene regulation and transport across membranes, immune response and enzyme catalysis.

A ligand or a hormone is a chemical messenger that carries a signal from one cell to another. Most hormones initiate a cellular response by interacting either with a specific intracellular or membrane associated receptor protein and subsequent stimulation of different signal transduction pathways.

In the absence of hormone, the intracellular localization of steroid receptors varies. For example the heterodimers of Androgen receptor (AR) with RXR are localized predominantly cytoplasmic (Tyagi et al., 2000); also the same localization has Glucocorticoid receptor (Htun et al., 1996). Other receptors like Estrogen receptor (ER) (Htun et al., 1999) and Ultraspiracle (Usp) are localized in nucleus (Nieva et al. 2005).

In the ligand-bound state most of receptors are in the nucleus. This reorganization within nucleus may be an important regulatory process since the pattern appears only upon agonists' treatment, but not in the presence of antagonists.

In the epithelial cell line of *Chironomus tentans,* EcR was partially present in the cytoplasm, and was shifted into the nucleus upon 20-hydroxyecdysone treatment (Lammerding-Köppel et al.,1998). Using

antibodies against EcR and Usp, Riddiford et al. (2000) showed that these proteins are localized in the nucleus.

In order to bind their cognate receptors, hormones must cross the plasma membrane. The hormone-receptor complex translocates then into the nucleus where it binds specific DNA sequences, augmenting or suppressing the action of target genes thus modulating protein synthesis (Figure 2).

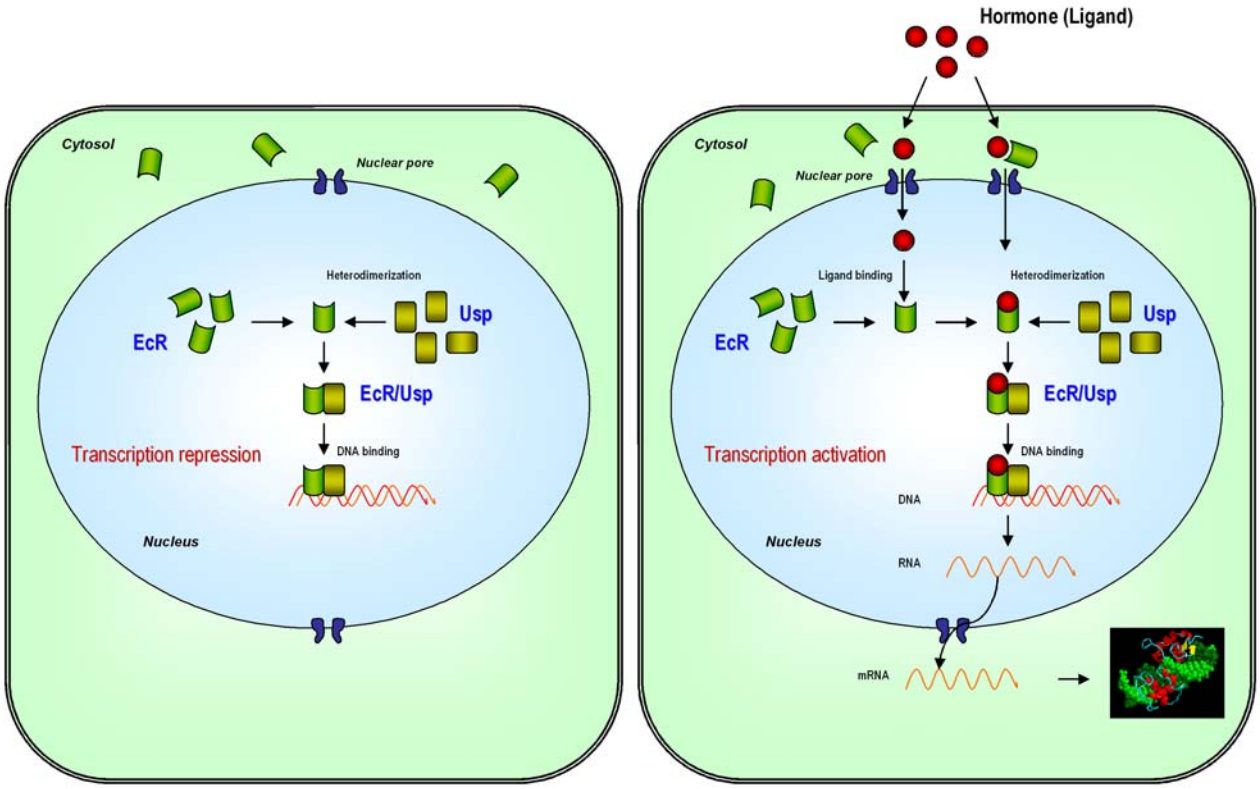


Figure 2 Schematic overview of classical signaling pathways of Ecdysone receptor. The Ecdysone receptor heterodimer is localized in the nucleus and represses transcription in the absence of the hormone. Upon ligand binding, the heterodimer comprised of EcR and its partner Usp, binds DNA motifs in the target genes, driving transcription activation.

Ligand binding domain (LBD) of Ecdysone receptor was found to play a major role during interaction of EcR with its cognate ligand. Furthermore, LBD mediates additional receptor functions including dimerization and transactivation (Weatherman et al., 1999; Bourguet et al., 2000).

Wild type and mutated LBDs of the EcR and its heterodimerization partner Usp from *Drosophila melanogaster*, fused to GAL4 domains were examined by hybrid assays (Lezzi et al.,2002), ligand-binding and gel mobility shift assays (Grebe et al., 2003 and 2004; Przbilla et al., 2004). These experiments showed that the minimal receptor fragment necessary for ligand binding of *Drosophila* EcR consists of the C-terminal part of the D-domain and the complete E-domain.

The general architecture of the EcR and Usp LBDs, composing each EcR/Usp heterodimer, is similar to that observed in the crystal structures of other nuclear receptors LBDs, with a general folding pattern consisting of a three-layered, anti-parallel, α-helical sandwich and a β-sheet. The region is comprised of 12 α-helices forming a ligand-binding pocket which holds the cognate ligand (Moras et al., 1996; Weatherman et al., 1999; Billas et al., 2005).

For EcR, a ligand-dependent transcriptional activation function (AF2) region is localized in the most carboxy-terminal helix 12, which folds over the pocket to hold the ligand molecule inside and prevent dissociation of the ligand. This folding creates an interactive surface with other proteins that finally modulates the transcriptional activity of the receptor. Perlmann et al., (1996) described along helixes 9 and 10, a dimerization interface Heterodimerization between EcR and Usp seems to be, at least in *Drosophila* the best studied example, however the interaction with other partners, such as seven-up (a homologue of vertebrate COUP-TF), can contribute to the diversity of ecdysteroid-regulated processes. Although *Drosophila* EcR is able to bind ligand in the absence of Usp, a considerable increase in ecdysteroid binding is observed after addition of the

heterodimerization partner (Lezzi et al., 2002; Spindler-Barth et al., 2003), which is accompanied by an allosteric change of the ligand-binding pocket of EcR (Grebe et al.,2003).

Some relevant insights were obtained from the structure of the Usp-LBD of *Drosophila* (Schwabe et al., 2000) and *Heliothis virescens* (Billas et al., 2001). The crystal structure is similar to the mammalian homologue RXR, except that the Usp structure of *Drosophila* shows a long H1-H3 loop and an insert between H5 and H6. Structural studies have yielded that helix 12 of the Usp-LBD is fixed in an antagonistic position, even in the absence of a specific ligand, because of the hydrophobic interaction between helix 12 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 the apo-position of helix 12, involving a different subset of amino acids residues than those associated with possible juvenile hormone binding.

The key hormone for the development of most insects is 20-OH ecdysone (20E), but experimental evidence indicates that ecdysone and other ecdysteroids coexist with 20E and have distinct roles at different insect developmental stages (Henrich et al., 1995; Gilbert et al., 2002).

The steroid Ponasterone A (PonA) is identical to 20E with the exception that it lacks the 25-OH group. It was chosen in binding experiments for its higher affinity compared with 20E. Furthermore, examination of the ligand-binding pocket shows that most residues interacting with PonA are conserved among species, rationalizing the promiscuous character of 20E. PonA is directly involved in the stabilization of the EcR-LBD structure (Graham et al., 2006). In fact, the helical conformation of H2 is stabilized by interactions between the PonA C2- and C3-hydroxyl groups and residues of the H1-H2 loop, helix 5 and β-sheet.

In general, ligand binding causes the release of associated corepressor proteins and allows receptor association with coactivator proteins that function to either modify the chromatin structure or link the nuclear receptors to the transcription machinery (Xu et al., 1999).

Like its vertebrate cognates (Evans et al., 1995), unliganted EcR/Usp can act as a repressor of transcription (Cherbas et al., 1991). It was shown that in a *Drosophila* Kc cell assay system, unliganded receptor depresses reporter gene expression 3-4 fold below the reference level (expression from a control plasmid lacking an EcRE), while liganded receptor stimulates expression to at least 20 times the same reference level.

Moreover the studies of Arbeitman and Hogness (2000) showed that functioning of EcR/Usp complex requires molecular chaperons, such as Hsp90 and Hsp70.

1.3. Receptor - DNA interaction

Nuclear receptors are characterized by a highly conserved DNA binding domain (DBD) containing two zinc fingers which enables the receptor to bind specific DNA sequences called hormone response elements (HREs). A hormone response element is a short sequence of DNA within the promoter of a gene that is able to bind a specific hormone receptor complex and therefore to regulate transcription. There are three types of such HREs, composed of consensus hexameric separated by 0 to 5 nucleotides used by essentially all nuclear receptors (Glass,1994), arranged in tandem as inverted, everted and direct repeats upon which nuclear receptors can bind as homodimers or heterodimers.

Functional analysis of nuclear receptors together with determination of the crystal structure of several complexes formed by their DNA-binding domain (DBD) bound to their cognate response elements have begun to explain the molecular basis for protein-DNA and protein-protein interaction,

essential for high-affinity and specific DNA binding by nuclear receptors. Specific recognition of the core half-site sequence is provided by three amino acid residues located in the base of the first zinc finger module (called P-box) (Evans et al.,1989; Chambon et al.,1989; Sigler et al.,1991), while recognition of the 5'-A/T-rich flanking sequence present in monomeric HREs is mediated by contacts between DNA and amino acid residues located in the carboxy-terminal extension (CTE) of the core DBD (Milbrandt et al., 1992 and 1993; Ozyhar et al., 2007). On the other hand, binding specificity for a given homodimer or heterodimer complex is dictated by DNA-dependent dimerization of the two DBD subunits. Spacing specificity is regulated by motifs located in the first and second zinc finger modules as well as in the CTE. The importance of an individual motif in determining half-site specificity depends on the configuration of the HRE (Evans et al., 1991; Lazar et al., 1998).

The vertebrate steroid receptors, such as Glucocorticoid receptor (GR), Estrogen receptor (ER), Progesterone receptor (PR) bind HREs as homodimers. Their response elements are inverted repeats with the same 3 bp (IR-3), causing their DBDs to form symmetric "head to head" interactions (Schwabe et. al., 1993). The RXR class binds HREs as heterodimers in which an RXR receptor is coupled with a ligand-specific nuclear receptor, whereas the orphan receptors bind DNA either as monomers or dimers. It was found that the heterodimer EcR/Usp was able to bind to inverted repeat sequences (palindromes, Pal; Riddihough and Pelham, 1987; Martinez et al.,1991; Antoniewski et al., 1993) as to direct repeat sequences (DR; Horner et al.,1995; Antoniewski et al., 1996) of the more or less conserved half-site AGGTCA. The best characterized EcRE is the pseudo-palindromic element of the heat shock protein 27 gene promoter (hsp27), which is composed of two heptameric half-site sequences separated by one central base pair. Detailed analysis of the interaction of EcR and Usp DNA-binding domains (EcR-DBD and Usp-DBD, respectively) with the hsp27 element,

suggested that Usp-DBD may act as a specific anchor that preferentially binds the 5' half-site of this element and thus locating the heterocomplex in a defined orientation (Ozyhar et al., 1993; Ozyhar et al., 2000).

The binding of the functional Ecdysteroid receptor to these various EcREs has been investigated for *Drosophila* (Antoniewski et al., 1994; Vögtly et al., 1998), *Chironomus* (Elke et al., 1997, 1999) and *Aedes* (Wang et al., 1998). The complexes between EcR/Usp and various HREs differ in their affinity. This was demonstrated by direct determination of the affinity between receptor and DNA (Wang et al., 1998) or by competition experiments using different EcREs (Elke et al., 1999).

In addition to binding of EcR/Usp complexes, either EcR or Usp alone can bind to DNA. *In vitro* translated dUsp (Antoniewski et al.,1994; Vögtly et al., 1998) and bacterially expressed cUsp as a fusion protein with GST (Elke et al.,1997) prefer direct repeats, whereas bacterially expressed cEcR-GST prefers Pal (Elke et al., 1997).

In vitro, the situation is more complex, since it was shown with *Chironomus* cell extracts that both Pal-1 and DR-1 bind EcR/Usp heterodimer strongly, but the complexes formed with these two motifs are not identical (Elke et al., 1999). It was shown that residues Arg51 and Lys52 from EcR and residue Asn51 from Usp are simultaneously involved in both dimerization and DNA binding functions. This implies that DNA binding and dimerization are mutually supportive (Devarakonda et al., 2003). The wide range of EcRs recognized by the EcR/Usp contributes to the complexity of the hormonal regulation.

1.4. Point mutation effects on Ecdysteroid receptor function

Gene switches are inducible gene regulation systems that are used to control the expression of transgenes. This is very important for various applications such as gene therapy, large-scale productions of proteins in cells, functional genomics, regulation of traits in transgenic plants and animals.

To develop such a gene switch several mutants in the LBD of EcR and Usp were generated by changing amino acids residues and thereafter evaluated in ligand binding and transactivation assays.

Palli et al. (2002) showed that a mutation of an alanine residue to proline (A110P), in the *Choristoneura fumiferana* EcR-LBD led to a selective disruption of both binding and transactivation with steroids, but not with non-steroidal ligands. This steroid-insensitive EcR mutant has potential gene switch applications in insects that have endogenous ecdysteroids. In addition, such mutant was reported to be useful for developing EcR-ligand pairs for regulation of multiple genes in the same cell (Palli et al., 2002).

Using yeast two-hybrid analysis, another study revealed that the EcR-LBD and Usp-LBD fused to GAL4 activation domain and GAL4 DNA-binding domain respectively, promote expression of a GAL4-inducible promoter and this response is enhanced significantly in a dose-dependent fashion by the addition of Muristerone A (Lezzi et al., 2002). This system has been used in conjunction with biochemical methods to analyze the effects of several site-directed mutations on the functionality of the EcR-LBD (Grebe et al., 2003). As expected, mutations of critical residues in EcR's helix 10 eliminate both dimerization and transcriptional activity.

Deletion or mutation of helix 12, which normally folds over the ligand-filled pocket, eliminates EcR's AF2 function, as do mutations that affect

ligand binding, but AF1 function is still detected in these mutant forms of EcR.

Substitution at a consensus cofactor-interacting residue, K497E, results in a strong elevation of basal transcriptional activity, in spite of the fact that this mutated receptor has a lower ligand affinity (Grebe et al., 2003; Bergmann et al., 2004).

Some point mutations in the Usp-LBD of Drosophila melanogaster eliminate transcriptional activity, though many retain the capability to dimerize with EcR.

These intra- and intermolecular effects suggest that the various regions of the LBDs are functionally linked and regulated across the entire heterodimer-hormone complex. They imply that ligand binding causes local and global transitions of the EcR-LBD to control the interaction with the Usp-LBD.

2. Aim of the study

Ecdysteroid hormones are major regulators in reproduction and development of insects including larval molts and metamorphosis which activate ecdysone receptor. In order to gain further insight into ligand binding properties of the ecdysone receptor heterocomplex (EcR/Usp) main focused was given on the effect of the A/B-domain of either EcR or Usp wild type, on the affinity of ^{3}[H]-Ponasterone A. Since the A/B domain of Usp wild type is routinely replaced by $VP16_{AD}$ fusions of Usp with $VP16_{AD}$ were investigated.

Some hormone-induced biological responses of EcR/Usp involve the DNA Binding Domain (DBD) of Usp, whereas others are already observed in the absence of a functional Usp-DBD. Therefore full length Usp and UspΔDBD were examined.

Thirdly, the influence of mammalian ortholog of Usp - RXR - on ligand binding was studied known the fact that it can replace Usp in several test systems.

Special focus was given to the influence on hormone binding of specific short DNA sequences (hsp27, DR-1 and Pal-1) recognized by the DNA-binding domains of EcR and Usp.

Taking into consideration EcR and Usp heterodimerization and inducibility by ligand, the influence of some point mutations in their Ligand Binding Domains (LBD) was investigated.

Finally, different normalization methods used to ensure that same amount of receptor was responsible for the observed effects were approached.

3. Results

3.1. Determination of receptor concentration

3.1.1. Determination of receptor concentration after normalization of transfection efficiency

For comparison of receptor functionality of EcR isoforms, like interaction with DNA or transcriptional activity it is essential that equal amounts of receptor protein complexes are used.

It is common practice to normalize data on the activity of a co-expressed reporter gene coupled to a constitutive promoter. Therefore, co-transfection experiments with *lacZ* expression vector (pCHIII-lacZ) were performed and subsequent quantification of β-galactosidase activity took place.

As shown in Table 1, receptor concentration calculated after transfection efficiency normalization deviates up to 40-fold from the concentration of the heterodimer calculated by Scatchard plot alone.

Relative EcR concentrations determined by Western blot correspond better with the Scatchard plot data (ratio of EcR concentrations determined by Scatchard plot/ Western blot =1,6±0,6). Deletion of C-domain of Usp (UspIII) impairs dimerization, which results in a decreased fraction of heterodimers indicated by a lower ratio: EcR/Usp (Scatchard plot)/EcR (Western blot). In order to compare functional properties of the various heterodimeric complexes *in vitro*, in addition care was taken that EcR was transferred nearly quantitatively as heterodimer.

Table 1 Comparison of heterodimer concentrations calculated by Scatchard plot, and transfection efficiency measured after cotransfection of constitutively expressed β-galactosidase, and EcR concentration determined by Western Blot.

Receptor	EcR/Usp Concentration (nM) (Scatchard plot)	EcR / Usp β-galactosidase Activity	EcR / UspEcR Concentration (RU) (Western Blot)
EcRA/Usp I	1,30	2,30	1,9
EcRB1/Usp I	0,95	1,54	1,1
EcRB2/Usp I	1,16	6,04	0,7
EcRA/Usp II	2,19	21,90	1,4
EcRB1/Usp II	2,54	16,60	2,2
EcRB2/Usp II	3,33	24,13	2,1
EcRA/Usp III	0,76	4,37	0,2
EcRB1/Usp III	2,74	41,52	0,6
EcRB2/Usp III	2,68	68,72	0,6

3.1.2. Determination of receptor concentration by Western blot

A prerequisite for quantitative determination of receptor concentrations by Western blot is a standard curve (Figure 3 and Figure 4A and 4B). Therefore the Quantity One® software was tested in an experiment using increasing amounts of whole cell extracts prepared from transfected CHO-K1 cells.

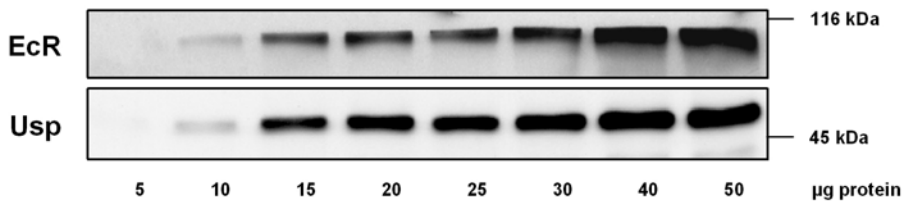


Figure 3 Western blot of lysates prepared from CHO-K1 cells transfected with either EcR or VP16-Usp constructs .Proteins were separated by SDS-PAGE and membranes were incubated with either anti-EcR or anti-VP16 antibodies and radiographed bands quantified

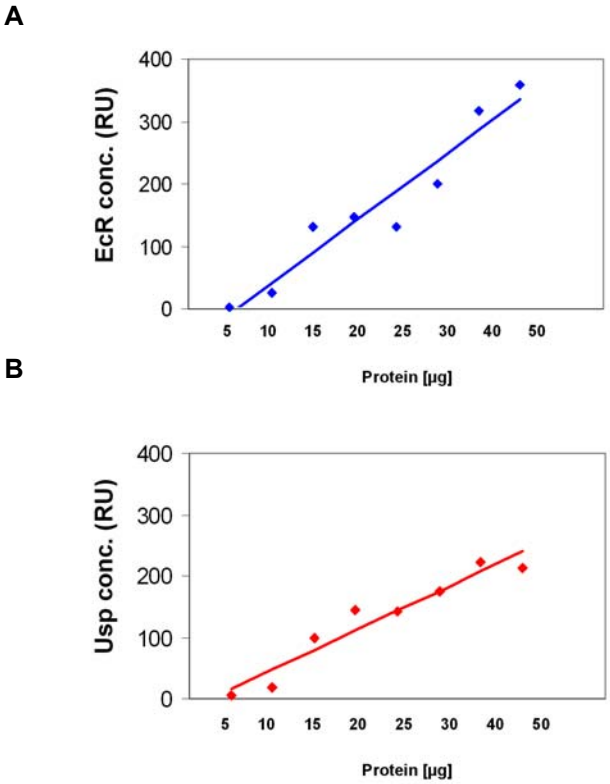


Figure 4 Relative receptor concentrations according to western blot analysis using different amounts of total protein as indicated. A= EcR concentrations; B= Usp concentrations.

The expression level of all EcR isoforms determined by Western blot in the absence of Usp is rather similar in mammalian CHO-K1 cells (Figure 5).

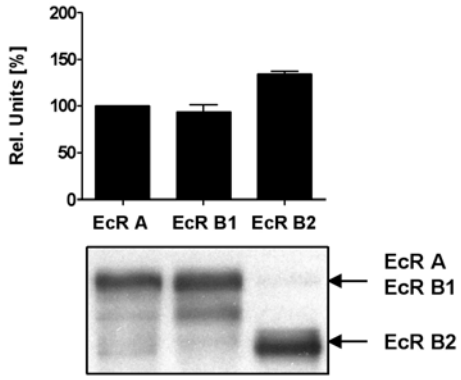


Figure 5 Quantitative determination of receptor concentration. Western blot of EcR isoforms in the absence of Ultraspiracle detected with DDA 2.7 monoclonal antibody. The intensity of the bands was calculated using BIO-RAD Quantity One v.4.4.0.

Coexpression with heterodimerization partner Usp enhances moderately the concentration of EcR isoforms as shown for heterodimers with UspI and UspII (Figure 6) but is considerably increased, if coexpressed with UspIII, in which the C-domain has been deleted. UspIII concentrations are also considerably higher compared to full length Usp (Figure 7).

The concentration of heterodimers depends not only on the amount of both nuclear receptors, but also on the affinity of the receptors, which is different for various receptor combinations and is especially low for complexes with UspIII, since the dimerization site in the C-domain of Usp is missing. Although is compensated partially by the higher expression levels (Figure 6 and Figure 7), calculation of the concentration of heterodimers is rather difficult since the affinity between EcR and Usp variants can only be roughly estimated.

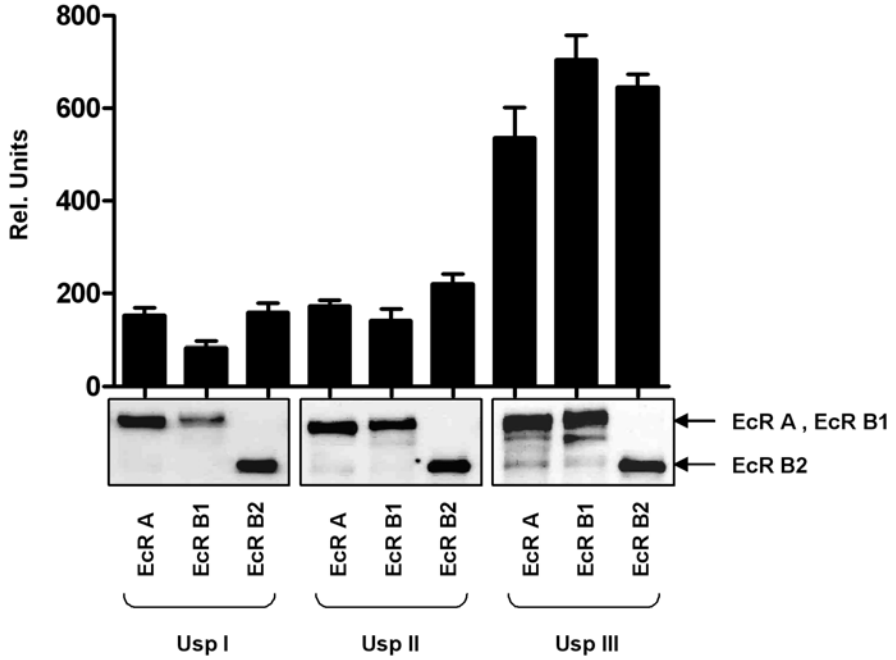


Figure 6 Quantitative determination of EcR concentrations coexpressed with Usp variants in CHO-K1 cells according to western blot analysis. EcR isoforms were detected with DDA 2.7 monoclonal antibody.

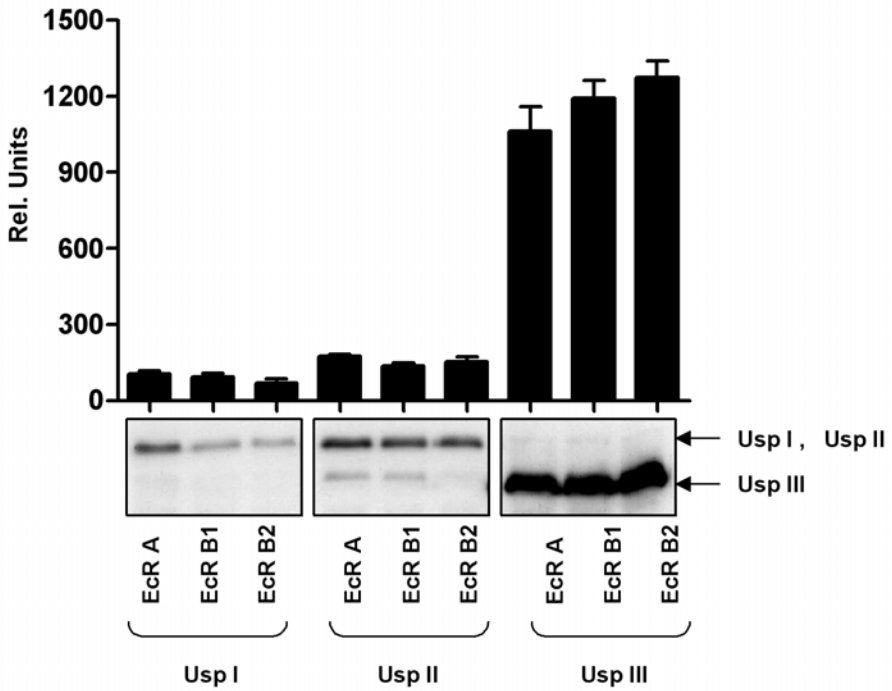


Figure 7 Quantitative determination of Usp concentrations coexpressed with EcR isoforms in CHO-K1 cells according to western blot analysis.Usp variants were detected with VP-16 antibody.

3.2. Estimation of the affinity between EcR isoforms and Usp

Even if the same concentrations of EcR and Usp are expressed, the concentrations of heterodimeric complexes (EcRs/Usps) vary according to the differences in the affinities between EcR isoforms and Usp variants. This is important especially in the case of UspIII, in which the dimerization interface in the C-domain is missing. In previous studies two hybrid assays were used to evaluate dimerization capability. Lezzi and co-workers (2002) assumed that the differences in transactivation capability are due to variations of the dimerization efficiency between EcR and Usp. Determination of EcR and Usp concentrations by Western blot and determination of heterodimer concentrations by Scatchard plot provide an alternative method to roughly estimate relative values for the affinity between both dimerization partners by the law of masses (Table 2):

$$K_{D\ dimer} = \frac{[EcR] \times [Usp]}{[EcR\ /\ Usp]}$$

Table 2 Interaction of EcR isoforms with Usp variants. The concentration of the heterodimer was determined by Scatchard plot. The concentrations of EcR and Usp were determined by Western blot.The relative affinities were estimated using the law of masses.

EcR A	Usp I	=	Usp II	>>>	Usp III
Rel.$K_{D\ (dimerization)}$	1	:	1	:	25
EcR B1	Usp I	=	Usp II	>>	Usp III
Rel.$K_{D\ (dimerization)}$	3	:	1	:	126
EcR B2	Usp I	>	Usp II	>>	Usp III
Rel.$K_{D\ (dimerization)}$	1	:	10	:	150

Although no exact calculation is possible in this way, it is evident that the affinity of Usp to all isoforms of EcR is reduced dramatically if the C-domain of Usp is deleted. This is most pronounced for EcRB1 and EcRB2. The dimerization capability of EcRA/UspIII is affected to a lesser extent, which may indicate that the hormone responses, which do not afford participation of Usp-DBD, are mediated more effectively by EcR A.

To determine the concentration of heterodimers for all receptor combinations ligand binding experiments were performed.

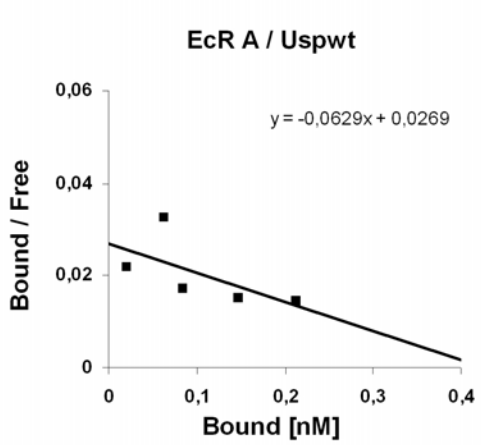
3.3. Ligand binding to Ecdysone receptor

Determination of receptor concentrations by ligand binding has the advantage that only the amount of functional receptor is determined.

In this study the whole receptor proteins were expressed and the affinity of the Ponasterone A to the receptor complex was determined by Scatchard plot analysis (Figure 8 - EcRs/Uspwt, Figure 9 - EcRs/UspI, Figure 10 - EcRs/UspII, Figure 11 - EcRs/UspIII). The A/B-domain of EcR isoforms had no significant influence on ligand binding. As is summarized and shown in Table 3, the activation domain of VP-16 (used in transactivation studies to overcome the inhibitory action of the A/B-domain of Usp - Henrich, 2005; Beatty et al., 2006) and the CDE-domains of Usp conferred the same ligand binding capability to all EcR isoforms as wild type Usp. No significant influence of the A/B-domain of Usp on ligand binding was observed. Deletion of the C-domain (DNA-binding domain) of Usp, which harbors a strong dimerization interface, did not affect ligand binding.

Scatchard plot analysis made also possible determination of the receptor concentrations for each cell extract in order to ensure that the same amount of ligand binding sites was used in each experiment. The number of the binding sites varies about 6 fold (Table 3).

A



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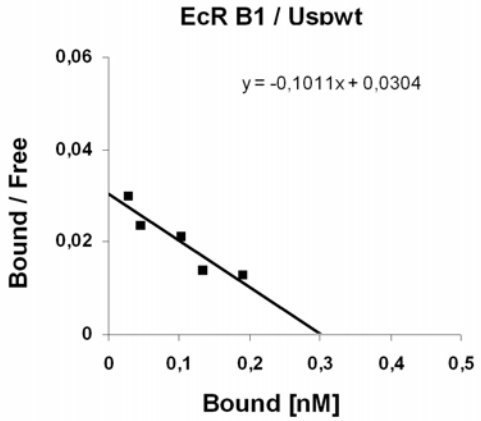

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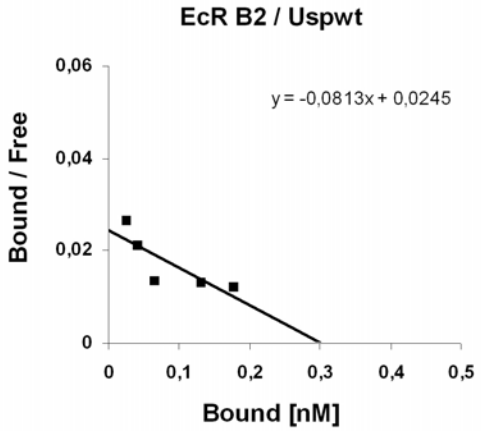
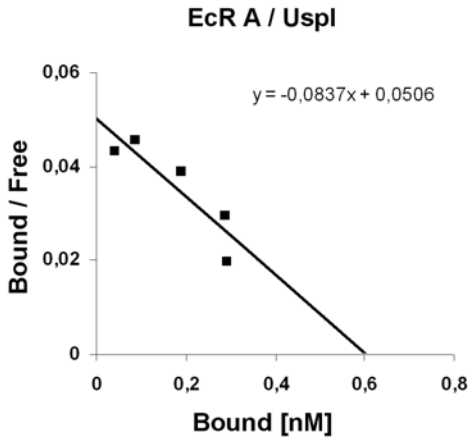


Figure 8 Ligand binding of EcRA/Uspwt **(A)**, EcRB1/Uspwt **(B)** and EcRB2/Uspwt **(C)** cotransfected in CHO-K1 cells. ^{3}[H]-Ponasterone A with specific activity 7.9TBq/mmol was used as ligand. Data were analyzed according to Scatchard (1949).

A



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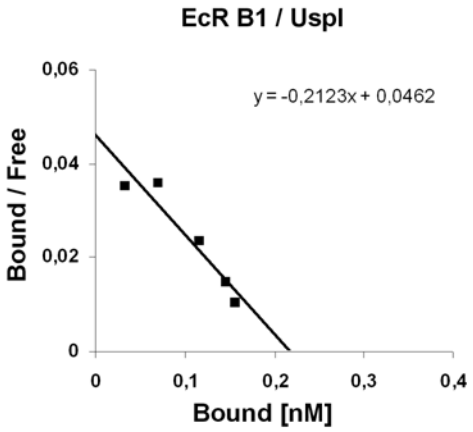

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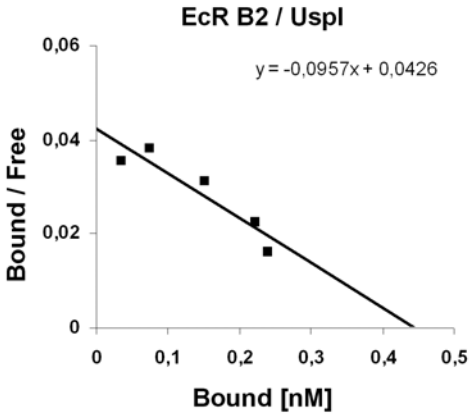
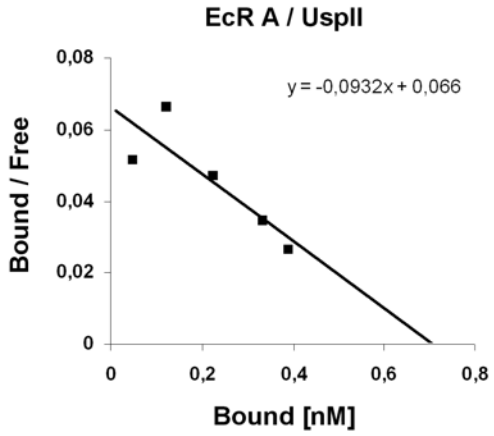


Figure 9 Ligand binding of EcRA/UspI **(A)**, EcRB1/UspI **(B)** and EcRB2/UspI **(C)** cotransfected in CHO-K1 cells. ^{3}[H]-Ponasterone A (specific activity 7.9TBq/mmol) was used as ligand. Data were analyzed according to Scatchard (1949).

A



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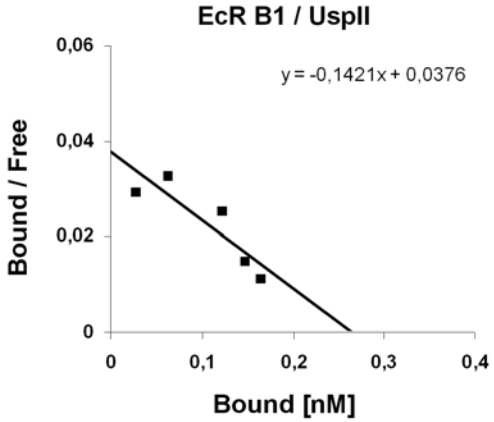

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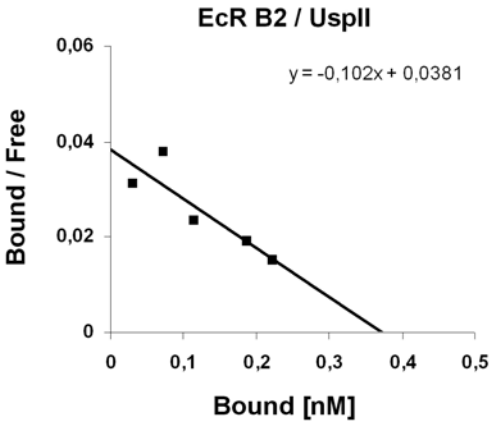
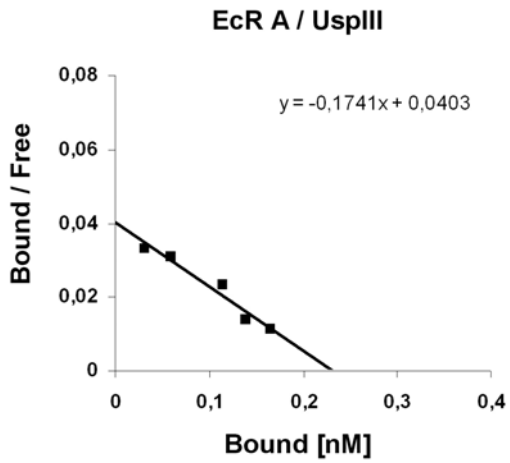


Figure 10 Ligand binding of EcRA/UspII **(A)**, EcRB1/UspII **(B)** and EcRB2/UspII **(C)** cotransfected in CHO-K1 cells. ^{3}[H]-Ponasterone A (specific activity 7.9TBq/mmol) was used as ligand. Data were analyzed according to Scatchard (1949).

A



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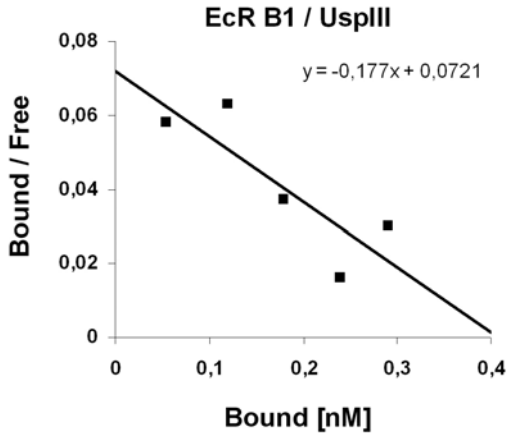

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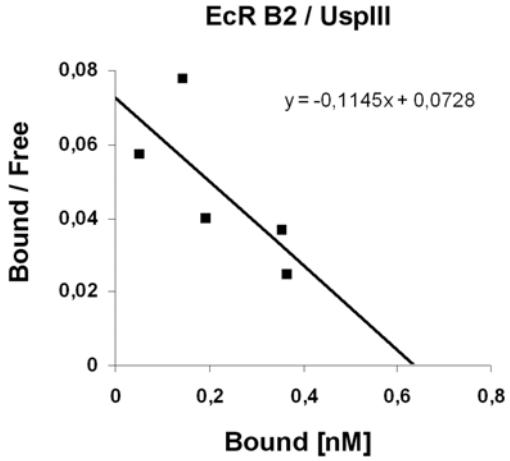


Figure 11 Ligand binding of EcRA/UspIII **(A)**, EcRB1/UspIII **(B)** and EcRB2/UspIII **(C)** cotransfected in CHO-K1 cells. ^{3}[H]-Ponasterone A (specific activity 7.9TBq/mmol) was used as ligand. Data were analyzed according to Scatchard (1949).

Table 3 Affinity of ^{3}[H]-Ponasterone A to EcR/Usp heterodimers and receptor concentration in the absence of DNA

Receptors	K_D (nM)	Receptor concentration (nM/L)
EcRA/Usp wt	7.0 ± 4.0	0.17 ± 0,02
EcRB1/Usp wt	7.1 ± 4.3	0.21± 0.11
EcRB2/Usp wt	4.4 ± 2.3	0.16 ± 0.04
EcRA/Usp I	6.6 ± 1.1	0.51 ± 0.23
EcRB1/Usp I	8.4 ± 4.1	0.16 ± 0.16
EcRB2/Usp I	8.9 ± 1.8	0.49 ± 0.12
EcRA/Usp II	10.7 ± 1.8	0.81 ± 0.64
EcRB1/Usp II	6.8 ± 1.7	0.39 ± 0.25
EcRB2/Usp II	11.0 ± 1.5	0.68 ± 0.58
EcRA/Usp III	6.6 ± 3.2	0.27 ± 0.14
EcRB1/Usp III	6.5 ± 4.1	0.96 ± 0.55
EcRB2/Usp III	7.8 ± 0.6	0.74 ± 0.30

3.4. Interaction of Ecdysone receptor isoforms with DNA

The natural ecdysone response elements (EcREs) discovered so far are asymmetric elements composed of either imperfect palindromes or direct repeats. Gel mobility shift assays have shown that both symmetric (perfect palindromes) and asymmetric (imperfect palindromes and direct repeats) elements can bind to the EcR and EcR/Usp complex (Braun et al., submitted). Several EcREs have been identified in *Drosophila* among which hsp27 has been best studied. Since Usp-DBD modifies ligand binding of the heterodimer it was speculated that presence of DNA may further modulate ligand binding affinity.

3.4.1. Ligand binding to EcR isoforms in the absence of Usp is reinforced by the presence of DNA

In contrast to ecdysone receptor from other arthropod species investigated so far, EcR from *Drosophila melanogaster* (Grebe et al., 2004) and *Leptinotarsa decemliniata* (Ogura et al., 2007) bind Ponasterone A specifically already in the absence of heterodimerization partner Usp.

Ligand binding of EcR is rather low in the absence of a heterodimerization partner (Table 4) and is different for the three isoforms. However, in the presence of ecdysone response elements (EcREs) especially, if oligomeric EcREs are used ligand binding is improved (Table 4). The stimulatory effect varies depending on the EcR isoform and is significant for EcRA and EcRB1 only, but not for EcRB2 indicating a modulatory effect of the A/B domain of EcR on the intramolecular interaction of the DNA- and ligand binding domains. Scatchard plot analysis revealed that specific high affinity binding is obtained in the presence of DNA especially with oligomers of EcRE DR-1 (Fig. 12).

Table 4 ^{3}[H]-Ponasterone A binding to EcR isoforms in the absence of heterodimerization partner

Isoform	Hormone Response Element	Bound (cpm)	% of the control	Statistically significant difference (p)
EcR-A	-	558,7 ± 19,0	100	p < 0,001
EcR-A	5x DR-1	909,7 ± 15,3	163	
EcR-B1	-	922,7 ± 97,9	100	p < 0,005
EcR-B1	5x DR-1	1312,0 ± 72,3	143	
EcR-B2	-	974,7 ± 132,5	100	p = 0,383
EcR-B2	5x DR-1	1100,7 ± 179,4	112	

***Note**:* Identical amounts of EcR as determined by quantification of specific by Western blot signals were used. 10 nmol Ponasterone A were applied in each test

Between EcRA in the absence or presence of 5x DR-1 there is a statistically significant difference ($p < 0,001$). For next two groups EcRB1 in the absence or presence of 5x DR-1 there is a statistically significant difference ($p < 0,005$) and for the third group EcRB2 in the absence or presence of 5x DR-1 there is not a statistically significant difference ($p=0,383$).

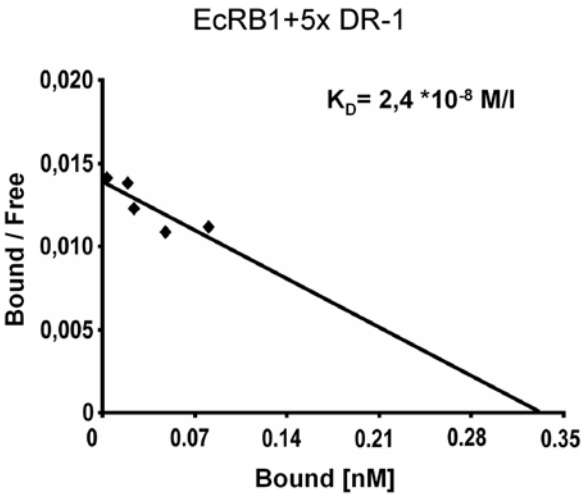


Figure 12 Ligand binding of EcRB1 (transfected in CHO-K1-cells) in the presence of the hormone response element 5x DR-1. ^{3}[H]-Ponasterone A (specific activity: 7.9 TBq / mmol) was used as ligand. Data were analysed according to Scatchard (1949).

3.4.2. Ligand binding of EcR/ Usp heterodimers is increased in the presence of DNA

In order to investigate whether interaction with DNA modulates ligand binding to EcR/Usp heterodimer, hormone binding experiments in the presence of hormone response elements were also performed. Monomers of the ecdysone response element hsp27 usually used for DNA binding studies like gel mobility shift assays (EMSA) increased ligand binding to all EcR isoforms in the presence of wild type Usp about 4-6 fold (Figure 13 A).

For determination of transcriptional activity tandemly arranged EcREs oligomers were used to enhance the hormonal response (Henrich, 2005). Therefore we also tested these oligomers. No difference between 1x hsp27 and 5x hsp27 was found with all heterodimers consisting of all EcR isoforms and wild type Usp. The increase in ligand binding was also the same for all hormone response elements tested (hsp27, Pal-1, or DR-1) (Figure 13 A, B, C).

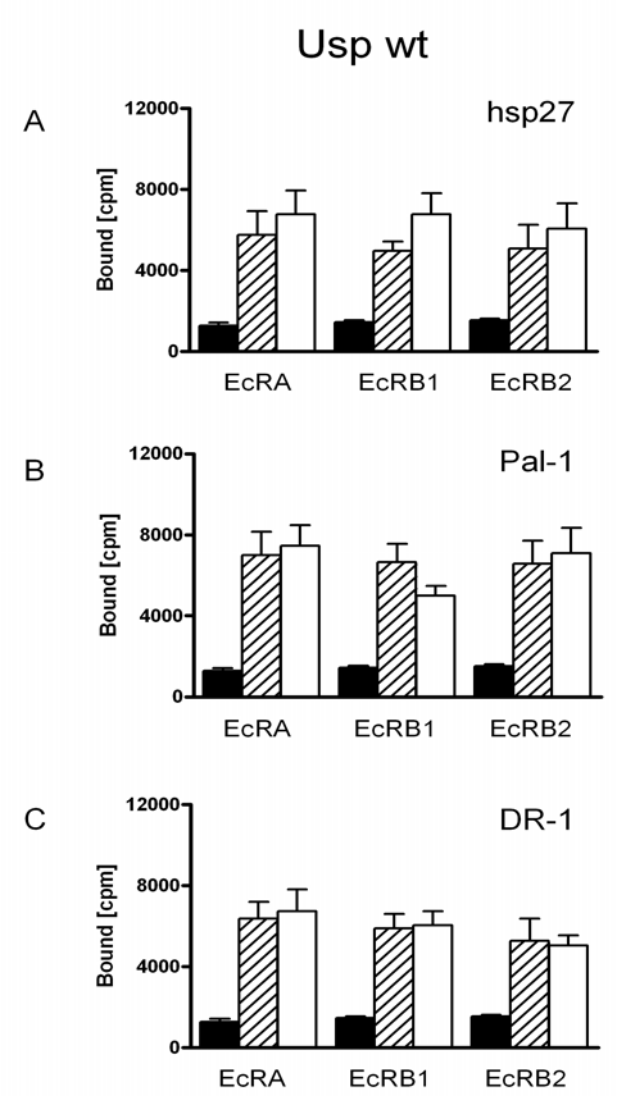


Figure 13 Influence of hormone response elements on ligand binding ([^{3}H]-Ponasterone A) to heterodimers of EcR isoforms with wild type Usp. Ligand binding experiments were performed with cell extracts containing 10nM receptor complex according to Scatchard plot analysis **A)** [^{3}H]-Ponasterone A binding in presence of 1x hsp27 and 5x hsp27. **B)** [^{3}H]-Ponasterone A binding in presence of 1x Pal-1 and 5x Pal-1. **C)** [^{3}H]-Ponasterone A binding in the presence of 1x DR-1 and 5x DR-1 (Mean $\pm$ SD, n= 3). Black bars: Ligand binding in the absence of DNA; hatched bars: Ligand binding in the presence of HRE monomers; white bars: Ligand binding in the presence of HRE pentamers.

3.4.3. The A/B-domain of Usp modifies interaction of the heterodimers EcR/Usp with DNA

As previously mentioned, the A/B domain of Usp is replaced by the activation domain of VP-16 in transcriptional assays. In this case, pronounced differences in ligand binding depending on the EcR isoform and the type of EcRE present were obtained (Figure 14 A,B,C). No influence on ligand binding was observed in the presence of 1x hsp27, whereas an about 4 fold increase was found with 5x hsp27 and EcRB1 and EcRB2 isoforms. The stimulatory effect of 5x hsp27 was less pronounced in case of EcRA. With the exception of EcRB1 in the presence of Pal-1, which showed up to 4 fold stimulation of ligand binding the influence of Pal-1 and DR-1 was rather modest. However, in contrast to 1x hsp27, monomers of Pal-1 and DR-1 already enhanced ligand binding.

A similar result was obtained with UspII (Figure 15 A,B,C) with the exception that the stimulatory influence of Pal-1 on ligand binding of EcRB1 was less pronounced. In contrast to UspI, the last six amino acids of the A/B domain and adjacent to the C-domain of wild type Usp (named “hexapeptide” in our experiments) were deleted in UspII and seemed to be responsible for this effect.

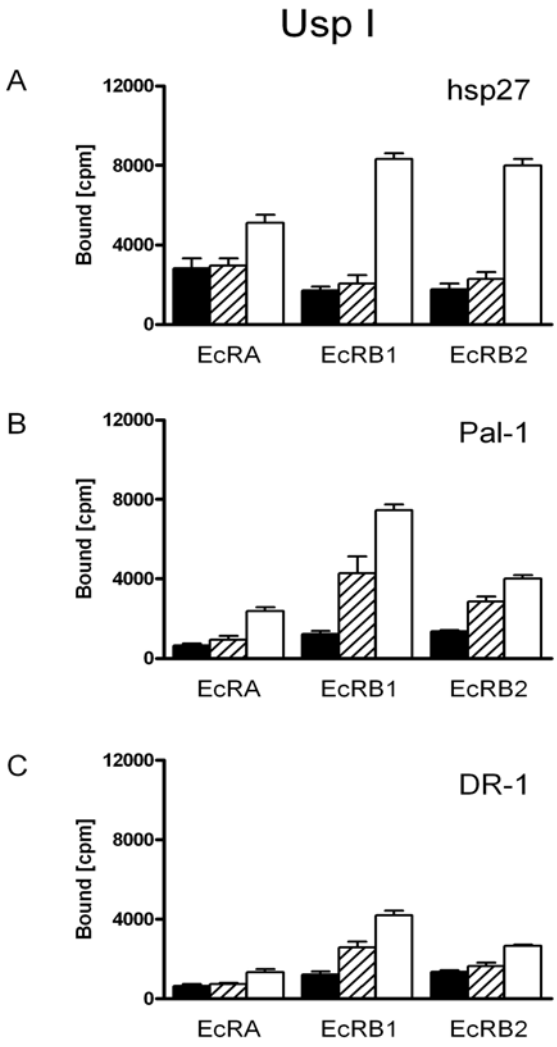


Figure 14 Influence of hormone response elements on ligand binding ([^{3}H]-Ponasterone A) to heterodimers of EcR isoforms with UspI. Binding experiments were performed with cell extracts containing 10nM receptor complex according to Scatchard plot. **A)** [^{3}H]-Ponasterone A binding in the presence of 1x hsp27 and 5x hsp27. **B)** [^{3}H]-Ponasterone A binding in the presence of 1x Pal-1 and 5x Pal-1. **C)** [^{3}H]-Ponasterone A binding in the presence of 1x DR-1 and 5x DR-1 (Mean $\pm$ SD, n= 3). Black bars: Ligand binding in the absence of DNA; hatched bars: Ligand binding in the presence of HRE monomers; white bars: Ligand binding in the presence of HRE pentamers.

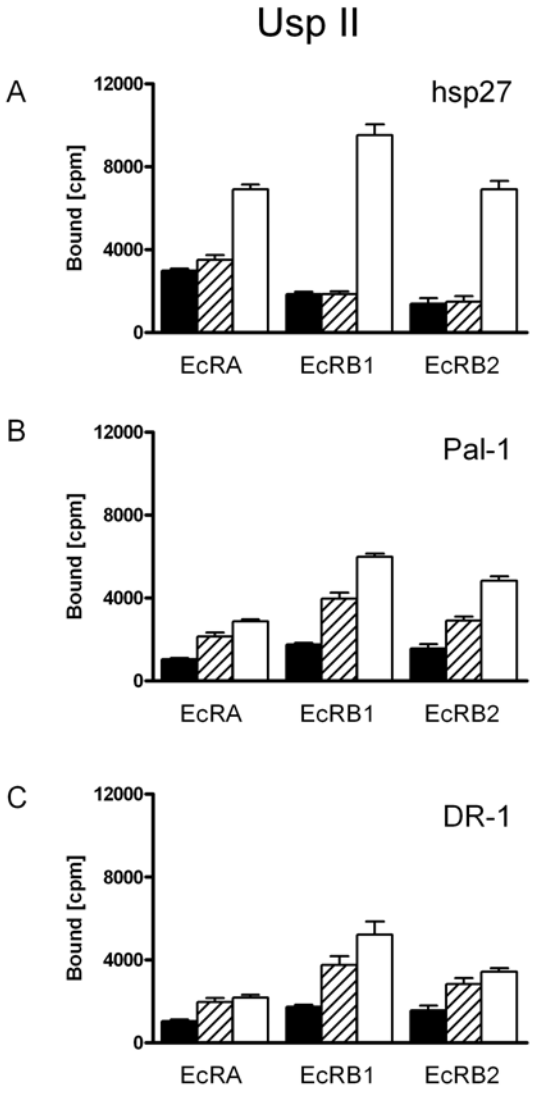
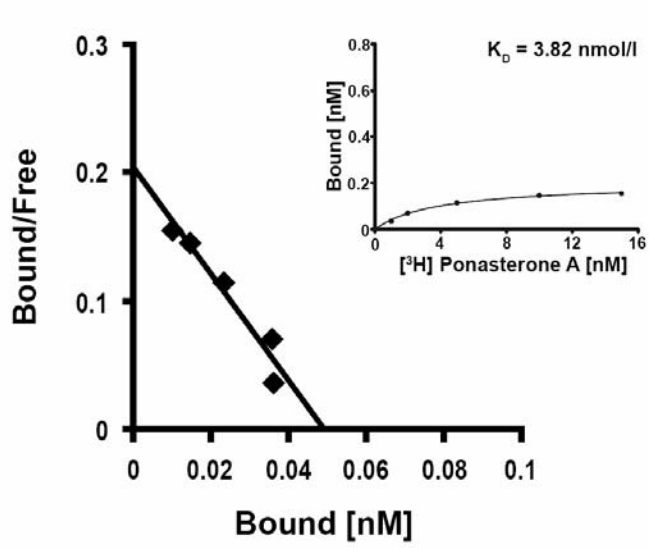


Figure 15 Influence of hormone response elements on ligand binding ([^{3}H]-Ponasterone A) to heterodimers of EcR isoforms with UspII. Binding experiments were performed with cell extracts containing 10nM receptor complex according to Scatchard plot. **A)** [^{3}H]-Ponasterone A binding in the presence of 1x hsp27 and 5x hsp27. **B)** [^{3}H]-Ponasterone A binding in the presence of 1x Pal-1 and 5x Pal-1 **C)** [^{3}H]-Ponasterone A binding in the presence of 1x DR-1 and 5x DR-1 (Mean $\pm$ SD, n= 3). Black bars: Ligand binding in the absence of DNA; hatched bars: Ligand binding in the presence of HRE monomers; white bars: Ligand binding in the presence of HRE pentamers.

To investigate, whether the increase in ligand binding is due to altered ligand affinity or increased number of binding sites due to enhanced heterodimerization in the presence of DNA, we determined K_D-values and receptor concentrations for some receptor complexes. As shown for EcRB1/UspII heterodimer, the number of binding sites increases from 0.19± 0.024 nM/l to 1.14±0.28 nM/l in the presence of 5x DR-1 and simultaneously, in the same conditions, the affinity of the ligand was slightly impaired (about 2 fold) (Figure 16 A and B).

A



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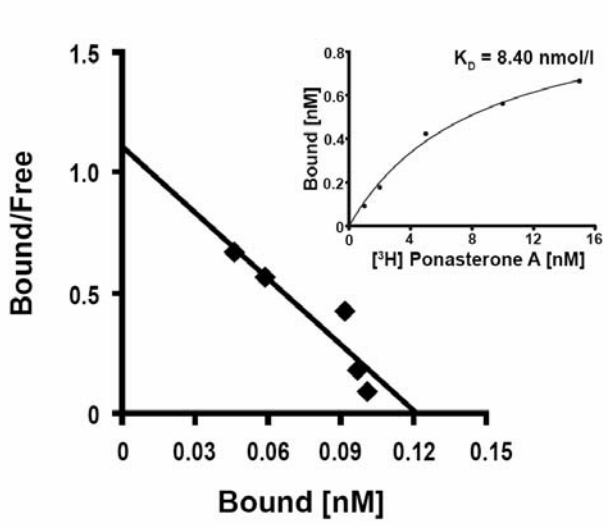

Figure 16 Ligand binding of EcRB1/UspII in the absence **(A)**, or presence **(B)** of 5x DR-1. ^{3}[H]-Ponasterone A (specific activity 7.9TBq/mmol) was used as ligand. Data were either analyzed using Scathard plot or Kaleida Graph software (insert) and comparable K_D values were obtained.

3.4.4. Deletion of the DNA binding domain of Usp improves ligand binding to EcR in the presence of DNA in an isoform specific manner

As mentioned above, in the absence of EcREs ligand binding was not changed if the C-domain of Usp was deleted and no influence of the EcR isoform was detectable (Table 3).

However, in the presence of EcREs deletion of the DNA binding domain of Usp selectively stimulated ligand binding. This effect was considerably higher in heterodimers containing EcRA compared to EcRB1 and EcRB2 both in the presence of DR-1 and Pal-1 monomers and pentamers (Figure 17 A,B,C).

According to Scatchard analysis (Figure 18 A and B) the number of binding sites was increased (5 fold) in the presence of the EcREs. Since the same cell extracts were used for determination of ligand binding with and without DNA, the only explanation is that the number of heterodimeric receptor complexes increased in the presence of a suited EcRE due to enhanced heterodimerization of the receptor molecules.

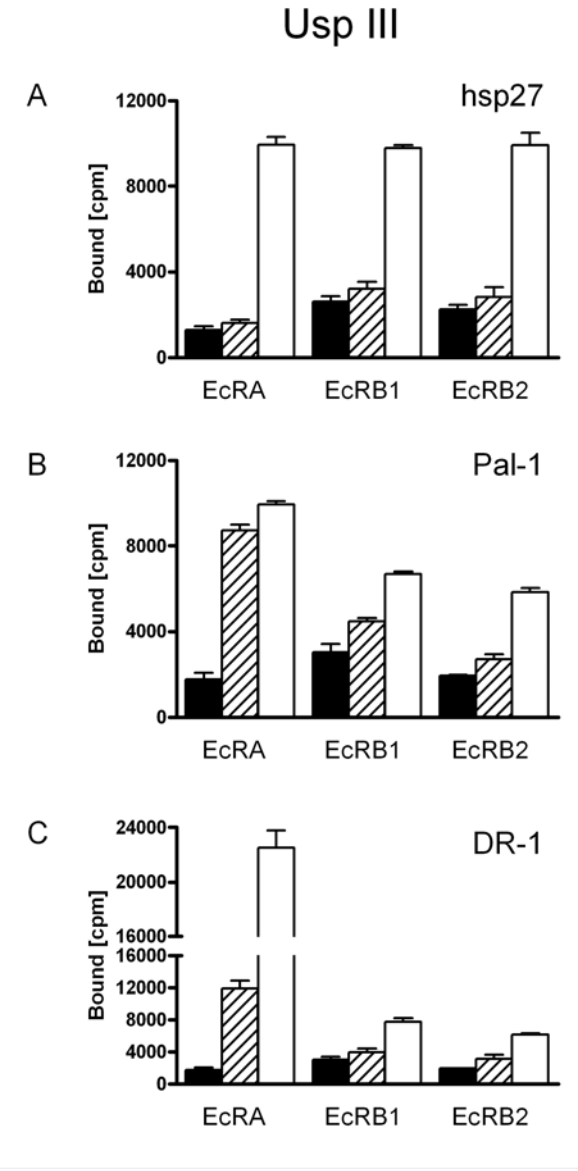

Figure 17 Influence of hormone response elements on ligand binding ([^{3}H]-Ponasterone A) to heterodimers of EcR isoforms with Usp III. Binding experiments were performed with cell extracts containing 10nM receptor complex according to Scatchard plot. **A)** [^{3}H]-Ponasterone A binding in the presence of 1x hsp27 and 5x hsp27. **B)** [^{3}H]-Ponasterone A binding in the presence of 1x Pal-1 and 5x Pal-1. **C)** [^{3}H]-Ponasterone A binding in the presence of 1x DR-1 and 5x DR-1 (Mean $\pm$ SD, n= 3). Black bars: Ligand binding in the absence of DNA; hatched bars: Ligand binding in the presence of HRE monomers; white bars: Ligand binding in the presence of HRE pentamers.

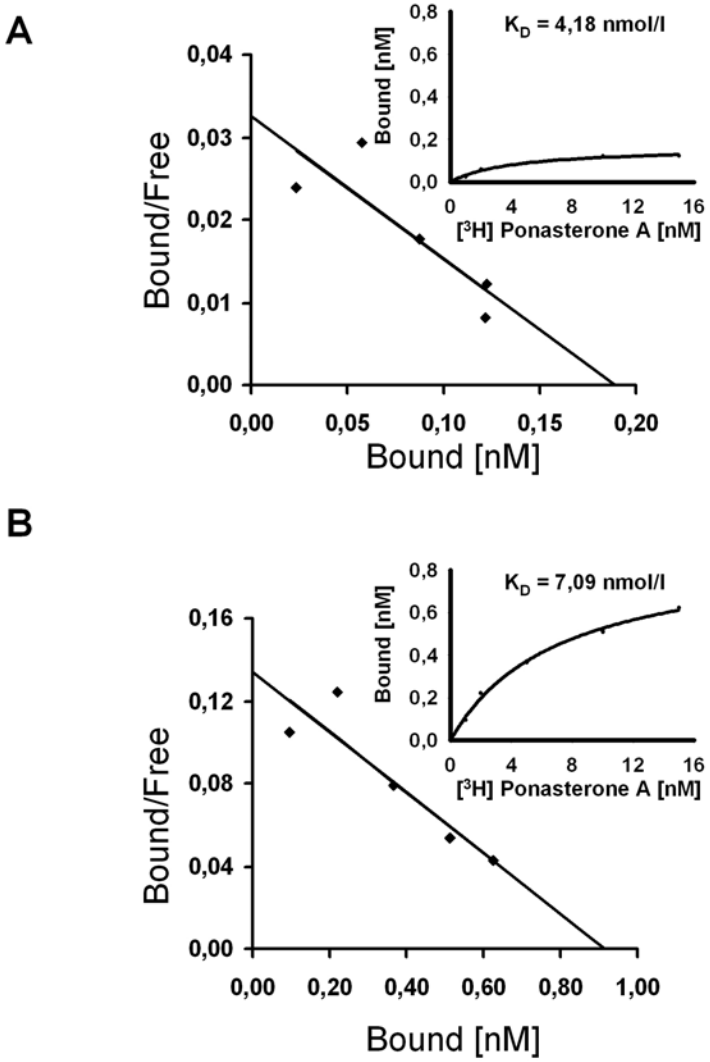

Figure 18 Ligand binding to EcR-A/UspIII in the absence **(A)** or presence **(B)** of 5x DR-1. ^{3}[H]-Ponasterone A (specific activity: 7.9 TBq/mmol) was used as ligand Data were either analyzed using Scathard plot or Kaleida Graph software (insert) and comparable K_D values were obtained.

3.4.5. RXR can partially replace Usp in ligand binding assays only in the presence of DNA

When Usp was replaced by RXR, no high affinity hormone binding was observed (Figure 19 A). However, in the presence of a hormone response element, RXR also conferred ligand binding to the heterodimer (Figure 19 B). Ligand affinity was in a similar range compared to heterodimers with Usp, but the number of binding sites seemed to be reduced, indicating that heterodimerization of EcR with RXR was less efficient compared to formation of EcR/Usp complexes.

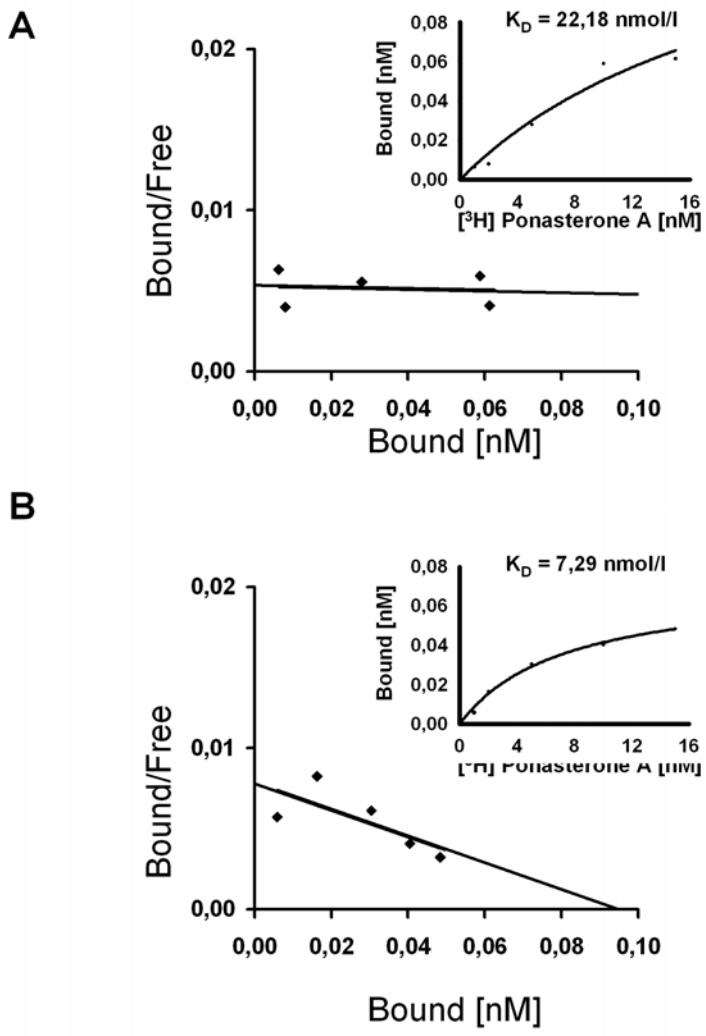

Figure 19 Ligand binding of EcRB1/RXR in the absence **(A)** or presence **(B)** of 5x DR-1. ^{3}[H]-Ponasterone A (specific activity: 7.9 TBq / mmol) was used as ligand. Data were either analyzed using Scathard plot or Kaleida Graph software (insert) and comparable K_D values were obtained.

3.5. Effects of EcR point mutations on ligand binding

Mutant EcR proteins produced by site-directed mutagenesis offer a strategy for developing hypotheses and considerations for studying *in vivo* functions. In these studies, two site-directed mutations in the EcR LBD were tested in each of the three full-length EcR isoforms to determine whether the substitutions evoked the same effect in whole receptors as they did in analogous yeast two-hybrid fusion proteins (Lezzi et al., 2002; Grebe et al., 2003; Przibilla et al., 2004).

The M504A substitution involves a residue associated with ligand-binding according to the crystal structure of the EcR from *Heliothis zea* (Billas et al., 2003) and shared by all reported EcRs in helix 5 of the LBD. Substitution of methionine at position 504 in the ligand binding domain of EcR with alanine destroys ligand binding (Figure 20).

The K497 residue lies in helix 4 and aligns with a consensus cofactor binding site in nuclear receptors. Substitution of Lysine at position 497 with alanine in the ligand binding domain of EcR impaired ligand binding (about 10 fold) by disruption of a salt bridge between helix 4 and helix 12 (Figure 21). This evident effect was observed for all three ecdysteroid receptor isoforms of *Drosophila* in the presence of heterodimeric partners Uspwt, UspII and UspIII.

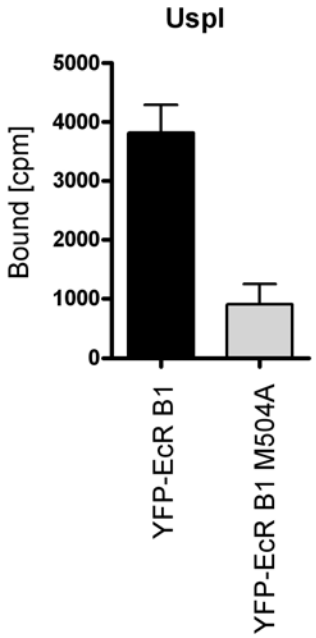

Figure 20 [^{3}H]-Ponasterone A binding to YFP-EcRB1 /UspI and YFP-EcRB1M504A/UspI. Values represent the Mean ± SD of three experiments.

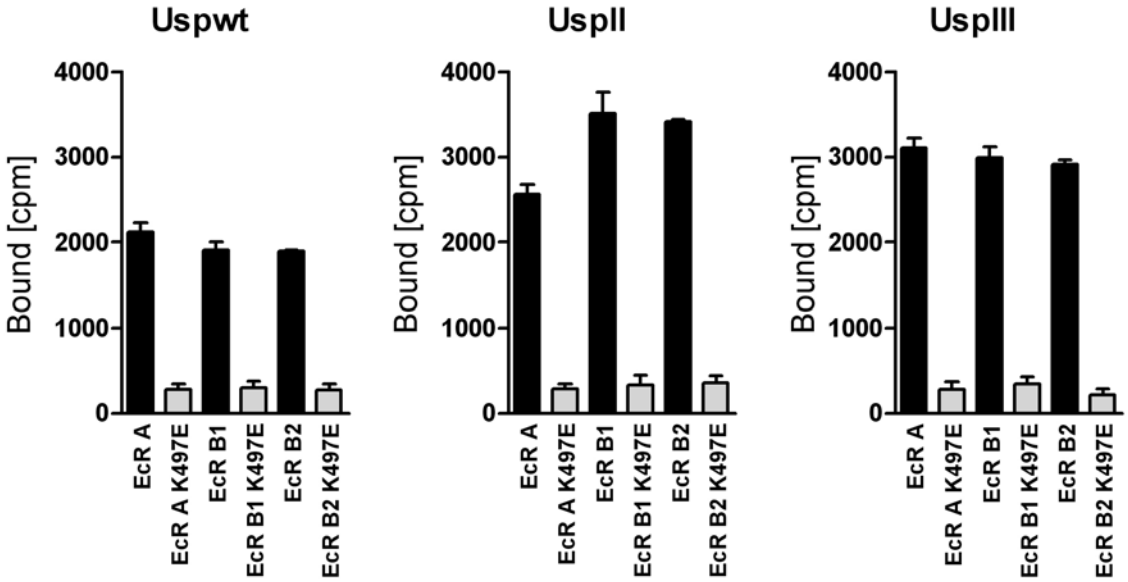

Figure 21 [^{3}H]-Ponasterone A binding to EcRs/Usps (black bars) and EcRsK497E/Usps (grey bars). Values represent the Mean ± SD of three experiments.

4. Discussion

Ligand binding of the Ecdysone receptor

The dogma of the 20 hydroxyecdysone (20E) hormone action during *Drosophila* development shows that EcR isoforms and Usp build an active heterodimer complex able to bind the EcREs of target genes responsible for directing the major developmental directions in insects. When not bound by ligand, the heterodimer associates with a co-repressor complex to prevent transcription of those genes. The ligand binding to EcR-LBD leads to a conformational change in the complex followed by the dissociation of the co-repressor complex and the recruitment of co-activators for high-level transcriptional activation (reviewed by Riddiford et al., 2000).

Although this model is well supported by evidence that both EcR and Usp are required to initiate events during the late-larval and prepupal periods, Constantino and co-workers (2008) showed that ecdysone signaling is mediated in a certain developmental stage without participation of Usp or any other RXR-like molecule.

Ecdysone receptors from arthropod species including *Drosophila melanogaster* and *Leptinotarsa decemlineata* bind Ponasterone A specifically even in the absence of a heterodimerization partner, although with reduced ligand affinity (Grebe et al., 2003; Ogura et al., 2005). Since affinity of the ligand to cell extracts *(in vitro)* containing only EcR is about 90 fold lower, the biological significance of hormone binding in the absence of Usp was often considered to be neglectable, although ligand-induced changes in receptor function in the absence of a heterodimerization partner were demonstrated repeatedly e.g. increased nuclear localization of EcR

(Nieva et al., 2007), enhanced interaction with DNA (Braun et al., submitted) and chromatin (Cronauer et al., 2007).

Altogether, determination of ligand binding under various experimental conditions gives insight on the impact of different receptor domains, DNA and dimerization partners on hormone-induced effects.

4.1. Influence of the heterodimerization partner in ligand binding

Ultraspiracle (Usp), as a partner for EcR, modulates the ecdysone receptor function in various ways. Usp increases the stability of EcR (Nieva et al., 2008), enhances nuclear localization of EcR (Nieva et al., 2005) and reduces ligand dissociation resulting in increased ligand affinity of the EcR/Usp complexes. Moreover, Usp affects interaction with DNA and enhances transcriptional activity of the EcR/Usp receptor complex.

The data of present work demonstrate that the A/B-domain of wild type Usp has no effect on ligand binding. Furthermore, additional deletion of the C-domain (DNA-binding domain) of Usp, which harbors a strong dimerization interface, does not affect this process.

The activation domain of VP16 fused to CDE-domains of Usp, (which is usually used for transactivation studies to overcome the inhibitory action of the A/B-domain of Usp)(Henrich, 2005; Beatty et al., 2006), confers the same ligand binding capability to all EcR isoforms as Uspwt.

4.2. Influence of the DNA in ligand binding

One of the most puzzling aspects of the Ecdysteroid receptor is its heterodimeric nature, typical for nonsteroid receptors of Nuclear receptor-superfamily and its preference for binding palindromic DNA sequences (PAL-1) (Vögtli et al., 1998), direct repeats (DR-1) or inverted palindroms (IPs).

The ability of binding to these different motifs implies that DBDs can spatially rotate with respect to the LBDs that are held together through the dimerization interface. Based on mutational analysis of androgen and progesterone receptors, the first zinc finger (which contains a region termed P-box) has been identified as the one responsible for the discrimination between DNA motifs (Chambon et al., 1988).

The arrangement and spacing between the motifs represent major determinants to confer selectivity and specificity. Palindromic (Pal) DNA repeats impose a symmetrical structure that results in a head-to-head arrangement of the DBDs of the dimmer. On the other hand, Direct Repeats (DRs) are inherently asymmetric and therefore heterodimeric complexes may bind to them with two distinct polarities.

Interaction of the ecdysone receptor with DNA was studied mainly with electromobility shift assays (EMSA). A palindromic sequence is preferred by EcR/Usp heterodimer, but direct repeats with different spacers and half-sites were also recognized (Spindler et al., 2001). Homodimers of EcR and Usp can also interact with DNA: while EcR prefers palindroms, Usp was reported to bind direct repeats (Spindler et al., 2001).

Interaction of EcR with DNA improves ligand binding in the absence of a heterodimerization partner

The increase in ligand affinity in the presence of an EcREs shows that not only dimerization via the ligand binding domains, but also via C-domains positively affects the ligand binding domain of EcR, presumably by stimulating homodimerization mediated by the strong dimerization interface in the DNA binding domain. Comparison with data reported by Grebe et al. (2004) revealed that ligand binding is enhanced about 3-4 fold in the presence of DNA, but is still about 20-25 fold lower compared to ligand affinity of the heterodimer EcR/Usp.

The A/B-domain of EcR does not affect ligand binding of EcR/Usp in the absence of DNA

As known from vertebrates, the stability of receptor proteins is dependent on the A/B-domain, which is post-translationally modified several fold by phosphorylation (Rauch et al., 1998) and ubiquitination (Picard et al., 2008). The influence of the A/B-domain of EcR on ligand binding is neglectable, but the presence of ligand and the heterodimer considerably improves receptor stability in an isoform-specific manner.

As described above, ligand binding of unpartnered EcR varies in an isoform-specific manner both in the absence or presence of DNA, depending on the A/B-domains of the receptor isoforms.

In the present thesis is shown that ligand affinity of EcR/Usp heterodimers is in the same range for all EcR isoforms in the absence of DNA. These results are in line with data reported by Perera et al. (1999) for ecdysone receptor of *Choristoneura fumiferana.* Moreover, the affinity of EcR/Usp in our experimental setup is in accordance with the data reported previously for heterodimers with the EcRB1 isoform (Yao et al., 1992) and is also comparable to heterodimers encompassing only the ligand binding domains of EcR and Usp fused to Gal4-AD and Gal4-DBD respectively (Grebe et al., 2003). Altogether, these data demonstrate that ligand binding of EcR/Usp is not influenced by other domains of the ecdysone receptor molecule.

However, in the presence of DNA the A/B-domain of EcR isoforms affects ligand binding of heterodimers. An influence of the A/B-domain of nuclear receptors on ligand-induced transcriptional activity was reported for different isoforms of vertebrate nuclear receptors (Bevan et al., 1999; Matthews and Gustafsson, 2003; Tian et al., 2006). This influence is likely

due to the interaction of N-terminus of the receptor with the ligand binding domain (LBD)(Takimoto et al., 2003). Ongoing experiments in our lab by Tremmel and colaboration partner – Prof. Schaefer (University of Leipzig) using FRET analysis will help revealing the isoform specific interaction of N- and C-terminus of EcR.

Influence of the A/B domain of the heterodimerization partner on ligand binding in the presence of DNA

Although ligand binding is stimulated in the presence of DNA, no influence of the type of EcRE or the A/B domain of EcR on ligand binding to EcR/Usp heterodimers was observed. However, this is true only if the A/B-domain of wild type Usp is present. Since transcriptional activity of heterodimers with wild type Usp and especially EcRB1 isoform is rather low even in the presence of hormone, the A/B domain was replaced by the activation domain of VP16 (Henrich, 2005). Surprisingly, this exchange has considerable consequences for ligand binding to the heterodimer in the presence of DNA. The A/B-domain of Usp does not only regulate the transactivation capability of the receptor complex, but affects also Ponasterone A binding (Azoitei et al., submitted). These results show that Usp is not only a passive partner as was previously proposed (Hu et al., 2003), but plays an active role in receptor-mediated hormone action. In contrast to heterodimers of all EcR isoforms with wild type Usp (EcRs/Uspwt), receptor complexes with $Vp16_{AD}$-Usp fusion proteins modify ligand binding of the heterodimers depending on the EcR isoform and type of EcRE suggesting that physiological consequences of isoform-specific hormone effects obtained with $Vp16_{AD}$-Usp fusion proteins should be interpreted cautiously since might not necessarily reflect the physiological situation.

Deletion of the C-domain of Usp selectively modifies ligand binding in the presence of DNA

Detailed analysis of the interaction of EcR and Usp DNA-binding domains with the pseudo-palindromic response element from the hsp27 gene promoter (Ozyhar et al., 1991; Riddihough et al., 1997) demonstrated that UspDBD can act as a specific anchor preferentially binding the 5' half-site on this element and thus locating the heterocomplex in a defined orientation.

According to Niedziela-Majka et al. (2000), the C-domain of Usp has a profound influence on DNA binding of the receptor complex directing EcR to the 5'-end of the HRE. Therefore we expected that deletion of the DNA binding domain of Usp also to have an impact on ligand affinity to the receptor complexes. However, in our experimental model we found that deletion of C-domain of Usp, which harbors a strong dimerization interface, does not affect the ligand binding.

On the other hand, heterodimerization through ligand binding domains of EcR and Usp was still present as demonstrated by EMSA experiments (Beatty et al., 2006). Although dimerization is considerably weaker in the absence of the C-domain of Usp (Azoitei et al., submitted), ligand binding in the absence of EcREs is not changed compared to heterodimers with full length Usp, as already mentioned above. Pronounced ligand binding in the absence of the C-domain of Usp after addition of EcREs demonstrates that dimerization through E-domains of the both nuclear receptors allows a more efficient ligand binding.

RXR confers ligand binding to EcR only in the presence of DNA

Retinoid X receptor (RXR) - the mammalian orthologue of the invertebrate Ultraspiracle (Usp) - plays a crucial role in many intracellular signaling pathways as a heterodimerization partner with other members of the nuclear receptor family as FXR, RAR, TR, VDR, PPAR. My experiments demonstrate that Usp replacement by RXR abolished ligand binding, indicating a weaker EcR/RXR heterodimerization as compared to EcR/Usp complex formation.

In presence of a strong heterodimerization partner like Usp, dimerization via the ligand binding domains seems to be sufficient to confer high affinity ligand binding to EcR even in the absence of DNA, since ligand affinity is not impaired if the C- domain of Usp is deleted. The weak dimerization (as in the case of that between either EcR homodimers or heterodimers with RXR) is reinforced in the presence of a suited EcRE which allows a high affinity ligand binding comparable to the ligand binding levels of that found for EcR/Usp complexes. This observation, explains why RXR can be successfully used to replace Usp in transcriptional assays (Henrich et al., 1993; Thomas et al., 1993; Nieva et al., 2005), but not in other functional tests such as nuclear import which do not involve interaction with DNA (Nieva et al.,2007).

The intra- and intermolecular interactions described here, which allow modulation of ligand binding by the A/B- and C-domains of the receptor molecule, the type and number of EcREs, and dimerization partners provide further examples of the high flexibility of the nuclear receptor complexes like the ecdysone receptor, offering multiple possibilities for fine tuning of the hormonal response. As outlined previously receptor domains like the ligand

binding domain (Billas et al., 2003) or the C-domain (Orlovski et al., 2004) are rather flexible, which certainly facilitates modification of receptor activity.

4.3. Influence of point mutations in ligand binding

The absence of an endogenous ecdysteroid response in CHO-K1 mammalian cells provides the opportunity to use mutated forms of EcR and Usp, so that the relationship between structure and individual receptor functions like 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 a baseline response and the presence of undefined insect comodulators (Henrich et al., 2008-Review).

According to the homology model of Wurtz et al. (2000), some amino acids within ligand binding domain are involved in ligand binding, while others do not show direct contact to the ligand. While EcR I463T does not impair ligand binding to EcR and EcR/Usp, EcR E476A was reported to reduce this process to a considerable degree (Grebe et al., 2003).

Many EcR mutants used in yeast two-hybrid system have been subsequently tested in full-length receptors in the CHO-K1 cell system. These also included point mutations corresponding to naturally occurring EcR mutations displaying a lethal larval phenotype (Bender et al, 1997). Such EcR mutants tended to show reduced basal as well as inducible transcriptional activity (Bergman et al, 2004) and also reduced ligand affinity (Grebe et al, 2003). Null *in vivo* mutations of EcR cause embryonic lethality and the partial activity seen in the two-hybrid and cell culture systems may underline the ability of mutants to survive to the larval stages.

Another class of mutations destroys specific receptor functions in both, yeast two-hybrid assays and the CHO-K1 cell culture system. For example,

the M504A substitution involves a residue associated with ligand-binding in the crystal structure of the EcR from *Heliothis zea* (Billas et al, 2003) and shared by all reported EcR isoforms in helix 5 of the LBD. M504 forms a hydrogen bond with the ligand according with the RAR-based model (Wurtz et al., 2000). This amino acid is essential for hormone binding and ligand-dependent dimerization, which is abolished after mutation to arginine (Grebe et al., 2003). Similarly, in our experimental setup M504A substitution effectively impaired the ligand binding.

A different substitution of a residue in helix 4 of the LBD shared by all known insect EcRs, namely K497E, further illustrated the multifunctional properties of individual residues within the ecdysteroid receptor sequence. This site was selected because it has been associated with cofactor interactions among several nuclear receptors (Wurtz et al, 1995). The effects of K497E on the ligand-binding domain have been initially described in the yeast two-hybrid system where GAL4(AD)-EcR(K497E)/GAL4(DBD)-Usp caused a higher basal transcriptional activity than in wild-type. Furthermore, K497E mutation was able to disrupt the salt bridge between helix 4 and helix 12 of EcR LBD resulting in decreased ligand binding (Grebe et al., 2003) which subsequently impaired ligand-dependent dimerization. Based on two-hybrid screen results, we sought to examine the properties of K497E in each of the three isoforms of EcR together with Uspwt, UspII and UspIII. Interestingly, despite considerably reduced ligand binding, hormone-induced transcriptional activity was not impaired. Therefore it is reasonable to assume that K497 is additionally involved in corepressor binding (Claessens et al., 2007), (Ruff et al., submitted).

4.4. Influence of receptor concentration on hormonal response of the Ecdysone receptor

Transcriptional activity of receptor proteins is routinely normalized on the activity of a constitutively expressed reporter like β-galactosidase, which compensate for differences in transfection efficiency. This is justified if the activity of the same protein or receptor complex is measured under different experimental conditions. As reported previously, ecdysone receptor protein is stabilized by hormone application, type and concentration of heterodimerization partners (Nieva et al., 2008), dimerization capabilities of receptor isoforms and accessory proteins like comodulators and heat-shock proteins (Cronauer and Spindler-Barth, unpublished observations). However, stability of receptor protein is different for all three EcR isoforms and the impact of hormone and dimerization partner also varies accordingly (Ruff et al., submitted).

For comparison of receptor functionality of EcR isoforms, like interaction with DNA or transcriptional activity it is essential that equal amounts of receptor protein complexes are used. It is common practice to normalize data on the activity of a co-expressed reporter gene coupled to a constitutive promoter. However, in our experimental setup co-transfection experiments with *lacZ* expression vector (pCHIII-lacZ) followed by quantification of β-galactosidase activity revealed a deviation of receptor concentration up to 40-fold as compared to the concentration of the heterodimer calculated by Scatchard plot. By contrast, quantification of specific Western blots signals of EcR isoforms, in the absence or presence of its heterodimerization partner, and ligand binding Scatchard plot tests lead to comparable results showing that dimerization of EcR isoforms with Usp was in a similar range.

These results bring a new insight with respect to a suitable approach / tool for calculation of receptor protein concentrations required in fine-tuned molecular mechanisms such as the interaction of ecdysone receptors EcR and Usp.

5. Material and Methods

5.1. Material

5.1.1. Biological material

5.1.1.1. Bacteria strain *E.coli* (Stratagene)

XL1-Blue – *end*A1 *gyrA*96(nal^R) *thi*-1 *rec*A1 *rel*A1 *lac gln*V44 F'[::*Tn*10 *pro*AB$^+$ *lac*I^q Δ(*lac*Z)M15] *hsd*R17(r_K^- m_K^+)

The function of genetic markers is in the table below:

Marker	Function
F’	Strain contains an F' episome.
*end*A1	Mutation in endonuclease I gene. Improves quality of isolated plasmid DNA.
*gln*V44	Suppression of amber (UAG) stop codons by insertion of glutamine.
*gyr*A96 (nal^R)	Mutation in DNA gyrase gene. Confers resistance to nalidixic acid.
*hsd*R17 (r_K^- m_K^+)	Restriction system that methylates host DNA (in) specific sites and cleaves DNA that is not methylated. The R gene codes for endonuclease, the M gene codes for methylase, and the S gene is required for the functionality of both enzymes. Thus hsdR mutants don’t have the endonuclease function, but can still methylate.

*lac*I^q	Mutation leads to high levels of the *lac* repressor protein, inhibiting transcription from the *lac* promoter.
Δ(*lac*Z)M15	Partial deletion of beta-galactosidase gene. Allows blue/white selection for recombinant colonies, when plated on X-Gal/IPTG.
*pro*AB$^+$	Mutation in genes involved in proline metabolism. Strains require proline for growth on minimal media.
*rec*A1	Mutation in general recombination gene.
*rel*A1	Relaxed phenotype, mutation eliminates stringent factor. Allows RNA synthesis in the absence of protein synthesis.
thi-1	Mutation in thiamine metabolism gene. Strains require thiamine for growth on minimal media.
*Tn*10	Transposon conferring resistance to tetracycline.

5.1.1.2. Chinese hamster ovary cells (CHO-K1)

Chinese hamster ovary cells (CHO-K1) (ATCC CCL-61) represent a subclone of the parental CHO cell line established by Puck et al. in 1958. They originate from ovary biopsy of an adult Chinese hamster. Morphologically, cells are adherent fibroblastoid. CHO-K1 cells have a low RXR content (Nieva et al., 2008).

5.1.2. DNA elements

5.1.2.1. Oligonucleotides

For some experiments ligand binding was performed in the presence of DNA. In this case 280 ng/100 µl (final concentration) of the following double stranded oligonucleotides were used:

1). 1x hsp27: AGCGACAAGGGTTCAATGCACTTGT
ATTGGACAAGTGCATTGAACCCTTGT

2). 5x hsp27: (AGCGACAAGGGTTCAATGCACTTGT
ATTGGACAAGTGCATTGAACCCTTGT)$_5$

3).1xDR-1:GATCTAGAGAGGTCAAAGGTCATGTCCAAG
GATCCTTGGACATGACCTTTGACCTCTCTA

4).5xDR-1(GATCTAGAGAGGTCAAAGGTCATGTCCAAG
GATCCTTGGACATGACCTTTGACCTCTCTA)$_5$

5).1xPal-1: GATCTAGAGAGGTCAATGACCTCGTCCAAG
GATCCTTGGACGAGGTCATTGACCTCTCTA

6). 5xPal-1: (GATCTAGAGAGGTCAATGACCTCGTCCAAG
GATCCTTGGACGAGGTCATTGACCTCTCTA)$_5$

Hsp 27 was purchased from Thermo (Ulm, Germany) and Pal-1 and DR-1 were obtained from Biomers (Ulm, Germany).

5.1.2.2. Plasmids:

pcDNA3-dEcR isoforms (pcDNA3-dEcRA, pcDNA3-dEcRB1 and pcDNA3-dEcRB2) kindly provided by Dr.V.C. Henrich (University of North Carolina, Greensboro, USA) were cloned between *BamHI – XbaI* sites in pcDNA3 vector (Invitrogen, Carlsbad, USA). The strategy of plasmid construction is described by Mouillet et al. (2001).

pEYFP-N1/Usp STOP:

Wild type Usp fused to the fluorescent protein YFP and inserted into pEYFP-N1 (Clontech Palo Alto, CA, USA) using *Eco*RI and *Sal*I restriction sites was provided by Prof. Ozyhar (University of Wroclaw, Poland) and modified further by Dr. S. Braun (University of Ulm, Germany) to allow expression of a fusion protein encompassing aa 1-507 of Usp.

pVP16-dUsp:

DNA constructs coding for different Usp variants (UspI, UspII and UspIII) were obtained from Dr.V.C.Henrich. All Usp variants were cloned between *EcoRI* and *HindIII* into pVP16 expression vector (BD Biosciences Clontech, Palo Alto, CA, USA) thereby replacing A/B transactivation domain of *Drosophila* Uspwt by VP16-AD .

The UspI variant includes the codons for 6 amino acids adjacent to the N-terminus of the C- domain of Usp that are conserved among all insect Usp sequences along with the C-domain (DNA binding domain), D-domain (hinge region) and E-domain (ligand binding domain). The UspII variant is identical to UspI, except that those 6 amino acids are not included. The UspIII variant carries only the hinge region and LBD of Uspwt (Figure 22).

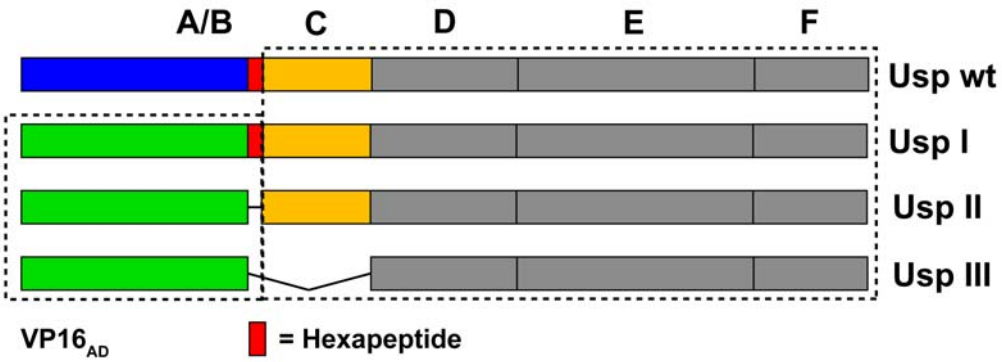

Figure 22 The UspI (VP16AD-UspCDE) variant includes the last 6 amino acids of the N-terminal domain and the remaining carboxy-terminal portion of the open reading frame (amino acids 98-507) which includes the DNA-binding domain, the hinge region and ligand binding domain. The shorter UspII variant (VP16AD-UspCDE) starts at the beginning of Usp DBD and codes for amino acids 104-507. A third variant VP16AD-UspDE, coding for amino acids 170-507, lacks the DNA binding domain, that means it carries only the hinge region and ligand binding domain.

pcDNA3-EosFP

The sequence coding for a small green fluorescence protein (EosFP, 25.9 kDa) was cloned into pcDNA3 vector (Invitrogen, Carlsbad, USA) and used for monitoring of mammalian cells transfection efficiency. The construct was kindly provided by Dr. J. Wiedemann at Department of General Zoology and Endocrinology, University of Ulm.

pCHIII-lacZ reporter construct coding for β-galactosidase was used for normalization of mammalian cells transfection efficiency.

pEYFP-EcR$_{K497E}$ isoforms were kindly provided by C. Tremmel (Department of General Zoology and Endocrinology) . They were obtained by direct mutagenesis (Stratagene, Heidelberg,Germany).

pEYFP-EcRB1$_{M504A}$ was provided by Dr. C. Nieva (Department of General Zoology and Endocrinology). It was obtained by direct mutagenesis (Stratagene, Heidelberg, Germany).

5.1.3. Kits and enzymes

Item	Company
Lipofectamine 2000	Invitrogen, USA
Lipofectamine LTX	Invitrogen, USA
PureYield Plasmid MidiPrep System	Promega, Germany
Trypsin-EDTA 1x	PAA, Germany

5.1.4. Chemicals

Item	Company
Acetic acid	Merck, Germany
Acrylamide solution 40%	Sigma, Germany
Agarose Ultrapure, Electrophoresis Grade	Gibco BRL , Scotland
Bio-Mager Milch Powder	Bio Heirler,Germany
Boric acid	AppliChem, Germany
BSA	Sigma, Germany
Chloroform	J.T.Baker, Holland
Coomasie Brillant Blue R250 / G250	Serva, Germany
DTT	Sigma, Germany
ECL, chemiluminescence detection reagent	Amersham, UK
EDTA – Dihydrate	AppliChem, Germany
Ethanol, absolute	Sigma-Aldrich, Germany
Glycin	AppliChem, Germany
HEPES	Carl Roth GmbH, Germany
Isopropanol	Merck, Germany
LSC-Cocktail Filter count	Packard, Germany

Methanol	Merck, Germany
Molecular weight standard SDS Page	Sigma
Non fat dried milk	AppliChem, Germany
O-Phosphoric acid	Carl Roth GmbH, Germany
Peptone from casein	AppliChem , Germany
Ponceau S Concentrate	Sigma
Potassium chloride	Carl Roth GmbH , Germany
Protease inhibitors (Aprotinin, Leupeptin, Pepstatin)	Sigma, Germany
SDS (sodium dodecyl sulphate)	AppliChem, Germany
Sodium Chloride	AppliChem, Germany
Sodium dihydrogen phosphate	Merck, Germany
Sodium hydroxide, pellets, technical grade	AppliChem, Germany
TEMED (N,N,N',N', tetramethyl ethylenediamine)	AppliChem, Germany
Thymerosal	Sigma, Germany
Tris, Ultrapure	AppliChem, Germany
Tween 20	AppliChem, Germany
Yeast extract	AppliChem, Germany
β-Mercaptoethanol	Sigma, Germany

5.1.5 Antibodies

Antibody	Dilution	Company
VP16 goat-polyclonal	1:1000	Sigma, Germany
AB11 mouse-monoclonal	1:2000	F.C. Kafatos, UK
YFP A.v.-peptide IgG mouse-polyclonal	1:500	Clontech, USA
Anti-Mouse IgG Peroxidase Conjugate	1:1000	Sigma, Germany
Anti-Rabbit IgG Peroxidase	1:1000	Sigma, Germany
DDA 2.7 mouse-monoclonal	1:3000	C. Thummel, Utah, USA

5.1.6. Radiochemicals

^{3}[H]-Ponasterone A of 7,9 TBq/mmol specific activity was kindly provided by Prof. H. Kayser (Syngenta, Basel, Switzerland). The purity was checked routinely by HPLC and radiolytic degradation products were removed by chromatography.

5.1.7. Cell culture

Item	**Company**
DMEM:F12(1:1)+L-glutamine	Gibco, Germany
FCS	Gibco, Germany
Trypan Blue	Gibco, Germany
Trypsin /EDTA 1x	Pan Biotech, Germany

5.1.8. Laboratory equipment

Item	**Company**
Centrifuge 5415	Eppendorf
Centrifuge 5804 R	Eppendorf
Spectrophotometer	Pharmacia
Thermomix	Eppendorf
Incubator	Hereus

5.1.9 Buffers

Lysis buffer

20mM TRIS

150mM NaCl

1mM EDTA

pH= 7,9

Assay buffer

60mM Na2HPO4

40mM NaH2PO4

5mM KCl

1mM MgCl2

50 mM beta-Mercaptoethanol

pH= 7,0

Ligand binding dilution buffer

20mM HEPES

15% (v/v) Glycerin

2mM EDTA

2mM DTT

pH= 7,9

Washing buffer

20mM HEPES

20mM NaCl

10% (v/v) Glycerin

1mM EDTA

1 mM beta-Mercaptoethanol

pH= 7,9

1xPBS

138 mM NaCl

2.7 mM KCl

1.8 mM KH2PO4

10 mM Na2HPO4 x 2H2O

pH = 7.2

Bradford solution:

0.1 g/l Coomassie Brilliant Blue G-250

5% (v/v) 96% (v/v) Ethanol

10% (v/v) 85% (w/v) Phosphoric acid (H3PO4)

Collecting gel buffer

1 M Tris / HCl (pH 6.8)

Running gel buffer

1.5 M Tris / HCl (pH 8.8)

Sodium dodecyl sulfate (SDS) solution

10% (w/v) Sodium dodecyl sulfate

Ammonium persulfate (APS) solution

0.4 g/ml Ammonium persulfate

Electrophoresis buffer (10x)

250 mM Tris-base

1920 mM Glycine

1% (w/v) SDS

pH = 8.3

2x Loading buffer

200 mM Tris-HCl

4% (w/v) SDS

6% (v/v) β-mercaptoethanol

20% (v/v) Glycerol

0.1% (w/v) Bromophenol blue

pH = 8.8

Ponceau solution

0.1% (w/v) Ponceau S (Sodium salt)

5% (v/v) Acetic acid

High Molecular Weight Standart Mixture (Sigma-Aldrich)

The mixture of the following 6 proteins:

Myosin, rabbit muscle 205.0 kDa

β-Galactosidase, *E.coli* 116.0 kDa

Phosphorylase b, rabbit muscle 97.4 kDa

Albumin, bovine 66.0 kDa

Albumin, egg 45.0 kDa

Carbonic Anhydrase, bovine erythrocytes 29.0 kDa

Tris buffered saline + Tween 20 (TBS-T)

20 mM Tris / HCl (pH 7.6)

137 mM NaCl

0.1% Tween 20

Transfer buffer

25 mM Tris

192 mM Glycine

20% Methanol

pH = 8.3

3% Non-fat milk

3% (w/v) Non-fat milk

1% bovine serum albumine (BSA)

0.02%Thymerosal

ECL Western blotting detection system (Amersham Biosciences)

Detection reagents 1 and 2. The composition is not known.

LB medium

10.0 g/l Pepton

10.0 g/l NaCl

5.0 g/l Yeast extract

1.5 % LB-agar

10.0 g/l Pepton

10.0 g/l NaCl

5.0 g/l Yeast extract

15.0 g/l Agar

Ampicillin solution

100 mg/ml Ampicillin Natrium

Kanamycin solution

50 mg/ml Kanamycin Monosulfate

Neutralization Solution

4090 mM Guanidine Hydrochloride (pH 4.8)

759 mM CH3COOK

2120 mM Glacial Acetic Acid

Column Wash solution

60 mM CH3COOK

60% (v/v) Ethanol

8.3 mM Tris-HCl

0.04 mM EDTA

Endotoxin Removal Wash

Pure YieldTM Clearing Column

Pure YieldTM Binding Column

Nuclease-Free Water

5.2. Methods

5.2.1. Maintenance of CHO-K1 cells and quantification of cells number

The Chinese hamster ovary (CHO-K1) cells were maintained in Dulbecco's modified Eagle medium (D-MEM/F12 with L-glutamine) supplemented with 5% fetal calf serum (FCS).

For quantification of cell number, a Neubauer chamber was used. Briefly, an aliquot of cell suspension was placed between glass slide and coverslip and cells were quantified on marked quadrate. Discrimination between living and dead cells was enabled by staining with Trypan blue, a dye, which enters into the cells upon membrane disruption.

Final number of cells is given by the formula:

$$\text{Living cells / ml media} = \text{average of quantified cells} \times 2^{(\text{dilution})} \times 10.000^{(\text{chamber factor})}$$

5.2.2. Cultivation of CHO-K1 cells

Cells were cultivated on tissue culture dishes at 37°C in a humidified atmosphere and 5% CO_2. Every three days CHO-K1 cells were washed once with 5 ml 1xPBS and trypsinized with 1ml of 1x Trypsin/EDTA (diluted 1:5), incubated at 37°C for 1-2 minutes and resuspended in pre-warmed fresh media (8-10ml). Finally, 1.2 – 1.5 x 10^6 cells /10 cm dish were plated.

5.2.3. Freezing and thawing of cells

Storage of cells at very low temperatures (e.g liquid nitrogen, -196°C) can drive formation of ice crystals triggering disruption of cell structure. In order to overcome this problem, a freezing agent, namely DMSO is routinely used. Shortly, cells in exponentially growing phase were trypsinized and

centrifuged for 5 minutes at 900 rpm. The cell pellet was resuspended in 1 ml freezing medium (90% culture media and 10% DMSO) and further incubated for 1 hour on ice, followed by 1 week at -80°C and finally stored in liquid nitrogen. Cells were thawed quickly at 37°C in a pre-warmed water bath, diluted with 10 ml fresh culture medium and plated in cell culture dishes.

5.2.4. Transfection of nuclear receptors in mammalian CHO-K1 cells

1,8 – 2 x 10^6 cells were plated on 10 cm cell culture dishes the day before transfection in order to reach 90% confluency at the start of the transfection procedure. 8,5 µg DNA consisting of 3,5 µg EcR, 3,5 µg Usp and 1,5 µg LacZ reporter were incubated for 5 minutes at room temperature with up to 750 µl serum free medium (mix A). In parallel, 25 µl either Lipofectamine 2000 or 20 µl Lipofectamine LTX were incubated for 5 minutes at room temperature with 725 µl serum free medium (mix B). Mix A and mix B were combined and further incubated for 20 to 30 minutes at room temperature. The resulting 1,5 ml cocktail (DNA + Lipofectamine + serum-free media) supplemented with additional 8,5 ml serum-free medium was added to the cells and incubated for 4 hours. During this time DNA-liposomal complexes fuse with the cell membrane enabling transfer of DNA. Thereafter the transfection mixture was replaced by fresh culture medium. After twenty-four hours extracts were prepared and used for subsequent experiments.

5.2.5. Reporter gene assay

Transfection of nuclear receptors was normalized upon co-transfection of CHO-K1 cells with a LacZ reporter followed by the measurement of β-galactosidase activity at 420 nm. Briefly, twenty-four hours after transfection, protein extracts were prepared and a volume of 5 µl of cleared lysate was

incubated with 500 µl assay buffer (see buffers formulation appendix 5.1.9) supplemented with 150 µl ONPG. The reaction mixture was incubated at 37°C in a water-bath until a light yellow colour arose. The reaction was then stopped with 300 µl of 1 M Na_2CO_3 and OD measured at 420 nm with a spectrophotometer. In some experiments the EosFP green fluorescence marker was involved for monitoring the positive transfected cells.

5.2.6. Preparation of protein extracts and determination of protein concentration (Bradford reaction, 1976)

The transfected cells were gently detached from the plate surface with a scraper. The cells were rinsed with 5 ml ice-cold 1xPBS and transfered to 15 ml tubes. After centrifugation (260g, 4 min, 4°C) the supernatant was discarded and the cell pellet was resuspended in three packed volumes of cold extraction buffer (see buffers formulation appendix 5.1.9). Lysates were subjected to three freeze/thaw cycles and centrifuged (18000g, 30 min, 4°C). The protein concentration of the cleared supernatant was determined according to Bradford (1976).

The concentration of protein in the samples was compared with BSA which was used to prepare a standard curve. The samples were supplemented with water up to 800 µl total volume. To each probe 200 µl of Coomasie dye was added, mixed and the absorbance of Coomasie dye protein complex was measured after 15 min. at OD_{595nm} using a Pharmacia Biotech Novaspec II spectrophotometer.

5.2.7. Ligand binding assay

Cell extracts, prepared as described above, were placed in siliconized tubes and then diluted with ligand binding dilution buffer (see buffers formulation appendix 5.1.9) supplemented freshly with ALP protease inhibitors (aprotinin,

leupeptin, pepstatin, final concentration 1µg/ml each) and 1mM DTT. Ligand-binding was determined with ^{3}[H]-Ponasterone A (with specific activity 7.9 TBq/mmol) using a filter assay (Turberg and Spindler, 1992). The total volume of the incubation mixture was 100µl. Aliquots of cell extracts were incubated with different concentrations of ^{3}[H]-Ponasterone A between 0,5-7,5 nM for 4-5hrs at 4°C. After this time the incubation mixtures were transferred to the filtration apparatus with NC45 nitrocellulose membranes, washed with 12ml (4ml three times) ice-cold washing buffer (see buffers formulation appendix 5.1.9), filtered immediately by applying vacuum. After washing the radioactivity absorbed at the filter was measured with a liquid scintiallation counter (TRicarb 1500, Perkin Elmer, Rodgau-Jügesheim,Germany) and the non specific binding determined by competition with 1µM 20-OH-Ecdysone subtracted. As additional control extract from untransfected cells was used (<100cpm).

For calculation of K_D -values the results were evaluated according to Scatchard (1949) or calculated with Kaleida Graph version 4.0 (Synergy Software, Reading, PA, USA). Comparable results were obtained with both methods.

The influence of different hormone response elements (280ng/100µl) was studied using the same amount of receptor protein and ligand. Nonspecific DNA of comparable size was used to eliminate nonspecific interaction with DNA and subtracted.

Statistical significance (P values less than 0,01) of differences among two groups was determined on the basis of a two-sided, matched paired Student's t test.

5.2.8. Purification of ^{3}[H]-Ponasterone A with High Performance Liquid Chromatography (HPLC)

To verify the purity of ^{3}[H]-Ponasterone A, routine analytical HPLC was performed and a purity <95% of Ponasterone A was achieved. Briefly, [^{3}H]-labeled Ponasterone A dissolved in methanol was dried by means of a Speed-Vacuum Concentrator (Bachhofer, Germany), resuspended in a water : methanol mix = 60:40 and then injected in a previously de-gassed HPLC system (Figure 23).

The separation of disintegration products was achieved using a reverse phase column (Radial-Pak, type 8NVC186, Water) in a time frame of 81 minutes and under a constant flow of 1 ml/min (600^{TM} controller, 600^{TM} pump, Waters). Absorbance of UV light was measured with a tunable absorbance detector and for radioactivity measurements was used a flow scintillation counter (Radiomatic 500TR, LSC Cocktail Ultimo FloM, Canberra Packard, Frankfurt).

Finally the fractions containing [^{3}H]-Ponasterone A were collected, the solvent evaporated and subsequently resolved in methanol. An additional analytic HPLC round was performed in order to confirm the purity of the probe (Figure 24).

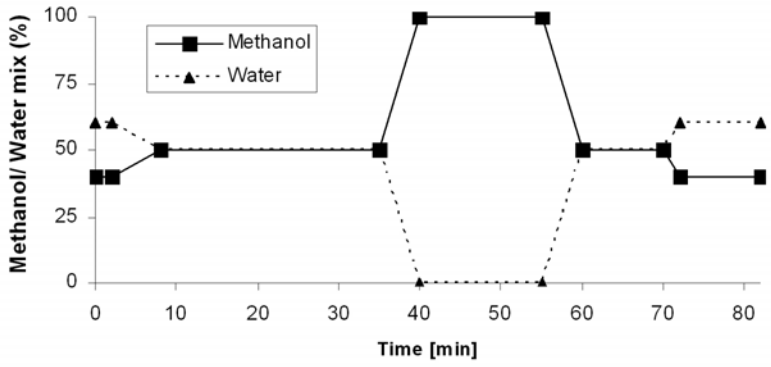

Figure 23 Water:Methanol mixture gradient for HPLC-separation of [^{3}H]-Ponasterone A.

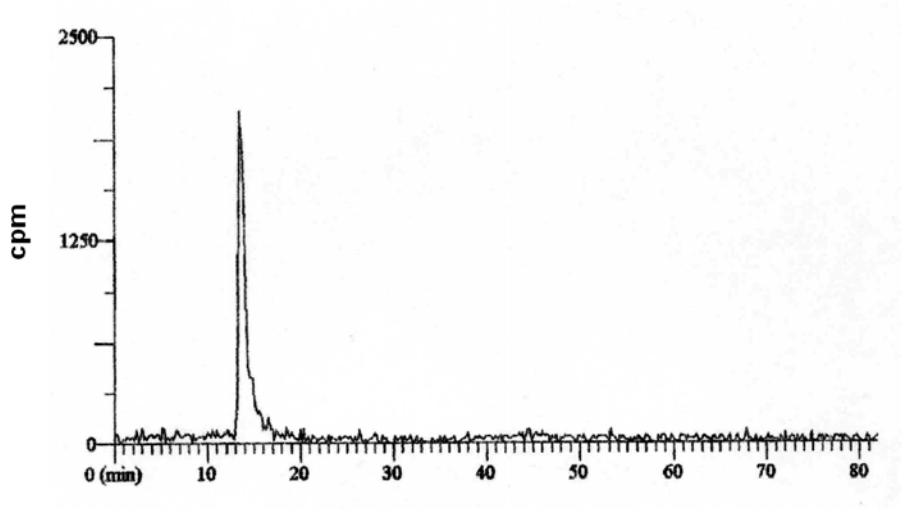

Figure 24 Chromatogram of HLPC-separation of [^{3}H]-Ponasterone A. Beside the main peak at a retention time of 14 minutes, a multitude of tiny peaks resulting from radioactive degradation of hormone are to be noticed.

5.2.9. SDS-PAGE (Laemmli,1970) and Western blot (Towbin et al., 1979; Burnette, 1981)

In order to determine the concentration of EcR isoforms cells were solubilized in extraction buffer. The lysate was mixed with the same volume of SDS 2x gel Laemmli buffer (see 5.1.9) freshly supplied with 2% β-mercaptoethanol and denatured for 5 minutes at 96°C in a heating block device. The proteins were separated by 10% SDS-PAGE (15mA for about 3 hours at room temperature) and transferred to a nitrocellulose membrane (NC45, Serva, Heidelberg, Germany) with a Mini Trans-Blot apparatus (Bio-Rad). The transfer of proteins from gel to the nitrocellulose membrane occurred under a constant current of ~300 mA and 20V for two hours at 4°C. In order to test the quality of the transfer the blotted membrane was immersed for 1 to 3 minutes in Ponceau solution (see 5.1.9) and

photographed. The blocking of proteins occurred by incubation of the membrane with 3% non-fat dry milk in TBS containing 0.1% Tween-20 for 1 hour at room temperature. For subsequent washes, 0.1% Tween-20 in TBS was used. Blocking was followed by incubation over night with specific primary antibodies (either DDA2.7 with a dilution 1:3000 or A.v.peptide with a dilution 1:500) in 3% non-fat dry milk in TBS-Tween 20 for desired proteins (EcR or YFP-EcR respectively), washed three times with TBS-Tween 20 and then incubated for 2 hours with a secondary antibody (either an anti-Mouse 1:1000 or anti-Rabbit 1:1000) (see also 5.1.5). Finally, after three consecutive washes, each of 10 minutes at room temperature, chemi-luminescence detection was performed by incubation of the membrane with equal amounts of ECL substrate (ECL western blotting detection reagent, Amersham Biosciences) and protein bands visualized by radiography with x-ray films (X-Ray, Fuji Medical) developed with an AGFA Curix unit.

Usp variants (UspI, UspII, UspIII) and **Usp wt** electrophoresis was performed as describe above.

The blocking of proteins was done by incubation of the membrane either with 5% BSA in PBS containing 0.1% Tween-20 or 3% non-fat dry milk in TBS-Tween 20 for 1 hour at room temperature. Blocking was followed by over night incubation with specific primary antibodies: either anti-VP16 (with a dilution 1:1000 in 5% BSA in PBS Tween 20) or AB11 (with a dilution of 1:2000 in 3% non-fat dry milk in TBS-Tween 20). After subsequent washes in corresponding buffers incubation with secondary antibodies (anti-Rabbit 1:1000 and anti-Mouse 1:1000 respectively) occurred for 2 hours at room temperature; after additional three consecutive washes, each of 10 minutes at room temperature, chemi-luminescence detection was performed as describe above.

The intensity of the bands was calculated using BIO-RAD Quantity One – 4.4.0 Biorad, Hercules, CA, USA.

The access to a new quantification system made possible an improved detection, therefore the membranes were incubated with Super Signal West Dura Extented Duration Substrate (Pierce, Rockford, USA)(for EcRs and YFP-EcRs, Uspwt) or with Super Signal West Pico Chemiluminoscent Substrate (Pierce, Rockford, USA)(for Vp-16Usps detection). Specific bands were visualized by the Chemi-Smart 5000 photo documentation system (Vilber Lourmat, Eberhardzell, Germany) and quantified relative to a standard probe using Bio-1D software. The linearity of the receptor quantification method was checked with calibration curves obtained by variation of the receptor concentration/lane as described by Przibilla et al., 2004).

5.2.10. Quantification of the intensity of protein bands

For quantifying of protein bands it was used a high resolution CCD (charged coupled device) camera as shown above, in which photographs/images (saved in 12-bit tiff file format) are translated by the computer software to digitized images/values. Ones the lanes were defined, next step was to detect the protein bands. For each band the volume, the height and the area were displayed in a table. Volume is the based of the spot quantification process and represents the sum of all the intensities included in the defined area (window + separation). The height is the maximum intensity and the area is defined for each peak by the width of the window and the separation lines. Quantification is based on the image in pixels whose intensities are coded on a scale which has 4 096 grey levels for a 12-bit image. The digital numbers obtained were the integrated density values of the intensity and the size of each band.

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ABBREVIATIONS

Aa	amino acid
AF-1	activation function 1
AF-2	activation function 2
Ala	alanine
APS	ammonium persulfate
AR	androgen receptor
Arg	arginine
ATP	adenosine triphosphate
BSA	bovine serum albumine
CHO-K1	Chinese hamster ovary cells
COUP	chicken ovalbumin upstream stimulator
CTE	carboxy-terminal extension
DBD	DNA binding domain
Dm	*Drosophila melanogaster*
D-MEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EcR	ecdysteroid receptor
EcRDBD	EcR DNA binding domain
EcRE	ecdysteroid response element
EDTA	ethylenedinitrilotetraacetic acid
ER	estrogen receptor
EYFP	enhanced yellow fluorescent protein
FCS	fetal calf serum
FXR	farnesoid X receptor
GFP	green fluorescent protein

Gly	glycine
GR	glucocorticoid receptor
GTP	guanosine triphosphate
HRE	hormone response element
JH	juvenile hormone
kDa	kilodalton
LBD	ligand binding domain
Leu	leucine
Lys	lysine
LXR	liver X receptor
Met	methionine
MR	mineralocorticoid receptor
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PKC	protein kinase C
PPAR	peroxisome prolifelator receptor
PonA	ponasterone A
PR	progesterone receptor
RAR	retinoic acid receptor
RXR	retinoid X receptor
S2	Schneider line-2 cell of *Drosophila melanogaster*
SDS	sodium dodecyl sulfate
Ser	serine
TEMED	N,N,N',N'-Di-(dimethylamino)ethane
TR	thyroid receptor
Tyr	tyrosine
UAS	upstream activating sequence
Usp	ultraspiracle
UspDBD	DNA binding domain of Usp

VDR	vitamin D receptor
WB	Western blotting
YFP	yellow fluorescent protein

ERKLÄRUNG

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Anca Azoitei

Ulm, den……

CURRICULUM VITAE

PERSONAL DATA

Name: Anca Azoitei

Birth date: 5.08.1970

Place of birth: Darmanesti – Suceava, Romania

Citizenship: Romanian

Marital status: married with one child

Current address: Institute of General Zoology and Endocrinology
University of Ulm
Albert-Einstein Allee 11
Ulm-89081, Germany
Phone: 0049 (0) 731 50 22582
e-mail: anca.azoitei@uni-ulm.de

EDUCATION

1995 – 1996 Master degree at Faculty of Biology
University "Alexandru Ioan Cuza" of Iasi, Romania
Defense: June 1996

1990 – 1995 License in Biology at Faculty of Biology
University "Alexandru Ioan Cuza" of Iasi, Romania
Defense: June 1995

1985 – 1989 Baccalaureate Diploma
High-School "Vasile Alecsandri", Bacau, Romania
Specialization: Chemistry-Biology
Final examination: June 1989

RESEARCH EXPERIENCE

since 08.2004	PhD student in the Department of General Zoology and Endocrinology, University of Ulm, Germany Prof.Dr. M.Spindler-Barth
02.2004 – 07.2004	PhD student in the Department of Internal Medicine III, Medical University of Ulm, Germany PD.Dr. S.Stilgenbauer
11.2000 – 01.2004	Research Assistant in the Department of Physiological Chemistry, University of Ulm, Germany Prof.Dr. T.Wirth
04.1996 – 08.1998	Biologist at Institute for Biological Research University "Alecsandru Ioan Cuza" of Iasi, Romania Team of Dr.G.Stefanescu

PUBLICATIONS

"DNA affects ligand binding of the Ecdysone receptor of Drosophila melanogaster"
Azoitei A. and Spindler-Barth M.,
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