Characterization of ecdysteroid receptor isoforms and their influence on other developmental pathways in *Drosophila melanogaster* cells

Dissertation

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Summary

Ecdysteroids are molting hormones that influence and control several processes like development, molting, reproduction or behavior in insects. Ecdysteroid action is mediated via a nuclear receptor heterodimer which is comprised of the ecdysteroid receptor (EcR) and Ultraspiracle (Usp). Previous studies on Ecdysteroid signaling were mostly performed in heterologous vertebrate systems to prevent the influence of endogenously present nuclear receptor proteins.

In the present work, a *Drosophila melanogaster* cell line (S2/H) was used, containing only a negligible amount of endogenous EcR, but still expressing the heterodimerization partner Usp. This offers the opportunity to study the ecdysteroid signaling with special focus on the three *Drosophila melanogaster* EcR isoforms within an insect specific environment.

It was shown that in the presence of Usp, the localization of the EcR isoforms is independent of the cell type, as EcR was located in the nucleus, same as previous studies have already shown for vertebrate cells.

The localization of Usp however, differed from results obtained in vertebrate cells as in insect cells, Usp was distributed between cytoplasm and nucleus in the absence of hormone instead of being located exclusively in the nucleus like in vertebrate cells. Transcriptional activity of the EcR isoforms is both isoforms-specific as well as cell type-specific as there are major differences in comparison to studies in vertebrate cells. Compared to vertebrate cells, where almost no hormone effect can be detected, a pronounced hormone effect can be seen in insect cells. Besides that, it was shown that the unliganded receptor complex can also have repressive functions.

The point mutation K497E within the ligand binding domain of EcR also exerted isoform-specific effects. Basal as well as hormone-induced transcriptional activity was increased for EcR$^{K497E}$ up to 6-fold compared to wild type, supporting its function as a corepressor binding site.

Deletion of the DNA binding domain of Usp resulted in decreased of transcriptional activity (up to 2-fold), again being isoform-specific and depending on the cell type. These results indicate the importance of the DNA binding of Usp for the proper function of the receptor complex.
Moreover, ecdysteroid signaling was studied for its interaction with three different pathways each having distinct functions like metamorphosis, cell differentiation and cell proliferation during insect development.

An interplay between ecdysone and juvenile hormone signaling was shown as juvenile hormone was able to potentiate isoform-specifically the transcription of the ecdysone-inducible genes BrC-Z1 and E74 A in combination with low concentrations of 20-hydroxyecdysone.

Additionally, expression of the EcR isoforms in the used S2/H cells lead to an altered expression of the two major signal-transducing proteins of the Toll pathway dorsal and relish, demonstrating that ecdysone signaling is also involved in cell differentiation via dorsal and probably innate immunity via relish, which however was found to be present in an inactive state.

Comparative studies in vertebrate cells with the homolog NF-κB pathway confirmed these data as localization and transcriptional activity of p65 was changed in an isoform-specific manner. This result shows that the interaction between these two pathways is independent of the cell type and influenced only by EcR.

Concerning cell proliferation however, no interaction between ecdysone and insulin signaling could be determined in the present study.
Zusammenfassung

Ecdysteroid sind Häutungshormone die verschiedene Prozesse wie Entwicklung, Häutung, Metamorphose, Reproduktion oder das Verhalten bei Insekten beeinflussen und regulieren.


In der vorliegenden Arbeit wurde eine Zelllinie von Drosophila melanogaster (Zelllinie S2/H) verwendet, in der EcR nur in vernachlässigbaren Mengen exprimiert wird. Der Heterodimerisierungspartner Usp liegt dagegen in ausreichenden Mengen vor.

Dieses Zellsystem bietet die Möglichkeit den Ecdyson Signalweg in seiner ursprünglichen zellulären Umgebung, besonders im Hinblick auf die drei unterschiedlichen Isoformen des Ecdyson Rezeptors, zu untersuchen.

Es konnte gezeigt werden, dass die Lokalisation von EcR in Anwesenheit von Usp zelltyp-unspezifisch ist, da EcR in den untersuchten Insektenzellen, gleich wie für Vertebratenzellen gezeigt, im Zellkern vorlag.


Die Punktmutation K497E innerhalb der ligandenbindenden Domäne von EcR zeigte ebenfalls eine isoformspezifische Wirkung. Sowohl die basale als auch die hormonabhängige transkriptionelle Aktivität von EcR_{K497E} war im Vergleich zum Wildtyp bis zu 6-fach erhöht, was auf eine Funktion dieser Position als Korepressor-Bindestelle schließen lässt.
Zusammenfassung


Des Weiteren wurde die Interaktion des Ecdyson-Signalwegs mit den für die Metamorphose, die Zellproliferation und die Zelldifferenzierung spezifischen Signalwegen der Insektenentwicklung analysiert.


1. Introduction

1.1 Organization of Nuclear receptors

Nuclear receptors are ligand-dependent transcription factors. The members of the nuclear receptor superfamily are ancient proteins that can be found in sponges, echinoderms, arthropods and vertebrates (Jones and Thummel 2005). Phylogenetic analysis revealed that the nuclear receptor superfamily consists of six distinct subfamilies from which the best known vertebrate nuclear receptors include all steroid receptors like the estrogen receptor (ER), the glucocorticoid receptor (GR), the thyroid hormone receptor (TR) and the retinoic acid receptor (RAR) (Laudet 1997). Nuclear receptors influence several processes like reproduction, development and various steps in metabolism by regulation of specific target genes (Chawla et al. 2001).

Compared to membrane-bound receptors that act via large signaling cascades, nuclear receptors have always been thought to use a shorter and more direct way to transduce their signals. However, during the last years it became more and more clear that also nuclear receptor action is modified in multiple ways including dimerization, ligand binding, co- or corepressors or the usage of different hormone response elements within the DNA. This versatile function can be explained by their unique structure as they show several receptor functions, like ligand and DNA binding capacity and a transcriptional activation function in the same molecule (Jones and Thummel 2005).

The organization of all nuclear receptors is very similar (Fig. 1). All of them contain a N-terminal A/B domain that harbors a ligand-independent activation function (AF-1) followed by the C-domain or DNA binding domain (DBD) that is comprised of two highly conserved zinc finger motifs allowing the receptor to bind to specific hormone response elements (Chawla et al. 2001). Moreover, the DBD harbors a DNA-dependent dimerization site as well (Perera et al. 2005). The D-domain is characterized by a hinge region responsible for intracellular trafficking and subcellular distribution. Additional to the ligand-dependent activation function (AF-2), the E-domain or ligand binding domain (LBD) contains a ligand dependent dimerization site. Some members of the nuclear receptor superfamily additionally imply an F-domain that is involved in cofactor binding (Spindler 1997).
1.2 Nuclear receptor action

The general scheme of nuclear receptor action is well-known. Signaling starts with an external ligand, the hormone, which binds to the nuclear receptor. This receptor is located either in cytoplasm or nucleus, depending on the type of receptor. After hormone binding, the hormone receptor complex either translocates to the nucleus, or, if already there, binds to specific hormone response elements within the promoter region of certain target genes thus modulating their transcription and gene expression (Fig. 1 2).

However, during the last years it became more and more evident that this model underlies a complex and specific regulation and shows a wide range of modifications on each level of the signal transduction pathway.
1.3 **Ecdysteroid signaling**

Molting and metamorphosis are crucial processes during the life cycle of arthropods. The regulation of these events is controlled by ecdysteroid hormones (molting hormones) and is highly complex. More than 100 genes are regulated by only one hormone signal, a fact that requires tissue-, developmental stage- and gene specificity.

Studies on ecdysteroid signaling are of great importance, first, as the molecular mechanism of ecdysteroid hormone action can be used as a model for steroid hormone action in general. Secondly, studies on ecdysteroid signaling are also of economic relevance as ecdysteroid hormone agonists are used successfully as insecticides due to their low vertebrate toxicity (PALLI ET AL. 2005). Moreover, the ecdysteroid receptor is already used as a gene switch in mammals and plants.

In insects, the biologically most active ecdysteroid is 20-hydroxyecdysone (20E); precursors like ecdysone or 3-dehydroecdysone elicit a lower biological response in *Drosophila* (SPINDLER ET AL. 1977). The concentration of the active hormone is regulated by the target cell specific metabolism (SPINDLER ET AL. 2001).

The ecdysteroid response is mediated by the nuclear ecdysteroid receptor. Phylogenetically, this receptor is the oldest steroid receptor and its ligand binding domain is known to be distantly related to the one of the vertebrate farnesol X receptor (FXR; FORMAN ET AL. 1995). The functional ecdysteroid receptor is known to be a heterodimer of the ecdysone receptor (EcR) and Ultraspiracle (Usp, YAO ET AL. 1992), which is an orthologue of the vertebrate retinoid X receptor (RXR, ORO ET AL. 1990). Both EcR and Usp are present in different phosphorylation isotypes (RAUCH ET AL. 1998), which offers on the one hand the possibility to adapt the activity of the receptor to the actual need of the target cell. On the other hand, the receptor thus is able to process signals from other hormonal pathways or from other signaling pathways in general (SPINDLER ET AL. 2001).

![Fig. 1](image)

**Fig. 1** Three different isoforms of the ecdysteroid receptor of *Drosophila melanogaster*. The length of the regions is true to scale (1 kb=0.5 mm).
In most insect species, different isoforms of the ecdysteroid receptor and Ultraspiracle are present. In *Drosophila melanogaster*, there are three different isoforms of EcR: EcR-A, EcR-B1 and EcR-B2, which differ only in the length and sequence of their A/B domain (Fig. I 3, TALBOT ET AL. 1993). The EcR isoforms are expressed in a tissue-specific manner, depending on the developmental stage (TALBOT ET AL. 1993), which again offers an additional possibility for modulation of the ec dysone response. Also the three different isoforms of the ecdysteroid receptor of *Drosophila melanogaster* exert their own functions and cannot replace each other (BENDER ET AL. 1997). In the mosquito (*Aedes aegypti*) for example, two isoforms of EcR are expressed (WANG ET AL. 2002). Both, isoforms of EcR and isoforms of Usp have different functions. In the tobacco horn worm *Manduca sexta* both Usp types can heterodimerize with EcR-B1 but only the complex EcR-B1/Usp-1 is able to activate the hormone-dependent MHR3 gene, whereas EcR-B1/Usp-2 represses this process (LAN ET AL. 1999).

As a heterodimer, EcR and Usp were shown to regulate a large amount of genes in a tissue- and time-specific manner. However, in *Drosophila*, EcR alone also controls some hormonal responses in the absence of Usp as it is able to bind hormone itself, however with low affinity (SPINDLER ET AL. 2009). Although most studies have focussed on EcR as the ligand binding receptor, while Usp was considered to be an orphan receptor (THUMMEL ET AL. 1995), during the last years it became more and more apparent that also Usp alone functions as ligand-dependent transcription factor (JONES ET AL. 2001). One potent ligand for Usp could be a member of the farnesolate family, juvenile hormone (JH) because of its structural similarity to 9-*cis*-retinoic acid, which is the natural ligand of the vertebrate RXR (JONES ET AL. 2001). Moreover, the binding of Usp and JH was already shown *in vitro*, however only with very low affinity (JONES ET AL. 2001). In addition, JH was shown to activate transcription via Usp (XU ET AL. 2002).

![Possibilities of the EcR/Usp heterodimer for binding to the DNA.](Image)
DNA binding of EcR/Usp is mediated via the two zinc fingers within the C-domain of EcR and Usp (ANTONIEWSKI ET AL. 1993). Moreover, both EcR and Usp can bind to DNA separately via their DNA binding domains (BRAUN ET AL. 2009) either as homo- or heterodimers (Fig. I 4).

Depending on the type of dimerization, different hormone response elements are preferred (SCHAUER 2010). Thus, the heterodimer EcR/Usp prefers palindromic sequences, whereas homodimers of Usp display a higher affinity to direct repeats. In vivo, the DBD of Usp was shown to be dispensable for the activation of some specific target genes but not for their repression (GHBEISH ET AL. 2001).

1.3.1 Mutations of EcR and Usp

For both, EcR and Usp different naturally occurring mutations are known that differ in their physiological impact (BENDER ET AL. 1997, SCHUBIGER ET AL. 2000). One efficient tool to study the molecular basics of nuclear receptor action is the usage of artificially created mutations.

For EcR, the point mutation K497E, leading to a substitution of the basic amino acid lysine to glutamine acid, has already been studied in heterologous systems (GREBE ET AL. 2003, BEATTY ET AL 2006, TREMMEL ET AL. 2010 SUBMITTED) but never in insect cells. By exchanging the above mentioned amino acids in helix 4 of the LBD of EcR, a salt bridge between helix 4 and helix 12 is disrupted (Fig. I 5). This leads to a conformational change of the ligand binding pocket. Moreover, the position 497 represents a highly conserved corepressor binding site for nuclear receptors (LEMPIÄINEN ET AL. 2005).

Concerning Usp, various approaches were already done to investigate the impact of its DNA binding domain on receptor function and modification (GHBEISH ET AL. 2001, BRAUN ET AL. 2009). Therefore, in many studies, mainly in vertebrate cells, Usp constructs lacking the DBD are used e.g. for transcriptional assays (RUFF ET AL. 2009).
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1.5 Interaction of the ecdysteroid receptor complex with other signaling pathways

The ecdysone signaling pathway is a key regulator during insect development and metamorphosis (KOZLOVA ET AL. 2000). As already described earlier, the signaling cascade of the ecdysone response offers many opportunities for a possible putative cross-talk with other signaling pathways. As ecdysone signaling excerts its functions throughout development, three pathways are of major interest for studying their interaction with ecdysteroid signaling. Juvenile hormone signaling, which is highly important during metamorphosis, Toll signaling for its influence on cell differentiation and insulin signaling which is responsible for cell proliferation during Drosophila development.

1.5.1 Juvenile hormone signaling

In Drosophila, molting and metamorphosis are regulated by mainly two hormones, ecdysone and juvenile hormone (JH, RIDDIFORD 1994). Although ecdysone or its biological more active form 20-hydroxyecdysone induces molting, JH plays a major role as it determines the character of the molt. At high JH titles, no change of the larval form occurs, whereas with low JH titles metamorphosis starts, making JH to a ‘status quo’ hormone (SOIN ET AL. 2007). However, neither a JH receptor, the exact molecular mechanism of action nor the molecular mechanism behind the interaction with ecdysone is known (BERGER ET AL. 2005).

As already mentioned above, one possible candidate for the JH receptor is Usp (JONES ET AL. 2001). Another candidate could be the transcription factor MET (methoprene-

### 1.5.2 The Toll pathway of *Drosophila melanogaster* and the mammalian NF-κB pathway

The Toll signaling pathway of *Drosophila melanogaster* is the insect homolog of the mammalian NF-κB (nuclear factor kappa B) signaling pathway (BELVIN AND ANDERSON 1996).

The Toll pathway is highly conserved and, like ecdysoid signaling, plays a crucial role in insect development. Moreover, both the ecdysone response and Toll signaling are important for cell differentiation (SCHUBIGER ET AL. 2005, ROTH ET AL. 1989) and in the regulation of innate immunity (FLATT ET AL. 2008, KIMBRELL AND BEUTLER 2001). In addition, multiple studies revealed interactions between the NF-κB signaling and steroid hormone signaling (MCKAY AND CIDLOWSKI 1998) in mammalian systems.

In the Toll pathway as well as in the NF-κB pathway, the main signal-transducing molecules are members of the NF-κB family. This family can be divided into two subgroups. The first is presented by the proteins of the Rel-family (in mammals: RelA [p65], RelB, c-Rel, in *Drosophila*: dorsal [dl], and dorsal-related immunity factor [dif]), which are characterized by a highly conserved rel-homology domain (RHD) and a C-terminal transactivation domain (TAD, GILMORE 2006).

Additional to the RHD, the second group of proteins of the NF-κB family is distinguished by a long C-terminal domain, consisting of several protein-protein interaction motifs (ankyrin repeats), which enable the binding of inhibitory proteins like the IκB (inhibitor of κB, LASAR ET AL. 2004). In mammals, these proteins are the precursor proteins p105/p50 (NF-κB1) and p100/p52 (NF-κB2), whereas in *Drosophila* only one member, relish (rl), is known.

A comparison of the two homologues pathways is shown in Fig. I. 6.

**The Toll signaling pathway of *Drosophila melanogaster***

In *Drosophila*, the major role of Toll signaling is the regulation of the dorso-ventral patterning in the embryo (ROTH ET AL. 1989). The formation of the dorso-ventral axis is regulated by a gradient distribution of dorsal (STEWARD ET AL 1987). In the syncytial blastoderm stage of the embryo dorsal is located at high concentrations in the ventral
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nuclei and with lower concentrations in the lateral nuclei (ROTH ET AL. 1989) where it acts as a transcription factor for region-specific genes and thus leads to dorso-ventral patterning.

Upon binding of the activated ligand spätzle to the Toll receptor, a protein close to the cell membrane called tube recruits the serine/threonine kinase pelle. Pelle then is supposed to phosphorylate the protein cactus, leading to the degradation of this protein (SILVERMAN AND MANIATIS 2001). In wild-type Drosophila embryos free cactus is complexed with the homodimer of dorsal in the cytoplasm due to the lack of stability of cactus. After the degradation of cactus, dorsal is released to enter the nucleus (BELVIN AND ANDERSON 1996).

In addition to this developmental function, dorsal, dif, and relish play an important role in the second major task of Toll signaling, the regulation of innate immunity. Upon activation by pathogens, dorsal, dif and relish can lead to the transcription of antibacterial peptides in the fat body, the functional equivalent of the mammalian liver (COCIANCICH ET AL. 1994).

**The mammalian NF-κB signaling pathway**

The mammalian NF-κB pathway more or less shares the same functions like its *Drosophila* homolog. Apart from the regulation of inflammatory processes, it is the key regulator of innate immunity, but plays also important roles in cell differentiation (LASAR ET AL. 2004). Moreover it was shown to be active in various types of cancer (RAYET AND GÉLINAS 1999). The mechanism of NF-κB/dorsal activation is highly conserved. In mammals, currently ten Toll-like receptors (TLR) are known. Ligand binding to TLRs, finally leads to the activation of an IL-1-receptor associated kinase (IRAK), which is a homologue to the *Drosophila* kinase Pelle (CAO ET AL. 1996). Via a signaling cascade, the IκB kinase (IKK) complex is supposed to phosphorylate the inhibitor of NF-κB (IκB, a homolog of the *Drosophila* cactus (GEISLER ET AL. 1992)) which is complexed with NF-κB in the cytoplasm. NF-κB itself is comprised of two members of the family of Rel-proteins. The ‘classical’ NF-κB is a heterodimer of p65 (Rel A) and p50. Due to phosphorylation, IκB is targeted for ubiquitinilation and subsequently degraded via the proteasome. With the degradation of IκB, NF-κB is released to enter the nucleus, leading to the activation of certain target genes.

1.5.3 **The insulin signaling pathway in *Drosophila melanogaster***

Apart from the control of cell differentiation, cell proliferation also plays a crucial role in development. The exact regulation of cell number and cell size is highly important for all metazoans. In this regulation, hormones play a major role, as they can mediate between exogenous factors such as nutrition and endogenous factors controlling growth rate for example (RIDDIFORD ET AL. 2000). One key element in this mechanism is insulin, which controls growth in mammals via the insulin receptor, but can also be found in *Caenorhabditis elegans* where it determines the life span of the animal (PUIG ET AL. 2003).
Also in *Drosophila* insulin signaling regulates cell growth and proliferation via the Drosophila insulin receptor (dInR, CHEN ET AL. 1996).

*In vivo* studies already revealed that in larvae the final size is determined by a complex interplay between the growth-promoting insulin signaling and ecdysone signaling, which is responsible for cell differentiation (COLOMBANI ET AL. 2003), thereby showing antagonistic actions.

Moreover, the interplay between vertebrate steroid receptors and vertebrate growth factors has been demonstrated manifold (OSBORNE ET AL. 2005, SADOWSKI ET AL. 1993).
2. Aim of the Study

During the last years, studies on ecdysteroid signaling were generally performed in heterologous systems due to the lack of endogenously present EcR and Usp and RXR, which can functionally replace Usp, at least partially. This offered the possibility to express the different EcR isoforms separately and under defined conditions. However, it became more and more apparent, that the cellular background strongly influences receptor action.

In this study, a Drosophila melanogaster cell line (S2/H) was used, containing only a negligible amount of endogenous EcR, but still expressing a sufficient amount of the heterodimerization partner Usp. This offers the opportunity to study the ecdysteroid signaling with special focus on the three EcR isoforms within an insect-specific environment.

Therefore the first aim of the study was to express the EcR isoforms separately and to characterize receptor functions like intracellular localization, DNA binding and transcriptional activity of wild type EcR isoforms and some selected mutated receptors. Isoforms of EcR containing the point mutation K497E were used to investigate the role of the ligand binding domain of EcR in detail.

To study the impact of Usp on receptor function, silencing of endogenous wild type Usp with siRNA was undertaken. Moreover, different variants of Usp and one mutated form were expressed and examined for their localization and influence on transcriptional activity.

To see possible interactions of ecdysone signaling with other developmental pathways, three pathways were selected for their distinct cellular functions: Juvenile hormone signaling for its role in metamorphosis, the Toll and NF-κB signaling pathway for its function in cell differentiation and the insulin signaling as a mediator of cell proliferation.
3. Material and Methods

3.1 Expression plasmids

3.1.1 Cloning of YFP-EcR isoforms

To express the three YFP-tagged EcR isoforms YFP-EcR-A, YFP-EcR-B1 and YFP-EcR-B2, that differ in length and sequence of their A/B domain in an insect cell line, the isoforms were cloned into the vector pAc5.1 B (Invitrogen, Karlsruhe, Germany), which harbors an insect-specific actin promoter.

The YFP-tagged EcR isoforms were amplified by PCR from the template vectors pEYFP-EcR-A, pEYFP-EcR-B1 and pEYFP-EcR-B2. At the 5’ end a *Not I* cutting site, and at the 3’ end an *Apa I* cutting site were introduced. With these restriction sites, the fragments were cloned into the pAc5.1 vector. As the reaction conditions were not the same for the used enzymes, the digests were done sequentially.

Primers (for all three isoforms):

*NotI* PCR: 5’ ttttgcggccgcGATCCGCTAGCGCTACCGGTCGCC 3’
*ApaI* PCR: 5’ ttttgggcccCAGGTTTCAGGGGAGGTGTGGGAGG 3’

For the PCR, the following conditions were used:

PCR reaction:
100 ng template DNA
5 µl 10 x *Pfu* Buffer
0.4 µl dNTPs (25 mM each)
1 µl primer 1 (100 ng)
1 µl primer 2 (100 ng)
1 µl (1 U) *Pfu* DNA Polymerase
H₂O ad 50 µl
3.1.2 Cloning of the YFP-K497E EcR isoforms

Additionally to the wild type EcR isoform constructs, these vectors were used as a template to insert the point mutation K497E into the three isoform constructs. The point mutation is located within helix four of the ligand binding domain of EcR (Spindler et al. 2001) and leads to a substitution of the basic amino acid lysine on position 497 of the EcR-encoding region with the acidic glutamine acid.

To introduce the point mutation K497E into the vectors pAc5.1 YFP-EcR-A, pAc5.1 YFP-EcR-B1 and pAc5.1 YFP-EcR-B2, direct mutagenesis was used (Site-Directed mutagenesis Kit, Stratagene, Waldbronn, Germany) according to the protocol of the manufacturer.

Mutagenesis primers to introduce the K497E point mutation:

K497E for: 5’ CAGATCACGTTACTAGAGGCCTGCTCGTCGG 3’
K497E rev: 5’ CCGACGAGCAGGCCTCTAGTAACGTGATCTG 3’

3.1.3 Cloning of Flag-EcR-B1

For immunoprecipitation studies, EcR-B1 was cloned from the construct pcDNA3 EcR-B1 into the pFlag CMV™ 5c vector (Sigma-Aldrich, Hamburg, Germany) containing a C-terminal Flag-tag. EcR-B1 was amplified by PCR adding the cutting site Pst I at the 3’ end. The fragment was cloned into the vector pFlag CMV™ 5c using Hind III and Pst I. To correct the reading frame, direct mutagenesis was performed (Site-Directed mutagenesis Kit, Stratagene, Waldbronn, Germany) according to the protocol of the manufacturer.
Material and Methods

Primers for amplification:

Flag for: \[5' \text{GTACATCAATGGGCGTGGATAG} 3'\]
Flag rev \(Pst\) I: \[5' \text{GAGGACGTCTGCAGTGAGTGCTCCG} 3'\]

For the PCR, the following conditions were used:

**PCR reaction:**
100 ng template DNA
5 µl 10 x \(Pfu\) Buffer
0.4 µl dNTPs (25 mM each)
1 µl primer 1 (100 ng)
1 µl primer 2 (100 ng)
1 µl (1 U) \(Pfu\) DNA Polymerase
H₂O ad 50 µl

**Program for PCR:**
\begin{align*}
95 \ ^\circ \text{C} & \quad 3 \text{ min} \\
95 \ ^\circ \text{C} & \quad 30 \text{ sec} \\
61 \ ^\circ \text{C} & \quad 45 \text{ sec} \\
72 \ ^\circ \text{C} & \quad 7 \text{ min} \\
72 \ ^\circ \text{C} & \quad 10 \text{ min} \\
8 \ ^\circ \text{C} & \quad \text{final step}
\end{align*}
35 cycles

Primers for direct mutagenesis:

Flag-a for: \[5' \text{GACGGATCCGATCAAGTGGAGACGACG} 3'\]
Flag-a rev- \[5' \text{CGTCGTCCTTGTAATCTGGTACCGGATCCGTC} 3'\]
3.1.4 Cloning of CFP-Usp variants

To study the influence of the A/B domain of Usp on the localization of Usp and EcR/Usp, three variants of Usp: Usp I, Usp II and Usp III were cloned into the insect-specific pAc5.1 B vector. In these Usp variants, the original A/B domain is replaced by the activation domain of herpes virus simplex (VP16\textsubscript{AD}) as the A/B domain of Usp\textsubscript{wt} is supposed to have an inhibitory effect, at least in mammalian cells. Usp II contains only the VP16 activation domain, Usp I contains the VP16 activation domain but still keeps a hexapeptide of the original activation domain of Usp\textsubscript{wt}. Usp III also starts with the activation domain of VP16, however, the complete DNA-binding domain is lacking (Fig. M 1).

To clone the three Usp variants into the insect-specific vector, direct mutagenesis was used. Thus, in the template vectors pECFP-Usp I, pECFP-Usp II and pECFP-Usp III, a new restriction site for the enzyme Acc65 I was created, whereas the original site for this enzyme was deleted to have a unique site. For both reactions the Site-Directed Mutagenesis Kit (Stratagene, Waldbronn, Germany) was used according to the protocol of the manufacturer. As second enzyme for the cloning, Sac II was used.

![Diagram](https://via.placeholder.com/150)

**Fig. M 1** The three different variants of Usp. Dark blue: activation domain of VP16 (VP16\textsubscript{AD}), light blue: common region of all three Usp variants, orange: original activation domain of Usp\textsubscript{wt}. The length for the regions is true to scale (10 bp=0.5 mm).
Material and Methods

Mutagenesis primers to create a new Acc65 I site:

Acc65 I for: 5’ GATCCGCTAGCGGTACCGGTCGCCAC 3’
Acc65 I rev: 5’ GTGGCGACCGGTACCGGATC 3’

Mutagenesis primers to delete original Acc65 I site:

Acc65 I del for: 5’ CTGCAGTCGACGGAACCGCGGGCCCGG 3’
Acc65 I del rev: 5’ CCGGGCCCGCGGTTCCGACTGAG 3’

For transactivation studies, another Usp construct, pAc5.1 UspΔDBD was used, also lacking the complete DNA-binding domain. However, in this construct the original A/B domain of the Drosophila Usp wt is present.

The template EcR constructs were provided by Dr. A Ozyhar (Technical University of Wroclaw, Wroclaw, Poland). The template constructs for the VP16 Usp plasmids as well as the insect-specific pAc5.1 UspΔDBD were kindly provided by Dr. V.C. Henrich (University of North Carolina, Greensboro, USA).

All constructs were subcloned in the chemo-competent E. coli XL1-blue strain (Stratagene, Waldbronn, Germany). DNA fragments were separated on 1 % agarose gels, stained with Ethidiumbromide and later visualized via the photo documentation system Chemi-Smart 5000 (Vilbert Lourmat, Eberhardzell, Germany). For determination of fragment sizes, GeneRuler™ 1kB DNA Ladder Plus (Fermentas, St. Leon-Rot, Germany) was used.

3.2 Cell Culture

3.2.1 Cell lines

Drosophila melanogaster Schneider cells (S2) were cultivated at 25 °C in Schneider’s medium (GIBCO, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal calf serum. Cells were split twice a week and seeded at a density of 1.2-2 million cells/ml medium in 10 ml of media/75 cm² flask (BD, Heidelberg, Germany).

Chinese hamster ovary cells (CHO-K1) were grown at 37 °C in a 95% air/5% CO₂ atmosphere. Cells were cultivated in D-MEM/F12 medium (1:1) supplemented with 5% heat-inactivated fetal calf serum. For passaging, cells were washed with 1 x PBS pH 7.2 and than trypsinized with Trypsine/EDTA 1:3 (PAA Laboratories, Pasching, Austria) for
Material and Methods

3 min at 37 °C. After dilution with medium, cells were counted and seeded with the desired density. The cells were split every second or every third day, respectively. For 25 cm² T-flasks (Sarstedt, Nümbrecht, Germany) a final volume of 5 ml was used with at least 200,000 cells.

3.2.2 Cell counting
To count the cells, an aliquot of the cell suspension was diluted 1:1 with Trypan blue, a dye that penetrates only the membrane of dead cells. Cells were counted with a Neubauer counting chamber. The number of living cells was determined by the formula:

\[
\text{Number of counted cells} \times \text{average over 4 squares} \times 2 \times \text{dilution with trypan blue} \times 10,000 \times \text{chamber factor} = \text{number of cells/ml medium.}
\]

3.2.3 Freezing and thawing of cells
Insect and vertebrate cells were cultivated till the end of the log-phase. In order to prevent formation of ice crystals during freezing, cells were centrifuged at 82 g for 5 minutes at room temperature and then resuspended in serum-containing culture medium supplemented with 10 % DMSO as a freezing agent. After 1 h incubation on ice, the cells were transferred to a polystyrene box at -80 °C. After one week, cells were finally stored in liquid nitrogen. Cells were thawed in a 37 °C warm water-bath and diluted with 5ml of medium. Then the suspension was centrifuged at 82 g in a 50 ml tube (Greiner bio-one, Frickenhausen, Germany) for 5 min at room temperature. The supernatant was removed; the pellet was dissolved in fresh medium and plated in T-flasks (BD, Heidelberg, Germany).

3.2.4 Transfection
Insect cells
S2 cells were transfected with Fectofly™ II (Polyplus transfection, Illkirch, France), a special transfection reagent for insect cells. Cells growing in suspension were seeded in six-well-plates at a density of two million cells in 1.6 ml of medium per well (BD, Heidelberg, Germany) and transfected immediately. Cells used for reporter gene studies were transfected with 9 µg of DNA according to the manufacturer’s instructions. For all
other experiments 7 µg of DNA were used. The medium was changed 4 hours after transfection. When cells were treated with hormone, Muristerone A, (first dissolved in ethanol, than diluted with 1 x PBS) was added 24 hours after transfection at a final concentration of 1 µM. Cells were harvested 48 hours after transfection. All experiments were performed in serum-containing medium.

**Transfection of siRNA**

For knock-down experiments in S2 cells, siRNA against the endogenous Usp wild type was obtained from MWG-Biotech AG (Ebersberg, Germany). An oligonucleotide was designed with the following sequence:

sense 5’ CGGUUCUGAUGACUUCAUGd(TT) 3’
antisense 5’ CAUGAAGUCUACAGAACCGd(TT) 3’

Cells were transfected with 3 µg siRNA/2 x 10^6 cells and 9 µl of Flyfectine (oz biosciences, Marseille, France). 24 h hours after transfection with siRNA, the cells were additionally transected with EcR constructs as described before.

**Mammalian cells**

CHO-K1 were seeded at a density of 1.5 million cells in 10-cm plates (Greiner bio-one, Frickenhausen, Germany) and transfected the other day with Nanofectin (PAA Laboratories, Pasching, Austria) according to the manufacturer’s instructions. The medium was changed after 4 hours. When cells were treated additionally with hormone, Muristerone A was added 6 hours after transfection at a final concentration of 1 µM. Cells were harvested 24 h after transfection. Transfections were performed in serum-containing medium.

**3.3 Reporter gene assay**

Cell extracts were prepared with a two-step method. The cell suspension was transferred to a 15 ml tube (Greiner bio-one, Frickenhausen, Germany). The plate was rinsed with 1 ml of ice cold 1 x PBS, which was combined with the cell suspension. After centrifugation (180 g, 3 min, 4 °C), the supernatant was removed and the pellet was resuspended in 40 µl of extraction buffer A. The suspension was incubated on ice for
Material and Methods

10 min. Then, the lysate was completed with 10 µl of a Tween 20 solution and incubated on ice for 10 min. After incubation, 50 µl of extraction buffer C were added. After incubation on ice for another 10 min, the lysate was centrifuged at 18000 g for 15 min at 4 °C. The protein concentration of the supernatant was then determined. The luciferase measurement was performed with a Dual-Luciferase-System (Promega GmbH, Mannheim, Germany).

3.4 Preparation of nuclear and cytoplasmatic fraction

For the extraction of cytoplasmatic proteins, cells were transferred to 15 ml tubes as described above. After centrifugation (1125 g, 3 min, 4 °C), the pellet was resuspended in 20 µl extraction buffer A and kept on ice for 10 min. Afterwards 5 µl of a Tween 20 solution were added and mixed thoroughly. After centrifugation (15 min, 18000 g, 4 °C), the supernatant was transferred to a fresh tube.

For the nuclear proteins, the pellet was resuspended in 25 µl of extraction buffer C and kept shaking for 10 min at 4 °C. Then the lysate was centrifuged as described above and the supernatant was transferred to a fresh tube.

**Extraction buffer A:**

- 10 mM HEPES
- 10 mM KCl
- 0.1 mM EDTA
- 0.1 mM EGTA
- pH = 7.9

**Extraction buffer C:**

- 20 mM HEPES
- 0.4 M NaCl
- 1 mM EDTA
- 1 mM EGTA
- pH = 7.9

Both buffers were completed with ALP protease inhibitors (aprotinin, leupeptin, pepstatin) to a final concentration of 1 µg/ml at the day of use.

**Tween 20 solution:**

- 10 % Tween 20
10 x PBS for insect cell culture:  
8 mM Na$_2$HPO$_4$ x 2 H$_2$O  
2.7 mM KCl  
1.5 mM KH$_2$PO$_4$  
137 mM NaCl  
pH = 6.7

10 x PBS for mammalian cell culture:  
10 mM Na$_2$HPO$_4$ x 2 H$_2$O  
2.7 mM KCl  
1.8 mM KH$_2$PO$_4$  
138 mM NaCl  
pH = 7.2

3.5 Harvesting of CHO cells

Cells were gently detached from the plate with a plastic scraper. The cells were rinsed with 5 ml of ice cold 1 x PBS and transferred to a 15 ml tube. After centrifugation (1125 g, 3 min, 4 °C), the supernatant was removed and the pellet was resuspended in cold extraction buffer (3 x pellet volume). The lysate was subjected to three freeze / thaw cycles and centrifuged (18000 g, 15 min, 4 °C).

Extraction buffer for CHO cells:  
20 mM Tris/HCl  
150 mM NaCl  
1 mM EDTA  
pH = 7.9

The buffer was completed with ALP protease inhibitors (aprotinin, leupeptin, pepstatin) to a final concentration of 1µg/ml at the day of use.

3.6 Bradford Assay (BRADFORD, 1976)

The protein concentration of the cell extracts was determined according to Bradford (1976) using a calibration curve prepared with BSA. Each sample was supplemented with water up to 800 µl. Then, to every probe 200 µl of Bradford solution was added, mixed
and incubated for 10 min. The absorbance was measured at 595 nm using a Pharmacia Biotech Novaspec II spectrophotometer.

**Bradford solution:**

- 0.1 g/l Coomassie Brilliant Blue G-250
- 5 % of 96 % Ethanol
- 10 % of 85 % Phosphoric acid (H₃PO₄)

### 3.7 SDS-PAGE (*LAEMMLI 1970*)

The concentration of the transfected and endogenous proteins of interest was determined by quantification of specific Western blot signals. A defined amount of protein was diluted in extraction buffer and denatured with either 2 x or 5 x loading buffer (*LAEMMLI 1970*) at 95 °C for 5 min. After a short centrifugation (30 sec, 1000 g), the samples were separated on a 10 % gel under denaturizing conditions in 1x electrophoresis buffer (running parameters: 15 mA/gel, 300 V) until the tracking dye bromphenol blue reached the bottom of the gel. The size of the separated proteins was determined using a high molecular weight standard mixture (Sigma Aldrich, Hamburg, Germany).

<table>
<thead>
<tr>
<th>Tab. 1 Composition of the SDS gels</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Running gel (10 %)</strong></td>
</tr>
<tr>
<td>Bidest</td>
</tr>
<tr>
<td>Acrylamide / Bisacrylamide</td>
</tr>
<tr>
<td>Running gel buffer</td>
</tr>
<tr>
<td>Collecting gel buffer</td>
</tr>
<tr>
<td>10 % SDS</td>
</tr>
<tr>
<td>TEMED</td>
</tr>
<tr>
<td>40 % APS</td>
</tr>
</tbody>
</table>

Collecting gel buffer:

- 1 M Tris/HCl
- pH = 6.8

Running gel buffer:

- 1.5 M Tris/HCl
- pH = 8.8
**Material and Methods**

**Sodium dodecyl sulfate (SDS) solution:**
10 % Sodium dodecyl sulfate

**Ammonium persulfate (APS) solution:**
0.4 g/ml Ammonium persulfate

**Electrophoresis buffer (10x):**
- 250 mM Tris/HCl
- 1920 mM Glycine
- 1 % SDS
- pH = 8.3

**2x Loading buffer:**
- 200 mM Tris/HCl
- 6 % SDS
- 4 % β-mercaptoethanol
- 20 % Glycerol
- 0.1 % Bromophenol blue
- pH = 8.8

**High Molecular Weight Standard Mixture** (Sigma-Aldrich, Hamburg, Germany):

The marker is a mixture of the following six proteins:
- Myosin, rabbit muscle: 205.0 kD
- 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

### 3.8 Western Blot ([Towbin et al. 1979; Burnette 1981](#))

After separation, the proteins were transferred to a nitrocellulose membrane (NC45, Serva, Heidelberg, Germany) with a Mini Trans Blot device (BioRad, parameters: 300 mA, 20 V, 2 h), the transfer efficiency was controlled by staining with Ponceau solution.
Material and Methods

After 1 h of blocking at room temperature with 3 % milk buffer, the gel was incubated with a specific first antibody over night at 4 °C. The subsequent incubation with the second antibody took 2 h at room temperature.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>1st Antibody</th>
<th>Dilution</th>
<th>2nd Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>YFP-EcR (A, B1, B2)</td>
<td>DDA 2.7</td>
<td>1 : 500</td>
<td>anti-mouse</td>
<td>1 : 1000</td>
</tr>
<tr>
<td>Usp wt</td>
<td>AB 11</td>
<td>1 : 1000</td>
<td>anti-mouse</td>
<td>1 : 1000</td>
</tr>
<tr>
<td>p65</td>
<td>p65</td>
<td>1 : 1000</td>
<td>anti-rabbit</td>
<td>1 : 1000</td>
</tr>
<tr>
<td>Dorsal</td>
<td>anti-dorsal 7A4</td>
<td>1 : 500</td>
<td>anti-mouse</td>
<td>1 : 1000</td>
</tr>
<tr>
<td>Relish</td>
<td>anti-relish-C21F3</td>
<td>1 : 500</td>
<td>anti-mouse</td>
<td>1 : 1000</td>
</tr>
<tr>
<td>Lamin A</td>
<td>H-102</td>
<td>1 : 500</td>
<td>anti-rabbit</td>
<td>1 : 1000</td>
</tr>
<tr>
<td>Flag-Tag</td>
<td>Anti-Flag® M2</td>
<td>1 : 5000</td>
<td>anti-mouse</td>
<td>1 : 1000</td>
</tr>
</tbody>
</table>

Between the incubation with first and second antibody and before the detection of the bands with substrate, the membrane was washed three times for 10 min with TBS-T. For the detection, the membrane was incubated with Super Signal West Dura Extended Duration Substrate (Pierce, Rockford, USA). The 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.

**Transfer buffer:**
- 25 mM Tris/HCl
- 192 mM Glycine
- 20% Methanol
- pH = 8.3

**Ponceau solution:**
- 0.1 % Ponceau S (Sodium salt)
- 5 % Acetic acid

**3% Milk buffer:**
- 3 % Non-fat milk powder
- 1 % bovine serum albumine (BSA)
- 0.02 % Thymerosal
- in TBS-T
Material and Methods

**Tris buffered saline + Tween 20 (TBS-T):**
- 20 mM Tris/HCl
- 137 mM NaCl
- 0.1 % Tween 20
- pH = 7.6

### 3.10 Immunoprecipitation

For immunoprecipitation, cells were transfected one day prior to the assay as described in 3.2.4. For one sample two 10 cm\(^2\) dishes were used.

Cell extracts were prepared as described in 3.5. The protein concentration was determined according to **Bradford** (1976). For each sample, 750 µg of total protein in 750 µl IP Lysis buffer were incubated with 50 µl of Anti-Flag® M2 Agarose beads, (Sigma, Hamburg, Germany) at 4 °C on a rotary shaker for 2 h. The probe was then centrifuged for 1 min at 1125 g at 4 °C. After centrifugation the supernatant was removed and the pulled-down beads were washed twice with 500 µl of 1 x PBS. Finally 25 µl of 4 x Laemmli Buffer supplemented with 5 % β-mercapto-ethanol were added and samples were denatured at 95 °C for 10 minutes.

All samples were later separated on a SDS gel and detected via Western Blot analysis.

**IP lysis buffer**
- 5 mM HEPES
- 1.5 mM MgCl2
- 0.2 mM EDTA
- 0.5 mM DTT
- 26 % Glycerol
- pH = 7.9

### 3.11 Electrophoretic Mobility Shift Assay (EMSA)

For EMSA, cell extracts were prepared as described in 3.4. The oligonucleotides were annealed and labelled with \([α^{32}P]-dCTP\) by fill-in reaction with Klenow polymerase.

**Oligonucleotides:**

- hsp 27 1 x for: 5’ AGCGACAAGGGTTCAATGCACTTGT 3’
- hsp 27 1 x rev: 5’ ATTTGACAAGTGCATTGAACCCTTGT 3’
Material and Methods

**PCR program for primer annealing:**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>88 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>65 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>37 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>22 °C</td>
<td>30 sec</td>
</tr>
</tbody>
</table>

For the fill-in reaction, the following mixture was prepared and incubated for 30 min at 37 °C:

1 x Ligation buffer
100 nM MgCl$_2$
5 µl dNTPs (dATP, dTTP, dGTP, 2.5 mM each)
10 µM annealed oligo
2 U Klenow polymerase
ad 45 µl H$_2$O
3 µl [$\alpha^{32}$P]-dCTP (30 µCi)

**10 x Ligation buffer:**
660 mM Tris/HCl,
50 mM MgCl$_2$
50 mM DTT
10 mM ATP
pH = 7.5

Oligonucleotides were separated from single nucleotides with Sephadex G-50 (Pharmacia Biotech, Freiburg, Germany) columns, equilibrated with a buffer containing 50 µl 1x TBE, 5 µl tRNA (10 mg/ml, Roche, Penzberg, Germany) and 5 µl salmon sperm (10 mg/ml, Böhringer, Ingelheim, Germany). The reaction was placed on the column and centrifuged for 2 min with 4000 g at room temperature.

For the binding reaction, an aliquot of protein was incubated with 400.000 cpm of labeled oligo, 5 x binding buffer, the non-specific competitor poly-dldC (1 µg, Roche, Penzberg, Germany), and BSA (10 µg) in a final volume of 20 µl. If indicated, Muristerone A was added to the samples (1 µM final concentration).
Material and Methods

10 x TBE buffer
- 890 mM Tris/HCl
- 890 mM Boric acid
- 20 mM EDTA
- pH = 8.0

5 x Binding buffer:
- 100 mM Tris/HCl
- 500 mM NaCl
- 10 mM EDTA
- 50 % Glycerol
- 5 mM DTT
- pH = 7.5

The reaction mixture was incubated at room temperature for 30 min. The samples were separated at 10 V/cm on a 4 % nondenaturing polyacrylamide gel in 0.5 x TBE (45 mM Tris, 45 mM boric acid, 0.5 mM EDTA, pH =8.0) for 3 h. The gel was dried on a filter paper (Whatman 3mm Chr, Dassel, Germany) in a vacuum dryer (Gel Dryer Model 583, BioRad, Munich, Germany) for 2 h. After drying, a x-ray film (Super RX, Fuji, Düsseldorf, Germany) was put on the gel for detection of the signals. The films were developed using Curix 60 (Agfa, Cologne, Germany).

3.12 Cell proliferation experiments

For proliferation studies of non-transfected cells, cells were seeded in 24-well-plates (Greiner bio-one, Frickenhausen, Germany) at a density of 500.000 cells per well in 1 ml medium and hormones were added. Every 24 h after hormone application, an aliquot of 0.5 ml of the cell suspension was taken out, diluted with 1 x PBS (pH 6.7) and counted with a Neubauer chamber. After 96 hours the experiment was stopped.

For proliferation studies of receptor-expressing cells, cells were transfected with EcR isoforms as described above. After 24 hours, the cells were centrifuged at 82 g for 5 min at room temperature. The supernatant was discarded and the pellet was resuspended in fresh medium. The cells were seeded as described already for the experiments with untransfected cells and hormone was added.

A non-treated control was prepared for each time interval.
3.13 Intracellular localization

To study the intracellular localization of EcR isoforms and Usp variants, fluorescence-tagged constructs of these proteins were transfected into S2/H cells. After 48 h cells were transferred to 35 mm µ-dishes (ibidi, Munich, Germany) and observed under a confocal laser scanning microscope (510 META, Zeiss, Jena, Germany), equipped with an argon ion laser. The YFP fluorescence of EcR was excited with a 488 nm filter, the CFP fluorescence of Usp with a 458 nm filter. The exact localization was determined with a C-Apochromat 63 x objective. The images were processed further with Zeiss LSM Image Browser software.

3.14 Isolation of genomic DNA and RNA from S2/H cells

In order to characterize the EcR isoforms and the ecdysone-inducible genes rp49 (ribosomal protein 49), BrC-Z1 (Broad-Complex isoform Z1) and E74 A (ecdysone inducible protein 74 A) of the used S2/H cell line also on the genomic level, DNA and RNA were isolated at the same time using peQGold TriFast (peqlab, Erlangen, Germany) according to the protocol of the manufacturer.

3.15 cDNA synthesis

To investigate whether all three isoforms of EcR and the above mentioned genes are transcribed in the subline of the S2 cell line which was used for all experiments, cDNA was synthesized.

cDNA synthesis:

2 µg RNA
1 µl Oligo(dt) Primer (500 µg/ml)
1 µl dNTPs (10 mM each)
H₂O ad 12 µl
65 °C for 5 min → ice, brief centrifugation
4 µl 5 x First Strand Buffer
2 µl DTT (0.1 M)
1 µl Rnasin® RNAse Inhibitor (40U/µl)
37 °C for 2 min
Material and Methods

1 μl M-MLV-Reverse Transcriptase (200 U/μl)
37 °C for 50 min
70 °C for 15 min

The presence of the specific RNA was checked via PCR using isoform-or gene-specific primers.

<table>
<thead>
<tr>
<th>gene</th>
<th>forward primer</th>
<th>reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcR-A</td>
<td>5’ CGGTGGCCGGAGGATC 3’</td>
<td>5’ CATGTACATGTCATTTCGCAG 3’</td>
</tr>
<tr>
<td>EcR-B1</td>
<td>5’ CGACTATTCGATCGAGACC 3’</td>
<td>5’ CATGTACATGTCATTTCGCAG 3’</td>
</tr>
<tr>
<td>EcR-B2</td>
<td>5’ GATACTTGTGGATTAGTAGCAG 3’</td>
<td>5’ GGCAGAGTGCACGATGCCCAG 3’</td>
</tr>
<tr>
<td>rp49</td>
<td>5’ TCTTCCAGCTTCAAGGTAC 3’</td>
<td>5’ GTGATATCCGACACGTTGCA 3’</td>
</tr>
<tr>
<td>E74 A</td>
<td>5’ AAGCTGGAGTACGCCCTCAT 3’</td>
<td>5’ GGGACTTGTCGATTGCTTGA 3’</td>
</tr>
<tr>
<td>BrC-Z1</td>
<td>5’ AACACACAGTTGCAGCAGTC 3’</td>
<td>5’ CCGAGGTTGATTGTGAG 3’</td>
</tr>
</tbody>
</table>

**Tab. 3 Primer sequences**

**PCR reaction:**

2 μl of cDNA
5 μl 10 x *Pfu* Buffer
1 μl dNTPs (10 mM each)
1 μl primer 1 (100 ng)
1 μl primer 2 (100 ng)
2-3 U *Pfu* DNA Polymerase
H₂O ad 50 μl

As the annealing temperature and the extension time for the PCR of the isoforms EcR-A and EcR-B1 as well as the genes rp49, BrC-Z1 and E74 A were identical, the same PCR program was used.
Material and Methods

PCR program:

95 °C  2 min  
95 °C  30 sec  
50 °C  45 sec  
72 °C  2 min  
72 °C  4 min  
8 °C   final step

29 cycles

The fragment expected for isoform EcR-B2 was about twice as big as the one for EcR-A and EcR-B1. Thus, 4 min of extension time for this PCR were used.

PCR program for EcR-B2:

95 °C  2 min  
95 °C  30 sec  
50 °C  45 sec  
72 °C  4 min  
72 °C  6 min  
8 °C   final step

29 cycles

3.16 Juvenile hormone experiments

For the experiments with juvenile hormone and 20E, S2/H cells were transfected as described in 3.2.4. Juvenile hormone III and 20-hydroxyecdysone were dissolved in DMSO to a final concentration of 0.1 % DMSO in the cell culture medium. 24 hours after transfection, hormones were added to the cells. As a vehicle control, one sample with DMSO only was prepared.
3.17 Statistical analysis

Statistical analysis was performed with the program SigmaStat 3.5 by Systat Software Inc. For normally distributed data Student’s t-test and ANOVA were used for data evaluation. For data that were not normally distributed Mann-Whitney Rank Sum Test or ANOVA on ranks was used.
### 3.15 Chemicals and Enzymes

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acc65 I</td>
<td>Promega GmbH, Mannheim, Germany</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Acrylamide solution 40%</td>
<td>Sigma-Aldrich, Hamburg, Germany</td>
</tr>
<tr>
<td>Agarose Ultrapure, Electrophoresis Grade</td>
<td>Sigma-Aldrich, Hamburg, Germany</td>
</tr>
<tr>
<td>Apa I</td>
<td>Promega GmbH, Mannheim, Germany</td>
</tr>
<tr>
<td>beta-Mercaptoethanol</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Boric acid</td>
<td>AppliChem, Darmstadt, Germany</td>
</tr>
<tr>
<td>Bromphenol blue</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>BSA</td>
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### Chemicals and Enzymes (continued)

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### 3.16 Laboratory equipment

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<td>Centrifuge 5415C</td>
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<td>Water bath type 1002</td>
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### 3.17 Kits for cloning

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<td>Wizard SV Gel and PCR Clean-up System</td>
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<td>Pure Yield™ Plasmid Midiprep System</td>
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### 3.18 Antibodies

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<td>Amino acid 335-393 KOELLE ET AL (1991), C-domain.</td>
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<td>Usp wt</td>
<td>AB 11</td>
<td>C-domain, (CHRISTIANSON ET AL. 1992)</td>
<td>AB 11 was kindly provided by Dr. Kafatos, Institute of Molecular Biology and Biotechnology, Heraklion, Crete, Greece</td>
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4. Results

4.1 Characterization of the subclone S2/H of the Drosophila melanogaster S2 cell line.

4.1.1 All EcR isoforms are present in the genome the cell subclone S2/H.

To test whether all three isoforms of EcR are encoded in the genome of the subclone S2/H of S2 cells, genomic DNA was isolated and multiplied by PCR with EcR isoform-specific primers. Comparison of these PCR products with EcR isoform fragments obtained with the same primer pairs and EcR isoform-encoding plasmids revealed that all isoforms are present in the genome of the cell subclone S2/H.

All forward primers are located within the variable region to ensure isoform-specificity. For isoforms EcR-A and EcR-B1, the same reverse primer was used. To amplify a clearly distinguishable fragment for isof orm EcR-B2, a reverse primer positioned farther downstream compared to the reverse primer for EcR-A and EcR-B1 was used.

Fig. 1 A Positions and fragment sizes obtained by PCR with isoform-specific primers on the three different EcR isoforms. Positions and fragment sizes are true to scale (10 bp=0.5 mm).
Results

<table>
<thead>
<tr>
<th>marker</th>
<th>EcR-A</th>
<th>EcR-B1</th>
<th>EcR-B2</th>
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<tr>
<td>marker</td>
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<td>plasmid</td>
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<td>genomic DNA</td>
<td>-</td>
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<td>+</td>
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As shown in Fig. 1 B, fragments specific for all three isoforms are detected via PCR in genomic DNA of S2 cells. As expected, the length of the fragment for Isoform A is between 500 and 700 bp, which is in accordance with the calculated length of 578 bp (Fig. 1A). For isoform B1, a fragment between 700 bp and 1000 bp was seen (calculated length 802 bp). The band specific for isoform B2 was located near the 1500 bp marker band (calculated 1458 bp, Fig. 1A). For all isoforms the detected fragment sizes and the calculated ones correspond well, which indicates, that all three EcR isoforms are present in the genome of the S2 cell line S2/H.

4.1.2 All three EcR isoforms are transcribed in the subclone S2/H of the S2 cell line.

To test whether the isolated RNA from S2/H cells might be contaminated by DNA, a test PCR was performed with EcR-A-specific primers.

As shown in Fig. 1C, a fragment with the expected length of 578 bp was obtained with the plasmid DNA (pAc5.1 encoding YFP-EcR-A), but not with RNA isolated from S2/H cells. This result proofs that the RNA was not contaminated by residual genomic DNA.
Results

Isoform-specific fractions of the expected size (Fig. 1 A) were amplified with all three primer pairs using cDNA obtained from RNA isolated from S2/H cells. For comparison, controls amplified with the same primer pairs and plasmids encoding EcR isoforms (pAc5.1) are used (Fig.1 D).

As shown in Fig. 1 D, fragments were amplified for all three isoform-specific primer pairs. The fragment sizes correspond to the calculated sizes (Fig.1 A) demonstrating that all three isoforms of EcR are actually transcribed.

Fig. 1 C Agarose Gel of a control PCR obtained with purified total RNA from S2 cells. PCR products obtained from extracted RNA were compared with fragments obtained from a plasmid encoding YFP-EcR-A. Marker: GeneRuler™ 1kB DNA Ladder Plus.
Fig. 1 D Agarose gel of PCR products obtained from cDNAs synthesized from extracted RNA from S2 cells were compared with products from EcR isoform-encoding plasmids. Marker: GeneRuler™ 1kB DNA Ladder Plus.

4.1.3 No EcR receptor protein is detectable in the subclone S2/H of Drosophila melanogaster cells.

To see, whether the EcR isoforms are also translated in the S2 subclone S2/H, Western Blot analysis was performed with S2 cell extracts. However, no EcR-specific bands were detected.

In contrast to that, the amount of endogenous wild type Usp was relatively high. Although no EcR is detectable in untransfected S2/H cells, Usp wt is present in sufficient amounts (Fig. 2).

Fig. 2 Western Blot analysis of untransfected S2/H cells. Whole cell extract (30 µg protein/lane) was detected with the monoclonal Usp wt antibody AB 11 which is directed against the very first part of the C-domain of Usp (CHRISTIANSON ET AL. 1992).
Results

**Determination of the detection limit of EcR in Western Blot analysis**

Previous experiments with the S2/H cells revealed that in some cases, the cells are still hormone sensitive. This fact suggests the presence of a very low, not detectable amount of endogenous hormone receptor. To grossly estimate the range of this amount, the detection limit of EcR was determined using a cell extract from CHO-K1 cells expressing EcR under the normally applied Western Blot conditions. For comparison, a cell extract from cells co-transfected with YFP-EcR-B1 and Usp wt was used.

As Fig. 3 shows, at an amount of 1.5 µg of total protein a weak band is still visible, whereas no band is detectable for 1 µg anymore (experiment was repeated twice).

The receptor concentration of this sample according to Scatchard Plot analysis (data from Dr. A. Azoitei) is 1.64 x 10^-10 mol/l. Taking the protein concentration and the volume representing 1.5 µg of protein into account, one can calculate the amount of receptor molecules in this 1.5 µg with the avogadro number (6.022 x 10^23 molecules per mol) resulting in 12.840.000 molecules.

To further estimate, one could draw a comparison to a Western Blot with extracts from S2/H cells transfected with YFP-EcR-B1, where the cells where counted prior to harvesting (Fig. 4).

With this cell extract, the last visible band represents 5 µg of total protein. Making the assumption that at this point, the amount of molecules should be the same in the extract from CHO-K1 cells and S2/H cells, one could calculate the concentration of receptor molecules per cell in the given sample with ~26 receptor molecules per cell.

![Western Blot analysis of CHO cells co-transfected with YFP-EcR-B1 and Usp wt. Different amounts of total protein were applied on the gel and detected with the monoclonal EcR antibody DDA 2.7.](image)

**Fig. 3** Western Blot analysis of CHO cells co-transfected with YFP-EcR-B1 and Usp wt. Different amounts of total protein were applied on the gel and detected with the monoclonal EcR antibody DDA 2.7.
Results

Fig. 4 Western Blot analysis of S2 cells transfected with YFP-EcR-B1 in the presence of endogenous Usp wt. Different amounts of total protein were applied on the gel and detected with the monoclonal EcR antibody DDA 2.7.

In previous experiments, Western Blot analysis with cell extracts from untransfected S2/H cells was performed to test for endogenous EcR. A concentration range of up to 100 µg of total protein was used, however, no endogenous EcR could be detected (data not shown). With the above mentioned receptor concentration, one could therefore assume that the concentration of endogenous EcR is less than ~26 molecules per cell, indicating that the biological activity of these endogenous receptor molecules can be neglected.

The fact that the endogenous EcR isoforms are only present only in low amounts in the used S2/H cell line offers the possibility to transfec and express them separately and to examine their isoform-specific function in their original insect cell environment. Because of the high level of Usp wt however, only EcR/Usp wt heterodimers can be analysed.

4.2 Intracellular localization of EcR and Usp in S2H cells

4.2.1 Microscopic analysis of intracellular localization

All three EcR isoforms were transfected successfully in the S2/H cells. In contrast to the endogenously present mRNA for EcR-A, EcR-B1 and EcR-B2, the transfected DNA was transcribed and translated in sufficient amounts. The presence of the transfected isoforms was proofed by microscopic analysis.

For the function of ligand-dependent transcription factors like EcR, the transport of the cytoplasmatically synthesized receptor protein to the nucleus is essential for its functionality. For this reason, the intracellular localization of the EcR isoforms and their heterodimerization partner Usp was examined first.

Therefore, fluorescence-tagged isoforms of EcR and three different variants of Usp, Usp I (VP16<sub>AD</sub>+CDE domain of Usp wt), Usp II (VP16<sub>AD</sub>+hexapeptide of Usp wt+CDE
domain of Usp wt) and Usp III (VP16\textsubscript{AD}+DE domain of USP wt) were transfected in S2/H cells. Localization was studied using laser scanning microscopy (LSM).

As shown in Fig. 5, all three EcR isoforms are present almost exclusively in the nucleus in S2/H cells containing endogenous Usp wt (Fig. 2). This nuclear localization can be seen already in the absence of hormone.

In order to see whether the different domains of Usp influence localization, the above mentioned variants of Usp were transfected in S2/H to determine their localization.

As shown in Fig. 6 all three variants of Usp are distributed equally within the cell in the presence of endogenous Usp wt (Fig. 2).

Fig. 5 Intracellular localization of yellow-fluorescence-protein (YFP)-tagged isoforms of the ecdysteroid receptor in the presence of endogenous wild type Usp. Scale 5 µm.
Results

4.2.2 Determination of intracellular localization of EcR and Usp by Western Blot analysis

The localization of the endogenous wild type Usp cannot be determined microscopically. Therefore, the localization was examined via Western Blot analysis. In addition, the results obtained by microscopy for the EcR isoforms were also confirmed via Western Blot analysis. After transfection, cytoplasmatic and nuclear fractions were separated and analyzed.

For the Western blot with the cytoplasmatic fraction and the one with the nuclear fraction the same amount of total protein was used. However, when the cytoplasmatic fraction of the S2 cells is detected with the EcR antibody DDA 2.7, if any, only very weak bands are visible (Fig. 7 A), independent of the presence of hormone. In contrast, all EcR isoforms can be clearly seen in the nuclear fraction (Fig. 7 B) and are expressed in similar

Fig. 6 Intracellular localization of cyan-fluorescence-protein (CFP)-tagged variants of Ultraspiracle. Scale 5 µm.

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concentrations. For isoform EcR-B2 the concentration was usually little higher than for EcR-A and EcR-B1. The treatment with Muristerone A has no effect on the concentration of EcR isoforms. Both, cytoplasmatic and nuclear fractions were extracted in the same buffer volume. Thus the same equivalent of cells was taken for each lane.

In both fractions, the endogenous Usp can be detected (Fig. 8 A and 8 B). However, the concentration of Usp in the nucleus is higher than in the cytoplasm (1.5 to 7-fold), independent of the present EcR isoform and hormone. Moreover, the presence of hormone has a stimulatory effect on the Usp concentration in the cytoplasm. This effect cannot be seen in the nucleus.

Both fractions were extracted in the same buffer volume. Thus the same equivalent of cells was taken for each lane.

To proof that the localization determined via Western Blot is not due to an incorrect separation of cytoplasm and nucleus and to ensure that the cytoplasmatic extracts were free from nuclear proteins, the same membranes were detected with a polyclonal antibody directed against Lamin A. Lamin A belongs to a group of intermediate filaments that are restricted exclusively to the nucleus and cannot be found in the cytoplasm.

**Fig. 7** Western Blot analysis of the three different EcR isoforms of transfected S2 cells. If indicated, the cells were treated with 1 µM Muristerone A. EcR-specific bands were detected with the monoclonal EcR antibody DDA 2.7 directed against the very last part of the C-domain of EcR, which is common for all three isoforms. A Cytoplasmatic fraction (30 µg protein/lane). B Nuclear fraction (30 µg protein/lane).
Results

Compared to the nuclear fractions, the cytoplasmatic fractions of the used cell extracts are free from Lamin A (Fig. 9), confirming that the method of separating cytoplasmatic proteins from nuclear proteins works out well.

**Fig. 8** Western Blot analysis of endogenously expressed Usp in S2 cells in the presence of the EcR isoforms. The detection was performed with the monoclonal Usp antibody AB11. A Cytoplasmatic fraction (25 µg protein/lane) detected with AB11. B Nuclear fraction (25 µg protein/lane) detected with AB11.

**Fig. 9** Western Blot analysis of endogenously expressed Lamin A in S2 cells in the presence of the EcR isoforms. The detection was performed with the polyclonal Lamin A antibody H-102. A Cytoplasmatic fraction (25 µg protein/lane) detected with H-102. B Nuclear fraction (25 µg protein/lane) detected with H-102.
4.2.3 Influence of wild type Usp on the localization of EcR

As reported previously for vertebrate cells, nuclear localization of heterologously expressed EcR is highly promoted by the heterodimerization partner Usp (Nieva et al. 2005).

To test if a reduction in Usp concentration impairs nuclear localization of EcR also in insect cells, Usp was partially silenced by siRNA. Moreover, in vertebrate cells, wild type Usp is located mainly in the nucleus (Gwozdz et al. 2007, Cronauer et al. 2007), which does not correspond to the present data from insect cells. This different localization could be due to the high amount of endogenous wild type Usp. Therefore, fluorescence-tagged Usp II (VP16\textsubscript{AD}+CDE domain of wild type Usp) was transfected to examine the intracellular localization after a reduction of the content of endogenous wild type Usp.

To see whether the transfection conditions were appropriate for the siRNA so that it in fact enters the cell, a non-specific AlexaFluor-stained control siRNA (Qiagen, Hilden, Germany) was used.

As Fig. 10 shows, the non-specific siRNA is taken up by the cells and is visible inside. The stained siRNA can be seen as small fluorescent spots within the cell. Therefore, the same transfection conditions were applied for transfection with specific siRNA.

If the cells are pre-treated with siRNA against wild type Usp, YFP-EcR-B1 is not located exclusively in the nucleus anymore, but can also be found in the cytoplasm. However, the localization of the later transfected CFP-Usp II is not changed. It is still distributed equally between nucleus and cytoplasm.

**Fig. 10** S2 cells transfected with an AlexaFluor-stained non-specific control
**4.4 Transcriptional activity of the EcR isoforms**

**4.4.1 Transcriptional activity of the wt EcR isoforms in the presence of endogenous Usp**

As a ligand-dependent transcription factor, EcR can bind to several response elements on DNA and thus modify the transcription of EcR-regulated genes. One of these response elements is in the promoter region of the EcR-dependent heat shock protein 27 (*hsp27*), which is most often used for studies on DNA binding or transcriptional potency. Therefore, in these studies, a reporter construct was used that is comprised of the *hsp27* response element coupled to the luciferase gene. The reporter construct was kindly provided by Dr. V. C. Henrich (University of North Carolina, Greensboro, USA).

First, DNA-binding to the *hsp27* response element was confirmed by electrophoretic mobility shift assay (EMSA).

As Fig. 12 shows, all three isoforms of EcR bind to 1 x *hsp 27*. The binding can occur already in the absence of hormone, however only very weak for isoform EcR-B1 and strongest for EcR-A. For all three isoforms an isoform-specific increase of DNA binding after hormone application can be seen.
Results

Fig. 12 EMSA of EcR isoforms and 1 x hsp 27 in the presence of endogenous wild type Usp. Identical amounts of all EcR isoforms determined by Western Blot were applied on each lane. If indicated (-/+), 1 µM Muristerone A was added 24 hours before the cell harvesting.

Fig. 13 Transcriptional activity of the EcR isoforms in the presence of endogenous Usp wt. EcR-A without hormone = 1. Values for luciferase activity are normalized on receptor protein concentration according to Western Blot. M (n=3) ± SD.
Results

The transcriptional activity varies among the three EcR isoforms (Fig. 13) with EcR-B1 having the highest basal transcriptional activity followed by EcR-A and EcR-B2. Concerning this basal, hormone-independent activity, there is a significant difference between the EcR isoforms (ANOVA, p<0.05). For all isoforms, an increase of transcriptional activity after application of 1 µM Muristerone A can be seen. The hormone effect varies significantly between the isoforms (fold induction: EcR-A: 3.4, EcR-B1: 26.7, EcR-B2: 13.6, ANOVA on ranks, p<0.05).

4.4.2 In the absence of hormone, EcR/Usp can have a repressive function

In in vivo studies on the sensory neurons in the Drosophila wing, it has already been shown, that the unliganded EcR/Usp can have a repressive function and thus trigger tissue differentiation during metamorphosis (Schubiger et al. 2005). To see if this is also true for the S2 subclone S2/H, the hsp27 reporter construct was transfected, and the transcriptional activity was determined for filler DNA as a control and for the EcR isoforms in the absence and presence of Muristerone A. As for the control no EcR was transfected, luciferase values were normalized only on the protein concentration. The transfection efficiency was checked microscopically and was about equal for all reactions.

The EcR isoforms EcR-A and EcR-B1 can exhibit a repressive effect on transcriptional activity in the absence of hormone (Tab. 4). For these isoforms, the reduction of transcriptional activity is statistically significant (EcR-A: p=0.02, EcR-B1: p=.001, Student’s t-test). For isoform EcR-B2 this decrease in transcriptional activity is not significant.

<table>
<thead>
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<th>EcR isoform</th>
<th>% inhibition ± SEM</th>
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<tr>
<td>EcR-A</td>
<td>48.6 ± 13</td>
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<tr>
<td>EcR-B1</td>
<td>76 ± 4.35</td>
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<tr>
<td>EcR-B2</td>
<td>45 ± 31.6</td>
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</table>

Tab. 4 Inhibition of transcriptional activity of the EcR isoforms in the presence of endogenous wild type Usp compared to basal transcriptional activity without EcR. Control = 100%. M (n=3) ± SEM.
4.4.3 Influence of the EcR mutation K497E on the transcriptional activity of the EcR isoforms

As already described, the point mutation K497E is located in helix four of the ligand-binding domain of EcR (Grebe et al. 2003). The substitution of the basic amino acid lysine with the acidic glutamine acid leads to a disruption of the salt bridge between helix four and helix twelve of the ligand binding domain and thus to an impaired hormone binding. In vertebrate cells, ligand binding of EcR<sup>K497E</sup>/wild type Usp is decreased about 10-fold (Azoitei 2009) compared to wild type EcR/wild type Usp.

Moreover K497E represents a conserved corepressor binding site of nuclear receptors (Lempainen et al. 2005). To see whether the influence of these co-modulators differs between insect and vertebrate cells, the EcR<sup>K497E</sup> isoforms were transfected in the S2/H cells and transcriptional activity was determined in the presence of endogenous wild type Usp.

As shown in Fig. 14, the basal transcriptional activity of the EcR isoforms EcR-A<sup>K497E</sup> and EcR-B1<sup>K497E</sup> compared to the wild type isoforms is not changed. For isoform EcR-B2<sup>K497E</sup>, the basal level is increased. The hormone induced transcriptional activity of EcR-A<sup>K497E</sup> is much lower compared to wild type EcR-A, whereas no effect is seen for

![Fig. 14](image_url) Comparison of the transcriptional activity of wild type EcR isoforms and the EcR<sup>K497E</sup> isoforms with endogenous wild type Usp. Wild type EcR-A without hormone = 1. Values for luciferase activity are normalized on receptor protein concentration according to Western Blot. M (n=3) ± SEM.
Results

EcR-B1$^{K497E}$ For EcR-B2$^{K497E}$, the increase after hormone application is even higher compared to the wild type EcR. Thus, the effect of the mutation K497E seems to be isoform-specific.

4.4.4 Transcriptional activity of the EcR isoforms in the presence of Usp$^{ΔDBD}$

The functional ecdysteroid receptor is a heterodimer of EcR and its partner Usp. For the binding of the heterodimer to the DNA, two possibilities are known (Ghbeish et al. 2001). The complex can bind directly to the DNA via the DNA binding domain of EcR, or, otherwise can bind to the DNA via the DNA binding domains of both receptor molecules (Ghbeish et al. 2001).

To see if, and to which extend the DNA binding of Usp influences the transcriptional activity of EcR/Usp, competition experiments were performed with the Usp mutation Usp$^{ΔDBD}$, which lacks the DNA-binding-domain but has still the original A/B domain of wild type Usp. Usp$^{ΔDBD}$ was transfected additionally to the endogenous wild type Usp, as the silencing with siRNA leads only to a reduction of about 40 % of wild type Usp (data not shown).

Basal and hormone-induced transcriptional activity highly depends on the concentration of endogenous wild type Usp and on the ratio between wild type Usp and Usp$^{ΔDBD}$. Therefore, for each experiment the transcriptional activity of the wild type EcR isoforms was determined as a control. For these controls, a non-specific filler DNA was transfected instead of Usp$^{ΔDBD}$.

When the endogenous wild type Usp has to compete with the Usp$^{ΔDBD}$ form, the basal as well as the hormone-induced transcriptional activity of all three EcR isoforms is reduced compared to the controls (Fig.15). This decreasing action of Usp$^{ΔDBD}$ on the transcriptional activity is highest for EcR-A, although this effect is not significant. This result clearly shows that the presence of the DBD of Usp influences the transcriptional activity in an isoform-specific-manner.
Fig. 15 Comparison of the transcriptional activity of the EcR isoforms with endogenous wild type Usp and the EcR isoforms with additional UspΔDBD. EcR-A without hormone = 1. Values for luciferase activity are normalized on receptor protein concentration of EcR according to Western Blot. M (n=3) ± SEM.

4.5 Interaction between ecdysone signaling and other signaling pathways

4.5.1 Influence of Juvenile hormone on ecdysone signaling

To see whether juvenile hormone (JH III) can influence ecdysone signaling in an isoform-specific way, its impact on the transcription of certain ecdysone-dependent genes was investigated for all three EcR isoforms. Therefore RNA was isolated from S2/H cells, each treated with different hormone concentrations and combinations of 20-hydroxyecdysone (20E) and JH III. Later on, PCR was done to amplify fragments for the genes BrC-Z1 (Broad Complex isoform Z1) and E74 A (ecdysone-inducible protein 74 A), which are already known to be regulated by ecdysteroids. As an internal control rp49 (ribosomal protein 49) was used.

With the BrC-Z1-specific primer pair, a 430 bp fragment was expected, with the primers for E74 A, a fragment with only 180 bp should be amplified.

The internal control gene rp49 encodes for a ribosomal protein of *Drosophila melanogaster* (O’CONNELL AND ROSBASH 1984). As the changes in the synthesis of ribosomal proteins are known to correlate with the physiological and developmental state
of the cell or organism, ribosomal proteins are well suited as internal controls (O’CONNELL AND ROSBASH 1984). With the primer pair used for this gene, a 526 bp fragment was expected. To see possible effects of JH, a concentration of 5 µM JH III was applied. This concentration was already proofed to work in previous experiments (BEATTY PERS.COMM). As previous studies in vertebrate cells showed that JH III is able to potentiate the response to 20E depending on the 20E concentration used, two different concentrations were applied in the present work that were already described in the mentioned study (BEATTY ET AL. 2006).

The presence of JH III clearly potentiates the transcription of the ecdysone-inducible gene BrC-Z1 when the lower concentration (0.1 µM) of 20E is used, reaching the same effect as the high concentration of 20E (Fig. 16). 0.1 µM 20E or JH III alone do not promote this effect. Transcription of E74 A however is stimulated only in a moderate way with low 20E concentrations (1.5-fold) whereas a higher stimulation can be achieved

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![Agarose gel of PCR products obtained with rp49-, BrC-Z1- and E74 A-specific primers obtained from cDNA of S2/H cells in the presence of EcR-A + endogenous wild type Usp and different concentrations and combinations of JH III and 20E. Both hormones were dissolved in DMSO (final culture medium concentration 0.1 %); therefore a control was done using only DMSO (Vehicle). M (n=3). Marker: GeneRuler™ 1kB DNA Ladder Plus.](image)

Fig. 16 Agarose gel of PCR products obtained with rp49-, BrC-Z1- and E74 A-specific primers obtained from cDNA of S2/H cells in the presence of EcR-A + endogenous wild type Usp and different concentrations and combinations of JH III and 20E. Both hormones were dissolved in DMSO (final culture medium concentration 0.1 %); therefore a control was done using only DMSO (Vehicle). M (n=3). Marker: GeneRuler™ 1kB DNA Ladder Plus.
with a combination of 0.1 µM 20E and JH III. The highest stimulation can be seen with 1 µM 20E. Moreover, also bands with a size of approximately 300 bp can be seen, which are probably due to unspecific PCR fragments.

In contrast to the above described results with EcR-A, no pronounced difference is seen in the presence of EcR-B1 for the transcriptional stimulation of BrC-Z1 when the combination of 0.1µM 20E and JH III is applied compared to situation with only 0.1 µM 20E (Fig. 17). Concerning the effect of 20E+JH III on the transcription of E 74A again no stimulatory effect can be seen. With 1 µM of 20E, a higher stimulatory effect on E74A transcription can be seen.

As shown in Fig. 18, in the presence of EcR-B2, the addition of JH III to 0.1 µM 20E leads to an only slight potentiation of the stimulatory effect on the transcription of BrC-Z1. Between the low concentration of 20E and the high concentration, no difference in the stimulation can be seen. Concerning the transcription of E74 A, no potentiation is visible. In this case, 1 µM 20E promotes the highest effect on transcription.

**Fig. 17** Agarose gel of PCR products obtained with rp49-, BrC-Z1- and E74 A- specific primers obtained from cDNA of S2/H cells in the presence of EcR-B1 + endogenous wild type Usp and different concentrations and combinations of JH III and 20E. Both hormones were dissolved in DMSO (final culture medium concentration 0.1 %); therefore a control was done using only DMSO (Vehicle). M (n=3). Marker: GeneRuler™ 1kB DNA Ladder Plus.
Comparing the stimulatory effects of all three isoforms, one can say that the two genes BrC-Z1 and E74 A respond differently to the applied hormone concentrations. Moreover, stimulation and potentiation depend on the EcR isoform.

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**Fig. 18** Agarose gel of PCR products obtained with rp49-, BrC-Z1- and E74 A- specific primers obtained from cDNA of S2/H cells in the presence of EcR-B2 + endogenous wild type Usp and different concentrations and combinations of JH III and 20E. Both hormones were dissolved in DMSO (final culture medium concentration 0.1 %); therefore a control was done using only DMSO (Vehicle). M (n=3). Marker: GeneRuler™ 1kB DNA Ladder Plus.

### 4.5.2 Interaction of ecdysone signaling and the insulin signaling pathway in S2/H cells

As shown for many invertebrates, insulin plays a major role as growth hormone also in insects, whereas 20-hydroxyecdysone (20E) initiates crucial developmental transitions (COLOMBANI ET AL. 2005) and cell differentiation of imaginal cells during metamorphosis. Moreover, it was found that insulin and 20E show antagonistic actions (COLOMBANI ET AL. 2005). According to GRAVES AND SCHUBIGER (1982), high concentrations of 20E lead to a proliferation arrest in S2 cell cultures. However it is reported that low concentration can also stimulate proliferation (KISSENBECK, unpublished results).
Results

For this reason, proliferation experiments were performed using S2 cells, expressing only a very low amount of endogenous EcR and S2 cells transfected separately with the different EcR isoforms.

To see which hormone concentrations are suitable for studying the antagonism between insulin and 20E, several preliminary experiments were performed. Different concentrations of 20E were tested within a range of 10^{-6} M and 10^{-9} M. At 10^{-6} M 20E the cells showed a very quick arrest in proliferation, cell membranes were disrupted and the cells could not maintain normal shape for more than 24 h. Cell conditions were improved at a concentration of 10^{-7} M; however the proliferation arrest was still visible. 10^{-9} M 20E showed a small stimulating effect. Insulin was used at a concentration of 1.7 x 10^{-12} M. This is the normal concentration used in Schneider’s Medium for insect cells as a growth factor. For the proliferation experiments however, cells were grown in insulin-free medium and hormone was added as additional factor.

Insulin concentrations were varied from 10^{-5} M to 10^{-10} M for non-transfected as well as for transfected cells. However, no insulin-dependent effect was seen (data not shown).

**Proliferation of untransfected S2/H cells**

As shown in Fig. 19 A, the treatment with 10^{-7} M 20E leads to a complete arrest in proliferation or cell death. After 72 h half of the cells have died. The lower concentration of 10^{-9} M 20E does not show any significant effect, compared to the control.

The application of 1.7 x 10^{-12} M insulin does not have any effect on cell proliferation (Fig 19 B). Moreover, the presence of additional insulin to 10^{-7} M 20E can not rescue normal proliferation (Fig. 19 B). The concentration of 10^{-9} M 20E with additional insulin does not have any effect.

These results demonstrate that in spite of the very low amount of endogenous EcR, the cells are still able to respond to hormone.
Results

Fig. 19 A Influence of the molting hormone 20-hydroxyecdysone on the proliferation of untransfected cells of the subclone S2/H of the S2 cell line of *Drosophila melanogaster*.

Fig. 19 B Influence of the growth hormone insulin and its antagonist 20-hydroxyecdysone on the proliferation of untransfected cells of the subclone S2/H of the S2 cell line of *Drosophila melanogaster*. Insulin was applied at a concentration of $1.7 \times 10^{-12}$ M.
Results

**Proliferation of S2/H cells transfected with the EcR isoforms**

To see whether a higher concentration of EcR leads to a higher sensitivity of the S2/H cells towards hormone, the cells were transfected with the EcR isoforms.

Fig. 20 A Influence of the molting hormone 20-hydroxyecdysone on the proliferation of *Drosophila melanogaster* S2 cells (subclone S2/H), transfected with YFP-EcR-B1.

Fig. 20 B Influence of the growth hormone insulin and its antagonist 20-hydroxyecdysone on the proliferation of *Drosophila melanogaster* S2 cells (subclone S2/H), transfected with YFP-EcR-B1. Insulin was applied with a concentration of $1.7 \times 10^{-12}$ M.
Results

The lower concentrations of 20E do not have any significant effect on cell proliferation. 20E at concentration of $10^{-7}$M however stops proliferation very quickly. Thus, already after 48 h almost half of the cells have died.

The presence of insulin does not change this fact significantly (Fig. 20 B). Same as for the untransfected cells (Fig. 20 B), insulin alone does not influence proliferation at the used concentration.

Compared to the untransfected cells, the inhibition of proliferation is more prominent for the transfected cells (1.5-fold).

The same experiment was performed for the isoforms EcR-A and EcR-B2 with the same results as for EcR-B1.

4.5.3 Interaction of ecdysone signaling with the Toll pathway in S2/H cells

In insects, three different proteins are present that are active as transcription factors in the Toll pathway: dorsal, relish and dif. In this study however, only dorsal and relish were taken into account as dorsal is involved mainly in cell differentiation, whereas relish is more responsible for the regulation of innate immunity.

Localization of the two Rel-family proteins dorsal and relish in S2/H cells

Like for NF-κB, localization of relish and dorsal is crucial for the transcriptional activity of these proteins. Therefore, localization of dorsal and relish in the used S2/H cell line was studied first. To see whether the stress of a transfection influences localization, the experiments were performed with untransfected cells as well as with cells transfected with a non-specific filler DNA.

Both relish and dorsal are located in the nucleus already without any stimulation (Fig. 21). This nuclear localization is independent of the transfection that could stress the cells and thus lead to a stress response via Toll signaling.
Results

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**Fig. 21** Western Blot analysis of endogenously expressed relish and dorsal in S2/H cells. A Membrane detected with the monoclonal antibody anti-relish C21F3 directed against the C-terminal fragment of relish (25 μg/lane). B Membrane detected with the monoclonal antibody anti-dorsal 7A4. M (n=3).

**Expression of relish and dorsal in the presence of the EcR isoforms in S2/H cells**

To study a possible influence of EcR on the expression of relish and dorsal, S2/H cells were transfected with EcR-A, EcR-B1 and EcR-B2 as well as with the empty pAc5.1 vector as a control. As the presence of EcR did not change the nuclear localization of the two proteins, Western Blot analysis was performed with nuclear extracts to see if there are changes in the expression of relish and dorsal.

Fig. 22 A clearly demonstrates that in the presence of the EcR isoforms the concentration of relish is changed in an isoform-specific manner. As no hormone effect could be detected for any of the isoforms the data were pooled for the following evaluation.

**Fig. 22** A Western Blot analysis of endogenously expressed relish in S2/H cells in the presence of the EcR isoforms compared to a non-specific control. Representative Western Blot. The membrane was detected with the monoclonal antibody anti-relish C21F3 directed against the C-terminal fragment of relish (25 μg/lane).
Fig. 22 B Concentration of relish in the presence of the EcR isoforms in S2/H cells compared to a non-specific control. The concentration of relish was determined via quantification of Western Blot bands detected with the antibody anti-relish C21F3. M (n=10) ± SD.

Compared to the control, the presence of the EcR isoforms EcR-B1 and EcR-B2 can stimulate the expression of relish (Fig. 22 A and 22 B) significantly (Mann-Whitney Rank Sum Test p<0.05) with EcR-B2 having the highest influence.

Same as for relish, the concentration of dorsal is changed in the presence of the EcR isoforms in an isoform-specific manner (Fig. 23 A). Again, no hormone effect can be detected. Therefore, the data were pooled for the following evaluation.

Fig. 23 A Western Blot analysis of endogenously expressed dorsal in S2/H cells in the presence of the EcR isoforms compared to a non-specific control. Representative Western Blot. The membrane was detected with the monoclonal antibody anti-dorsal 7A4 (25 µg/lane).
In the presence of the three EcR isoforms, the concentration of dorsal is increased (Fig. 23 A and 23 B) significantly (Mann-Whitney-Rank-Sum Test p<0.05).

Same as shown before for relish, EcR-A shows the lowest effect, whereas EcR-B2 shows the highest one.

**Influence of the EcR isoforms on the transcriptional activity of dorsal in S2/H cells**

To test whether apart from concentration also the transcriptional activity of dorsal is changed by an interaction with EcR, a NF-κB reporter construct containing three κB-motifs (GGGGACTTTCC) coupled to a luciferase gene was transfected into the S2/H cells and reporter gene activity was measured in the absence and presence of the EcR isoforms and hormone.
Results

Fig. 24 Transcriptional activity of a NF-κB reporter construct in the presence of the EcR isoforms and endogenous Usp wt compared to a non-specific control. Values for luciferase activity are normalized on the concentration of dorsal according to Western Blot. M (n=6) ± SEM.

To include the fact that the concentration of dorsal is affected by the presence of the EcR isoforms, luciferase values were normalized on its concentration according to Western Blot quantification. As previous results showed that relish is inactive (see discussion), relish concentrations were not taken into account. However, after normalization, no significant effect of any EcR isoform on the transcriptional activity of dorsal can be determined (Fig. 24).

4.5.4 Interaction of ecdysone signaling with the NF-κB signaling pathway in CHO-K1 cells

As a control to see whether the interactions between the two pathways are cell-type specific, the experiments were performed in parallel in the mammalian CHO-K1 cell line. In this cell line, p65, the most common protein of the Rel-family was studied. Moreover, compared to insect cells, in CHO-K1 cells the influence of endogenous Usp affecting the behavior of the EcR/Usp complex can be excluded.
Results

Localization and expression of p65 in the presence of the EcR isoforms in CHO-K1 cells

To study the influence of EcR on the localization of the ‘classical’ NF-κB, which is a heterodimer comprised of the factors p65 and p50, Western Blot analysis was performed with a p65 antibody. In contrast to relish and dorsal, p65 was not always found exclusively in the nucleus but was detectable in cytoplasm and nucleus in about equal amounts, independent whether the cells were transfected or untransfected (data not shown). Therefore, cytoplasmatic as well as nuclear extracts were used to see possible changes in localization due to the EcR isoforms. The experiments were done for all three EcR isoforms. However, for EcR-A no influence on p65 localization was seen (data not shown). The presence of EcR-B1 clearly influences the localization of p65 (Fig. 25). Compared to the control, nuclear localization of p65 is increased for about 30 %, whereas cytoplasmatic localization is decreased. This effect can be seen in the absence and in the presence of the heterodimerization partner Usp wt, however, with no statistical significance.

![Fig. 25 Localization of p65 in the presence of the YFP-EcR-B1 and Usp wt compared to a non-specific control. The localization of p65 was determined via quantification of Western Blot bands detected with the antibody sc-372. M (n=3) ± SD.](image-url)
Fig. 26 Localization of p65 in the presence of the YFP-EcR-B2 and Usp wt compared to a non-specific control. The localization of p65 was determined via quantification of Western Blot bands detected with the antibody sc-372. M (n=3) ± SD.

Unlike for isoform EcR-B1, the presence of EcR-B2 does not stimulate the nuclear localization of p65, but leads to an increase in the cytoplasm (Fig. 26). This effect seems to be independent of Usp wt.

The present data clearly show that although the Toll pathway is a homolog of the NF-κB pathway, the proteins involved in this signaling are regulated differently in insect cells compared to vertebrate cells. However, in both cell types no hormone dependence could be detected and in both cell types regulation seems to depend exclusively on EcR not on EcR/Usp.

Influence of the EcR isoforms on the transcriptional activity of NF-κB in CHO-K1 cells

To see if EcR also influences the transcriptional activity of NF-κB, reporter gene studies were performed with the same reporter construct already used for the insect cells.
Results

As shown in Fig. 27, the EcR isoforms influence the transcriptional activity in an isoforms-specific way. The presence of EcR-A leads to a significant induction of transcriptional activity (ANOVA, p<0.05), whereas for EcR-B1 and EcR-B2 only slight increases can be seen. For all isoforms no influence of hormone can be detected.

The comparison between insect cells and vertebrate cells concerning transcriptional activity demonstrates that especially in the case of EcR-A there must be pronounced differences between the two studied cell types.

Direct interaction of EcR-B1 and p65 in CHO-K1 cells

As for some other steroid hormone receptors like the progesterone receptor (KALKHOVEN ET AL. 1996), the glucocorticoid receptor (RAY ET AL. 1994) and the estrogen receptor (QUAEDACKERS ET AL. 2007) direct interactions with p65 have already been reported, immunoprecipitations were performed to see whether there is also a direct physical interaction with EcR.

As Fig. 28 shows, the immunoprecipitation for Flag-EcR-B1 with Flag beads works very well, as no band can be detected in the supernatant, but only in the IP itself (A). In
Results

contrast to that, in the untransfected cells, endogenous p65 binds unspecifically to the Flag-beads already in the absence of any other Flag-tagged protein (B). Therefore the beads were blocked with several different blocking solutions like milk buffer, BSA, Blocking Solution (Candor Biosciences, Weißenberg, Germany) or Low Cross Buffer (Candor Biosciences, Weißenberg, Germany). However, the unspecific binding of p65 to the beads could not be prevented.

As a positive control, the direct interaction of EcR and Usp was confirmed (C).

**Fig. 28** Western Blot of immunoprecipitation with Flag-beads in CHO-K1 cells. A Cells transfected with Flag-EcR-B1, B untransfected cells, C Cells cotransfected with Flag-EcR-B1 and Usp wt. E Cell Extract (25 µg total protein), Sn Supernatant, IP immunoprecipitation.
5. Discussion

5.1 The S2/H subclone of the Drosophila melanogaster S2 cell line

In this work, a subclone of the Drosophila Schneider cell line S2 (SCHNEIDER 1972) was used to study the function of the three Drosophila isoforms of EcR and their heterodimerization partner Usp. The origin of S2 cells is not completely clear; however, they are supposed to be derived from imaginal disc cells, out of which the adult structures of the later animal can develop (SCHNEIDER 1972).

In vivo studies revealed that the EcR isoforms are expressed tissue-specific and are depending on the developmental stage (TALBOT ET AL. 1993). The isoforms EcR-B1 and EcR-B2 are expressed mainly in larval cells, whereas isoform EcR-A is predominantly present in developing adult cells (TALBOT ET AL. 1993).

As expected, all three EcR isoforms were found in the genome of the S2/H cells. Although also mRNA was found for all isoforms, the endogenously expressed EcR was under the detection limit. The fact that no endogenous EcR could be detected was already described for S2 cells by TALBOT ET AL. 1993 where it was found that mRNA was present for EcR-A, EcR-B1 and EcR-B2 but only EcR-B1 was expressed in detectable amounts.

As demonstrated, Usp is present in the S2/H cell line in rather high amounts. In Drosophila, Usp transcripts were shown to be present throughout development whereas translation of the Usp protein is regulated, depending on the number of larval instars (HENRICH ET AL. 1994). As mentioned above, this regulation of nuclear receptors on the level of translation was shown also for EcR in the present study with S2/H cells.

5.2 Intracellular localization of the EcR isoforms and Usp in S2 cells

Up to now, most studies on the localization of EcR and Usp were performed in mammalian cell lines to prevent the influence of the endogenous receptor proteins. In CHO-K1, COS-7 and HeLa cells, the localization of EcR in the absence of Usp and hormone was found to be dependent on the used EcR isoform as well as on the cell line
(Gwozdz et al. 2007). Therefore, in this work, fluorescence-tagged isoforms of EcR and variants of Usp were analysed for their localization in the insect cell.

In CHO-K1 cells, EcR-A and EcR-B1 are located exclusively in the nucleus, whereas EcR-B2 is distributed equally between cytoplasm and nucleus (Gwozdz et al. 2007). For these two isoforms a nuclear localization signal (NLS) was found in the A/B domain, which is lacking in EcR-B2. Proteins containing NLS are able to interact with importin α binding to importin β which then acts as a cargo to the nucleus (Laudet and Gronemeyer 2002).

Additional to the NLS in the A/B domain, all isoforms contain a NLS within the DBD and a nuclear export signal (NES) in the LBD.

Analogous to the nuclear import, NES can interact with specific exportins, leading to the export of the protein to the cytoplasm (Wen et al. 1995). Additionally, studies in vertebrate cells with the DNA binding inhibitor nitric oxide (NO) showed that Usp and EcR can enter the nucleus also separately and that neither receptor interacts with DNA in the absence of hormone (Cronauer et al. 2007).

The present data in insect cells reveal that after formation of the EcR/Usp complex, the heterodimer is located predominantly in the nucleus as shown microscopically and by Western Blot analysis. However, EcR-B2 could be determined to a rather low extend also in the cytoplasm, possibly due to the lack of a NLS in the A/B domain.

One explanation for this is that heterodimerization sterically impairs the interaction with exportins as Usp masks the NES of EcR in the LBD (Betanska et al. 2007).

As the used S2/H cell line endogenously contains wild type Usp, the localization of the EcR isoforms alone could not be determined. However, as the experiments with siRNA showed, a silencing of wild type Usp resulted in a rather equal distribution of EcR within the cell.

According to the Western Blot results, the presence of hormone in this work did not stimulate the expression of EcR but the expression of Usp was always higher in the cytoplasm after hormone application as was shown by Western Blot with S2/H cell extracts. Stimulation of EcR by 20E was reported for a Chironomus tentans cell line (Lammerding-Köppel et al. 1998) and for mammalian CHO-K1 cells (Ruff et al. 2009).

Contrary to the fact that in the present study wild type Usp was located in the cytoplasm and in the nucleus, in mammalian cells wild type Usp alone was present only in the nucleus (Nieva et al. 2005), what can be explained by the fact that Usp harbors a
NLS signal in the DBD, but no NES (GWOZDZ ET AL. 2007). Therefore, once in the nucleus, it remains there. This was confirmed by the finding that in CHO cells the Usp variant VP16$_{AD}$ Usp III, which is lacking the DNA-binding domain, is located mainly in the cytoplasm (CRONAUER ET AL. 2007).

In the present work however, all three VP16$_{AD}$ variants of Usp, also Usp III were distributed within the whole cell. Also the endogenous Usp was located in both fractions, in the cytoplasm and in the nucleus, which was shown using Western Blot analysis. Moreover, the influence of the endogenous Usp on the localization of the transfected variants can be excluded, as experiments with siRNA showed that a silencing of endogenous Usp influences the localization of EcR, but not of the Usp variant.

One explanation for the different localization of Usp in the *Drosophila* cells, compared to the mammalian cell system could be the cellular background. This thesis would be confirmed by the finding that in the insect cell line from *Chironomus tentans* Usp was located to a high degree in the cytoplasm (RAUCH ET AL. 1998). Another reason could probably be that the shuttling of the proteins between cytoplasm and nucleus is much slower in insect cells compared to mammalian cells. This hypothesis would be supported by the fact that with a doubling time of 24-30 h the complete metabolism in the insect cells is substantially slower than for example in CHO cells (doubling time 6 h). If the produced proteins are retained longer in the cytoplasm and are produced on a constantly high level, it would be more likely to find them there. This thesis would also correlate with the fact that in the presence of hormone, more Usp can be found in the cytoplasm compared to the situation without hormone as hormone could stimulate the Usp expression.

In summary, concerning the localization of EcR in the absence and in the presence of its heterodimerization partner Usp, the data obtained from mammalian cells were confirmed by the present data from insect cells. With a lower amount of Usp, EcR seems to be distributed within the complete cell. After formation of the EcR/Usp complex Usp promotes the nuclear localization of the EcR isoforms. In contrast to heterologously expressed Usp, insect Usp is located in the cytoplasm as well as in the nucleus, independent of the Usp variant. The result that in the case of localization, no pronounced differences between insect and vertebrate cells could be determined can be explained by the fact that both NLS and NES are molecular characteristics of the receptor proteins themselves and thus independent of the cellular context.

In general, the localization of nuclear steroid receptors in the absence of hormone mainly depends on the type of receptor. Homodimers of the androgen receptor (AR) are
located predominantly in the cytoplasm (TYAGI ET AL. 2000), same as homodimers of the glucocorticoid receptor (GR, HTUN ET AL. 1996). Only after hormone binding the dimers translocate to the nucleus. In contrast, the thyroid receptor (TR) is located already in the nucleus, independent of hormone (HTUN ET AL. 1999).

5.3 Transcriptional activity of EcR/Usp

The transcriptional activity of a nuclear receptor can be regulated and modified in many ways. The probably most crucial factor is given by the ligand and the dimerization partner. However, also the hormone response element, phosphorylation or the interaction with other comodulators can play an important role. These modification possibilities allow the specific regulation of the hormone response as well as the adaptation of the receptor activity to the actual needs of the target cell.

In case of the ecdysteroid receptor from Drosophila, another possibility for the modulation of the ecdysone response is offered by the presence of the three isoforms of the ecdysteroid receptor that differ in their hormone-independent activation domain (A/B domain). The first prerequisite for the activation of transcription is of course the binding to the DNA. In the used S2/H cells, DNA binding, as well as the induced hormone effect differs, depending on the used EcR isoform as shown by EMSA. Moreover, this hormone effect in the presence of wild type Usp is more pronounced in the used insect cells compared to data from vertebrate cells (BRAUN ET AL. 2009) showing the dependance on the cell type.

Most studies on transcriptional activity of the EcR isoforms expressed separately were performed in heterologous systems to overcome the influence of endogenously present EcRs. Therefore data on transcriptional activity of the EcR isoforms in insect cells are scarce. In the studies on heterologously expressed EcR, mostly the different variants of Usp (Fig. M1) were used as the original wild type Usp seems to have an N-terminal inhibitory effect in mammalian cells (HENRICH ET AL. 2003).

In the present work, all experiments on transcriptional activity of the EcR isoforms in insect cells were performed in the presence of endogenous Usp.
5.3.1 In the absence of hormone EcR/Usp has repressive function on the hsp27 response element

The fact that nuclear receptors cannot only activate transcription but also repress basal transcription in the absence of hormone is long known (HU AND LAZAR 2000). It shows once again how complex gene regulation and modification of the hormone response can be. Repressive functions have already been described for thyroid hormone receptor (TR) and retinoid acid receptor (RAR, HU AND LAZAR 2000).

Repression is mediated via protein factors such as N-CoR (Nuclear receptor corepressor), SMRT (silencing mediator of retinoic acid and thyroid hormone receptor), its Drosophila variant SMRTER (SMRT-related ecdysone receptor-interacting factor) or Alien.

These factors are able to form large complexes on the one hand via the LBD or the A/B domain of nuclear receptors and, on the other hand, with factors like HDACs (histone deacetylases) leading to hypoacetylation which is associated with transcriptionally silent chromosomes (HU AND LAZAR 2000).

As reported recently from in vivo studies, unliganded EcR/Usp can inhibit transcription concerning the differentiation of the sensory neurons in the Drosophila wing (SCHUBIGER ET AL. 2005). One molecular mechanism behind this repression in the absence of hormone is given by an interaction of unliganded EcR/Usp with SMRTER, which is impaired after the binding of hormone (TSAI ET AL. 1999).

The present results show that without hormone, all isoforms of EcR can exhibit repressive functions in the presence of the hsp27 response element in an isoform-specific manner. This repression is relieved after the addition of hormone and an isoform-specific hormone effect can be seen that will be described later. Such effects were already reported from an EcR-deficient cell line, the Drosophila Ke L57-3-11 (HU ET AL. 2003), where transcriptional activity was also determined for all three isoforms via reporter gene assay and are probably due to an interaction with corepressors as described above.

As recent studies with heterologously expressed EcR/Usp showed, inhibition of transcriptional activity can also be mediated via an intramolecular interaction of the A/B domain of EcR with specific binding motifs within the LBD (TREMMEL ET AL. SUBMITTED).

Moreover, the repressive function seems to depend highly on the cellular context and thus on the composition of cofactors, as, although many cofactors are conserved among
insects and vertebrates, these effects were not determined in a vertebrate cell system (RUFF ET AL. 2009).

5.3.2 Transcriptional activity of separately expressed EcR isoforms

In the above mentioned experiments, transcriptional activity of the EcR isoforms was determined compared to a control, where no EcR was transfected and expressed. Therefore, luciferase values could be normalized only on protein concentration. Thus, not the specific transcriptional activity of the EcR isoform, but the sum of the receptor concentration and the transcriptional activity was determined. To measure the specific transcriptional activity of the respective EcR isoform, in all other experiments the luciferase values were normalized on the concentration of the EcR isoforms according to Western Blot.

As described by HU ET AL (2003), two activation functions are located within the EcR molecule. The first, activation function 1 (AF-1), is located within the A/B domain of EcR and is therefore isoform-specific. Activation function 2 (AF-2) is associated with the ligand binding domain and is thus regulated by ligand binding.

Moreover, in contrast to EcR-B1 and EcR-B2, for EcR-A no AF-1 could be detected in the work by HU (2003). Thus, the transcriptional activation of EcR-A was supposed to be only regulated by AF-2. However, as mentioned before, the A/B domain of EcR-A is also reported to act as an intramolecular repressor (TREMMEL ET AL. SUBMITTED).

These findings could explain the fact that in all transcriptional assays in the present work, EcR-A showed the weakest hormone response.

Compared to the repression study, transcriptional activity of EcR-B2 is lower than the one of EcR-A, which is due to the fact that the concentration of EcR-B2 usually was weakly higher than the one of EcR-A. Therefore, normalization on Western Blot results in lower luciferase values for EcR-B2. This could explain why in the study from HU (2003) transcriptional activity of EcR-A was also lower compared to EcR-B2 as the data there were normalized on transfection efficiency.

Additionally, the data obtained here in insect cells demonstrate well that concerning transactivation, there are crucial differences between the insect cell system and the mammalian system. From studies in CHO-K1 cells it was known that after normalization on receptor concentration, basal activity is highest for EcR-B1 followed by EcR-B2 and EcR-A (RUFF ET AL. 2009), which would correspond well to data from insect cells. However, in CHO-K1 cells, no significant hormone effect is detectable for EcR-A and
EcR-B2; EcR-B1 shows only a very weak hormone effect compared to the situation in insect cells (RUFF ET AL. 2009). This clearly underlines the importance of the original cellular context at least in the case of transactivation studies.

5.3.3 The point mutation K497E affects the transcriptional activity of the EcR isoforms

In contrast to vertebrate steroid receptors, the three dimensional structure of the LBD of EcR without Usp is still unknown due to the lack of stability of the purified receptor. Therefore only the structure of the heterodimer can be used to study the conformation of the LBD of EcR. These data revealed that the LBD of nuclear receptors determined by X-ray analysis consists of 11 to 12 helices and a β-sheet arranged as an antiparallel sandwich, forming the ligand binding pocket of the hormone receptor (WURTZ ET AL. 1995).

In the present work, special attendance was drawn to the function of the last helix, helix 12, which, after ligand binding is known to flip inward and thus to lock the hormone within the ligand binding pocket (MORAS AND GRONEMEYER 1998, Fig. I 5). Studies on the LBD of vertebrate RARγ showed that this conformational change of helix 12 offers a new surface that allows other coactivators to bind, thereby mediating the activity of AF-2 (WURTZ ET AL. 1995). On the other hand, changing of the position of helix 12 after hormone binding plays also an important role for the release of corepressors like SMRT or N-CoR.

To study the interaction of EcR, Usp and hormone on the molecular level, site-directed mutagenesis was used to determine the function of single amino acids within the LBD of EcR. As already shown for vertebrate receptors, one important factor for the stabilization of the LBD is given by a salt bridge between a lysine on position 497 in helix 4 and a conserved glutamine acid in helix 12.

Ligand binding studies with recombinant EcR and Usp in yeast strains showed that substitution of this basic amino acid by an acidic one and the resulting disruption of this salt bridge leads to impaired ligand binding (GREBE ET AL. 2003).

As mutation of the other partner of the salt bridge (E648) showed only moderate changes in these studies, the effect of the K497E mutation cannot only be explained by the destabilization of the ligand binding pocket by disruption of the salt bridge (GREBE ET AL. 2003), but probably also by effecting cofactor binding, especially because K497E represents a conserved cofactor binding site (LEMPIÄINEN ET AL. 2005).
The data presented in this work would support an influence of cofactors, mainly of corepressors, as the basal transcriptional activity of the EcR$^{K497E}$ isoforms is increased, especially for EcR-B2 compared to the wild type isoforms.

One explanation for this effect could be that by mutating the conserved corepressor binding site, corepressor binding is impaired resulting in an increased transcriptional activity as described.

Additionally, this effect would support the data concerning the repressive function of EcR in the absence of the ligand. Compared to the control, unliganded wild type EcR isoforms are able to repress transcriptional activity, probably due to the interaction with corepressors as already mentioned above. The fact that the basal transcriptional activity is higher for the EcR$^{K497E}$ isoforms compared to wild type EcRs confirms the hypothesis, that the point mutation predominantly influences corepressor binding.

These results demonstrate that the composition of cofactors complexed with EcR/Usp is isoform-specific, as for EcR-A$^{K497E}$ no such increase compared to wild type EcR is detectable. Moreover, the higher hormone induction of EcR$^{K497E}$ compared to the wild type receptors is not only isoform-specific, but also depending on the cell type as in studies with vertebrate cells this effect is not seen (TREMMEL ET AL. SUBMITTED). As the EcR isoforms differ only in their A/B domain, one explanation for the isoform-specificity is that the three dimensional structure is influenced by the A/B domain and offers thus different possibilities for the binding of external cofactors as well as for intramolecular interactions (TREMMEL ET AL. SUBMITTED).

### 5.3.4 The C-domain of Usp influences the transcriptional activity of the EcR isoforms

As already mentioned, the S2/H cells do not express endogenous EcR (or only in very negligible amounts) but do express a rather high level of endogenous Usp.

Therefore, the data on the transcriptional activity of EcR and the Usp mutation UspΔDBD do not reflect only the effect of the mutation, but are competitive experiments between UspΔDBD and endogenous wild type Usp. However, the influence of the lacking DNA binding domain can be seen clearly as basal transcriptional activity of all three isoforms was reduced in the presence of UspΔDBD with the used hsp27 response element compared to wild type Usp.
As shown before (Fig. I 4), the EcR/Usp complex can bind to the DNA in two different ways: via the DBD of EcR or via the DBDs of both receptors (GHBEISH ET AL. 2001).

Depending on the type of DNA binding, the receptor complex conformation is changed and can thus modulate transcriptional activity as for example, different sites for comodulator binding are presented or not. Moreover, also the dimerization and the orientation of the complex on the DNA can be modulated (DEVARAKONDA ET AL. 2003). The difference in comodulator binding could also explain why the present results in insect cells differ from the ones obtained in vertebrate systems. For mammalian cells, cotransfection of the EcR isoforms and UspΔDBD leads, depending on the type of hormone response element, to a rather small decrease, or even to an increase in transactivation (SCHAUER, PERS. COMM., GHBEISH ET AL. 2000).

Except DNA binding, the DBD of nuclear receptors also harbours a dimerization site (PERERA ET AL. 2005), which is highly important for the dimerization with EcR. Therefore, the present results demonstrate also, that not only in the presence, but already in the absence of hormone dimerization via the LBD of both partners is effective. Thus, the decreased transcriptional activity of EcR/UspΔDBD cannot be directly related to the lack of DNA binding of Usp complexed with EcR, but can also be a result of decreased dimerization with EcR.

5.4 Interactions with other signaling pathways

As described earlier, ecdysone signaling is known to be involved in a large variety of processes during insect development. As in development many different signaling pathways are involved, it was therefore interesting to study these possible interactions more in detail. To cover a wide range of ecdysone-dependent processes, three different pathways were chosen for their distinct functions.

First, the juvenile hormone pathway, which is already known to interact with ecdysone signaling during metamorphosis and differentiation (RIDDIFORD 1994), especially as differentiation plays a major role for future adult tissues from which the used S2/H cell line likely is derived. However, the character of this interaction and the question whether it is dependent on the EcR isoforms has not been addressed so far.

Second, the Toll pathway which is involved in embryonal cell differentiation and later on in the differentiation of imaginal discs, which is also known to be influenced by ecdysteroids.
Unlike in vertebrates, insulin does not act as regulator of blood sugar in insects, but as a growth hormone and offers thus also the possibility of an interaction with ecdyson signaling.

5.4.1 Interaction with juvenile hormone signaling

According to ASHBURNER (1974) different sets of genes are regulated after hormone binding in a distinct time flow. After hormone binding, a first set of genes is activated (‘early genes’). The synthesized proteins then on the one hand repress their own expression; on the other hand, they stimulate the expression of a second set of genes (‘late genes’).

Both E74 A and BrC-Z1 belong to the group of early genes being directly stimulated by ecdysone (Huet et al. 1993). As mutant analysis revealed, the Drosophila transcription unit Broad Complex (BrC) is essential for the onset of metamorphosis (Kiss et al. 1976). The Broad Complex locus produces multiple transcripts, however only four types of proteins are produced, that differ in their C-terminal domains: BrC-Z1, BrC-Z2, BrC-Z3 and BrC-Z4 (DiBello et al. 1991). Like the BrC-Z1, E74 A is also part of a large ecdysone-dependent transcription complex, consisting of two overlapping transcript units, E74 A and E74 B (Burtis et al. 1990). As described earlier, E74 A is crucial for metamorphosis of larval and imaginal tissues and for proper pupariation (Fletcher et al. 1995).

The fact that both genes can be activated at a high concentration of (1 µM) of 20E is not surprising. Lower concentrations of 20E lead to a different induction of both genes, depending on the EcR isoform.

In hsp27 reporter genes assays, juvenile hormone alone failed to mediate any response via EcR and Usp in vertebrate cells (Henrich et al. 2003). In contrast, when low concentrations of 20E were used, JH was able to potentiate the ecdysone response to restore a maximal induction level comparable to the response at high 20E titres (Henrich et al. 2009).

Previous studies in Drosophila S2 cells where juvenile hormone and several juvenile hormone analogs in combination with 20E were tested could not support these results (Soin et al. 2008). However, in this study, 20E concentrations were much higher (1-10 µM) compared to the low concentration used here. Therefore, probably no potentiation could be observed as the hormone response was already at maximum.
One hypothesis for the potentiation effect of juvenile hormone on the ecdysone response is that it represents an interface between nutritional state and molting (Beatty et al. 2006).

As already mentioned above, in reporter gene assays, the simultaneous presence of JH III and 20E in vertebrate cells leads to a potentiation of the transcriptional activity by reducing the concentration of ecdysteroids necessary for a maximal ecdysone response for about 10% (Beatty et al. 2006). As during larval-larval molts, ecdysteroid titres are generally low this ecdysone-reducing effect of JH III could trigger molting at good nutrient supply (Beatty et al. 2009).

### 5.4.2 Interaction with the insulin signaling pathway

In *Drosophila*, a family of proteins, the *Drosophila* insulin-like peptides (DILP) are able to bind and activate one unique insulin receptor (InR). This activation initiates a signaling cascade leading finally to the phosphorylation of the *Drosophila* forkhead transcription factor dFOXO by the protein kinase Akt (Jünger et al. 2003).

In the absence of nutrients, and therefore DILPs, dFOXO is located in the nucleus in a constitutive active form, stimulating the transcription of InR and the translational inhibitor 4EBP (Puig et al. 2008). 4EBP inhibits growth, whereas the increased amounts of InR sensitize the cells for small changes in the concentration of DILP (Puig et al. 2008). Upon high nutrient concentrations, DILP increase, leading to the phosphorylation of dFOXO and its shuttling to the cytoplasm. The subsequent decreasing amounts of 4EBP can then promote growth and cell proliferation (Puig et al. 2008).

However, in the cell culture system used here, no stimulating effect of insulin on cell proliferation was determined. One reason for the missing stimulation could be that not all factors of the insulin signaling cascade are expressed or are only present in lower amounts. Moreover, the insulin concentration is crucial for cell stimulation. Although a wide range of concentrations was tested, it cannot be excluded that the cells do not respond to the applied concentrations as data from insect cell studies are scarce.

Also 20-hydroxyecdysone is known to have a strong effect on proliferation and differentiation during development (Graves and Schübiger 1982). *In vivo*, rising ecdysteroid titers during larval development stimulate DNA synthesis, accompanied by a mitotic block, where cells arrest in G2 phase (Graves and Schübiger 1982) of the cell cycle. Decrease of the ecdysteroid titer results in the release of the cells from this block,
thereby synchronizing the cells for following cell divisions (GRAVES AND SCHUBIGER 1982).

The present results clearly confirm this proliferation arrest at higher hormone concentrations for untransfected and for S2/H cells transfected with the EcR isoforms. One explanation why the untransfected cells still show the typical mitotic block could be, that even the very low concentrations of endogenous EcR are sufficient to mediate this developmental effect. This hypothesis is supported by the observation, that in other experiments like reporter gene assays, untransfected cells do not show any hormone effect as for the transcriptional activation the amount of endogenous EcR is probably not sufficient for the used reporter construct. However, it can also not be excluded that 20E triggers the cell cycle arrest via additional factors such as cross-talk with other pathways or for example. Additionally, also some non-genomic effects of ecdysone signaling are reported and could probably explain the present results (TOMASCHKO 1999).

Moreover, recent studies showed that 20E inhibits larval growth in Drosophila by promoting the nuclear localization of dFOXO (COLOMBANI ET AL. 2005) and therefore having antagonistic effects to insulin-mediated growth stimulation.

This antagonistic effect could not be demonstrated in the present results. The presence of insulin does not influence the arrest of proliferation in the S2/H cells. Obviously these antagonistic effects of the two signaling pathways cannot be monitored with the used system. As the S2/H cells are likely derived from imaginal disc cells, they are probably more sensitive towards high concentrations of 20E inducing cell differentiation compared to the growth-promoting effects of insulin.

5.4.3 Interaction with Toll signaling in S2/H cells

Originally, in Drosophila, relish and dorsal are located predominantly in the cytoplasm (STÖVEN ET AL. 2000, WHALEN AND STEWARD 1993). After stimulation by exogenous factors, these proteins translocate to the nucleus, where they can activate gene transcription.

In the present study however, dorsal was found to be located exclusively in the nucleus already under normal culture conditions. One possible explanation could be that in the used S2/H cell line, signaling via dorsal is already activated. Another reason for this contradictory finding could derive from the molecular mechanism behind the Toll pathway (Fig. 16) and thus the localization of dorsal. This mechanism of activation differs
between the two studied proteins dorsal and relish. Unlike dorsal, which translocates to the nucleus after stimulation, relish does not translocate as a full-length protein to the nucleus, but is cleaved from 110 kD into two fragments (49 kD and 68 kD) from which only the 68 kD one enters the nucleus and activates transcription (Støven et al. 2000).

In the present work however, it is not clear whether really all components of this pathway are expressed in sufficient amounts, if they are expressed at all. Therefore, the lack of cactus or pelle for example could result in the strictly nuclear localization of dorsal.

Also the protein relish was reported to be predominantly cytoplasmatic without any stimulation (Støven et al. 2000). After LPS treatment of *Drosophila* mbn-2 cells, full-length relish was shown to be cleaved into two smaller fragments from which only one was able to enter the nucleus (Støven et al. 2000). These results could not be found in the present data, as full-length relish was found to be located in the nucleus. One explanation for this different finding could arise from the origin of the mbn-2 cells as they are a haemocyte-like cell line (Gateff 1978). In *Drosophila*, haemocytes play an important role in host defense and are thus very sensitive for bacterial factors like LPS (Johansson et al. 2006), whereas S2 cells are more involved in ecdysone-dependent cell differentiation. This fact might explain the different response to LPS as relish is more involved in the regulation of the immune response and is therefore probably not completely functional in the differentiating S2/H cells.

However, the present data show that the expression of relish and dorsal is stimulated slightly by the presence of the EcR isoforms.

Previous studies on hormonal control of the innate immune response in *Drosophila* cells showed a high stimulatory effect of 20E on the transcription of several antimicrobial peptides (AMP) like Diptericin for example via the Toll pathway (Flatt et al. 2008), which was proofed *in vivo* and *in vitro* by reporter gene assays.

In the reporter assay used here, such stimulating effects on Toll signaling cannot be seen clearly. As reported by Pan and Corey (1992), DNA binding of dorsal was extremely high with nearly the same response element (GGGACCTTCC) in *Drosophila* cells, confirming the functionality of the reporter gene construct. One possible explanation could be the normalization of luciferase values on the dorsal concentration, which was not done in the study by Flatt et al. (2008). This explanation is supported by the fact that in the presence of the EcR isoforms, the expression of dorsal is slightly increased. Moreover, as dorsal is already located in the nucleus, one could expect that probably the basal level
of transcriptional activity is already rather high and that very pronounced stimulatory effects can thus not be achieved anymore.

### 5.4.4 Interaction with NF-κB signaling in CHO-K1 cells

Interactions between steroid hormone receptors and NF-κB signaling have been known already since a long time. Especially studies on the glucocorticoid receptor (GR) are of major interest due to the fact that glucocorticoids act as potent suppressors of immune and inflammatory responses. However, only little is known about the molecular mechanism behind this repression.

The data presented here demonstrate that also the EcR/Usp complex is able to interact with NF-κB signaling as localization of p65 is changed in an isoform-specific manner. Moreover, this change in localization seems to be independent of Usp. As the isoforms differ only in their A/B domain, it is likely that interaction with p65 is mediated via this part of the molecule. However, up to now, no direct interaction was determined for technical reasons. Recent studies revealed a direct interaction between GR and NF-κB, resulting in a mutual repression of transcriptional activity (MCKAY AND CIDLOWSKI 2009).

For AR mutual effects concerning transcriptional activity were described, which were independent of a change in the expression pattern of p65 (PALVIMO ET AL. 1996). In the case of ER, transcriptional activity of NF-κB is repressed, however, these effects are not mutual (QUAEDACKERS ET AL. 2007). Concerning transcriptional activity, the data here again show a clear isoform-specificity as only in the presence of EcR-A an increase can be seen. Interestingly, only for this isoform no change in the localization of p65 could be determined. Moreover, all experiments indicated that the described interaction is independent of Usp. The fact that also hormone does not seem to have any influence supports the thesis that the cross-talk between both pathways is mediated only via EcR. Additionally the results show that in the absence of hormone EcR/Usp cannot only repress transcription, as was shown for the hsp27 response element but transcription can also be enhanced.

For the interaction between steroid hormone signaling and the NF-κB pathway or the Toll pathway respectively, two major scenarios are possible. First, steroid receptor monomers or dimers can impair the transcriptional potency by binding directly to the NF-κB complex as has already been shown for the ER (QUAEDACKERS ET AL. 2007), the GR (RAY ET AL. 1994), and the PR (KALKHOVEN ET AL. 1996). Second, also the competition of the two transcription factors for the same DNA binding site is possible. In the case of the
GR this second possibility seems to play a major role (Novac et al. 2006, Luecke et al. 2005). Additionally to these two scenarios, also the interplay between steroid hormone receptors, NF-κB/Toll signaling and other, more common cofactors might be a potential mechanism for cross-talk.

The data of the present work clearly demonstrate that one major point in the modulation the ecdysone response is given by the presence of the EcR isoforms. Although the isoforms differ only in their N-terminal part, each of them exhibits its own behavior in hormone induction, the ability to repress transcriptional activity, compensation of the mutations UspΔDBD and K497E, DNA binding and in the cross-talk with other signaling pathways.

Moreover, the comparison with heterologously expressed EcR/Usp in vertebrate cells reveals that the cellular background influences various factors of ecdysone signaling like the localization of Usp, DNA binding of EcR/Usp and the transcriptional activity of wild type EcR as well as EcR^{K497E}.

Finally, the diverse actions of the three EcR isoforms in Drosophila melanogaster offer the possibility for a wide range of modifications of the ecdysone response.
6. Abbreviations

Aa amino acid
AF-1 activation function 1
AF-2 activation function 2
AMP antimicrobial peptides
APS ammonium persulfate
AR androgen receptor
ATP adenosine triphosphate
BrC Broad Complex
BrC-Z1 Broad Complex isoform Z1
BSA bovine serum albumine
CFP cyan fluorescent protein
CHO-K1 Chinese hamster ovary cells
DBD DNA binding domain
dFoxy Drosophila forkhead transcription factor
dif dorsal-related immunity factor
DILP Drosophila insulin-like peptide
dInR Drosophila Insulin receptor
dl dorsal
Dm Drosophila melanogaster
D-MEM Dulbecco’s modified eagle medium
DMSO dimethylsulfoxide
DNA deoxyribonucleic acid
DTT dithiothreitol
E74 A ecdysone-inducible protein 74 A
20E 20-hydroxyecdysone
EcR ecdysteroid receptor
EcRE ecdysteroid response element
EDTA ethylenedinitrilotetraacetic acid
EGTA ethyleneglycoltetraacetic acid
ER estrogen receptor
FCS fetal calf serum
FXR farnesoid X receptor
GR glucocorticoid receptor
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HDAC</td>
<td>histone deactelyase</td>
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<tr>
<td>HRE</td>
<td>hormone response element</td>
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<tr>
<td>hsp27</td>
<td>heat-shock protein 27</td>
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<tr>
<td>IKK</td>
<td>IkB kinase</td>
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<tr>
<td>IRAK</td>
<td>IL-1 receptor associated kinase</td>
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<tr>
<td>IkB</td>
<td>Inhibitor of κB</td>
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<tr>
<td>JH</td>
<td>juvenile hormone</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>LBD</td>
<td>ligand binding domain</td>
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<tr>
<td>MET</td>
<td>methoprene tolerant</td>
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<tr>
<td>N-CoR</td>
<td>nuclear receptor corepressor</td>
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<td>NES</td>
<td>nuclear export signal</td>
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<td>NF-κB</td>
<td>nuclear factor κB</td>
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<td>NLS</td>
<td>nuclear localization signal</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PR</td>
<td>progesterone receptor</td>
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<td>RAR</td>
<td>retinoic acid receptor</td>
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<td>rel</td>
<td>relish</td>
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<td>RHD</td>
<td>Rel-homology domain</td>
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<tr>
<td>rp49</td>
<td>ribosomal protein 49</td>
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<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>S2</td>
<td><em>Drosophila melanogaster</em> Schneider cell line 2</td>
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<tr>
<td>S2/H</td>
<td><em>Drosophila melanogaster</em> Schneider cell line, subtype H</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SMRT</td>
<td>silencing mediator of retinoic acid and thyroid hormone receptor</td>
</tr>
<tr>
<td>SMRTER</td>
<td>SMRT-related ecdysone receptor-interacting factor</td>
</tr>
<tr>
<td>TAD</td>
<td>transactivation domain</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Di-(dimethylamino)ethane</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<td>TR</td>
<td>thyroid receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>Usp</td>
<td>ultraspiracle</td>
</tr>
<tr>
<td>UspDBD</td>
<td>DNA binding domain of Usp</td>
</tr>
<tr>
<td>VDR</td>
<td>vitamin D receptor</td>
</tr>
<tr>
<td>VP16\textsubscript{AD}</td>
<td>transactivation domain of herpes virus simplex</td>
</tr>
<tr>
<td>WB</td>
<td>Western blotting</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
</tr>
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</table>
7. References


**References**


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8. Erklärung

Ich versichere hiermit, dass ich die vorliegende Arbeit selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt sowie wörtlich oder inhaltlich übernommene Textpassagen als solche gekennzeichnet habe.

Céline Hönl

Ulm, den……………………
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