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**Interaction of the N-terminus of ecdysone receptor isoforms with the ligand
binding domain**

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Abbreviated title: N/C interaction of EcR isoforms

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Abstract

Ecdysone receptor (EcR) isoforms exert different biological functions, although they vary only in their N-terminal domain. Despite identical C-termini, which mediate hormone-induced activity, the influence of ligand is isoform specific, which indicates an N/C-interaction. The position of helix 12 with and without hormone varies among isoforms and modifies N/C–interaction determined by fluorescence resonance-energy transfer (FRET), which depends on the salt bridge between helices 4 and 12 of the ligand-binding domain (LBD). Disruption of the salt bridge by mutation of K497 (helix 4) had no effect on basal N/C interaction, but prevented the hormone-induced increase, which was partially restored by a salt bridge with reversed polarity. The heterodimerization partner Ultraspiracle (Usp) can compensate for the disruption of the salt bridge. Without ligand the AB-domains of EcR-A and EcR-B1, but not EcR-B2, interact with the LBD via K497 and repress transcriptional activity. This intramolecular cross talk between N- and C-terminus along with the position of helix 12 stabilized by K497 regulates transcriptional activity of EcR isoforms.

Keywords: Corepressor, *Drosophila*, N/C-interaction, FRET, insect, nuclear receptor.

Abbreviations used

Aa: amino acid, AF: activation function, AR: androgen receptor, CFP: cyan fluorescent protein, DBD: DNA binding domain, EcR: ecdysone receptor, EcRE: ecdysone response element, ER: estrogen receptor, FRET: fluorescence resonance energy transfer, LBD: ligand binding domain, NTD: N-terminal domain, PPAR: Peroxisome proliferator – activated receptor PR: progesterone receptor, RAR: Retinoic acid receptor, RXR: retinoid X receptor, Usp: Ultraspiracle, VP16: herpes simplex transcriptional activator protein, wt: wild type, YFP: yellow fluorescent protein.

1. Introduction

The functional ecdysone receptor, which mediates the signal transduction of moulting hormones of arthropods, is generally considered as a heterodimer of ecdysteroid receptor and Ultraspiracle, the invertebrate orthologue of RXR (Yao et al., 1993) although there are several indications that EcR (Costantino et al., 2008) and Usp (Jones and Sharp, 1997; Spindler et al., 2009) also exert separate functions in the absence of the heterodimerization partner.

In *Drosophila melanogaster* three isoforms of EcR are known, which differ in length and sequence of their N-terminal AB - domain (Talbot et al., 1993). The isoforms EcR-A, EcR-B1, and EcR-B2 exert different functions during development and cannot substitute for each other (Bender et al., 1997; Schubiger et al., 2003; Davis et al., 2005). EcR - A is predominantly expressed in cells that proliferate and differentiate during metamorphosis, while EcR-B1 and EcR-B2 are present in larval cells, which are eliminated by apoptosis during metamorphosis. In *Drosophila melanogaster*, only one isoform of the heterodimerization partner Usp is found, widely expressed during development and involved in the activation of ecdysone-dependent genes (Hall and Thummel, 1998).

As expected, basal transcriptional activity of EcR isoforms varies (Dela Cruz et al., 2000; Mouillet et al., 2001; Hu et al., 2003; Beatty et al., 2006; Ruff et al., 2009) due to the different AF-1 sequences present in the AB-domains of EcR isoforms.

Although AF-2, located in helix 12 of the ligand- binding domain (Hu et al., 2003), is identical for all EcR isoforms, hormone-induced transcriptional activity also varies considerably among EcR isoforms expressed in vertebrate and in insect cells (Spindler et al., 2009). Therefore, we speculated that the NTD of EcR may interact

with AF-2 to regulate the transcriptional capability of the receptor complex as proven previously for several vertebrate nuclear receptors, e.g. AR (Doesburg et al., 1997; Schaufele et al., 2005), ER (Cvoro et al., 2007) and PR (Tetel et al., 1999), either by determination of transcriptional activity (Gianni et al., 2003) or by direct measurements of fluorescence resonance energy transfer (FRET) of receptor proteins tagged with fluorescent receptor fusion proteins (Grant et al., 2001; van Royen et al., 2009).

The amino acid lysine in helix 4 (K497) of the ligand-binding domain of EcR plays an essential role for the function of the ecdysone receptor and is involved in the formation of the salt bridge between helix 4 and helix 12 (Bourget et al., 2000; Renaud and Moras, 2000) and in ligand binding (Grebe et al., 2003). Since mutation of K497 has more severe effects on transcriptional activity compared with mutation of the partner amino acid of the salt bridge (E648 in helix 12), we assumed that an additional function is associated with lysine 497. We therefore studied the functional role of K497 in more detail. In order to separate the impact of the salt bridge from other receptor functions mediated by K497, we mutated not only K497 but also the partner of the salt bridge located in helix 12 E648. This allowed the formation of a salt bridge, although with opposite polarity, whereas other functions associated with K497 were still disrupted.

In our experiments, we mainly used vertebrate cell lines to study the functionality of the receptor complexes, ensuring that the test system was devoid of endogenous EcR and Usp interfering with the function of heterologously expressed nuclear receptor proteins (Beatty et al., 2006; Henrich et al., 2009). The subclone of CHO cells used in our experiments contained no detectable amounts of RXR (Nieva et

al.,2008; Azoitei et al., 2009), which ruled out that this vertebrate receptor may functionally have replaced Usp (Thomas et al., 1993). We used fluorescence resonance energy transfer (FRET) technique, which allows monitoring of intramolecular interactions and conformational changes that are crucial for receptor - mediated signal transduction with non-invasive techniques, while preserving the cellular protein network, compartmentalization and spatial cell arrangement (Eidne et al., 2002).

2. Material and methods

2.1. Plasmids:

Plasmids coding for wild type and mutated isoforms of EcR tagged with CFP at the N-terminus and with YFP at the C-terminus (Fig.1) were obtained by subcloning the sequences coding for EcR isoforms lacking the F-domain (aa 654–878 of EcR-B1) into the pECFP-C1 vector (Clontech, Mountain View, USA).

To this end, the open reading frame of DNA sequences coding for the EcR isoforms were amplified by PCR from the plasmids pEYFP-EcR-A, pEYFP-EcR-B1 and pEYFP-EcR-B2. If necessary, restriction sites (*Hind*III, *Bam*HI) were introduced by PCR. The following forward primers were used (*Hind*III restriction site underlined): 5'-CGG CAT GGA CGA GCT GTA CAA G-3' (EcR-A, EcR-B1, EcR-B2, CFP-EcR-A); 5'-GGA AAGCTT CG ACC TTA CCT AGC-3' (EcR-AΔ1-14aa); 5'-GGA AAGCTT CT GCA GGC CCA TC-3' (EcR-AΔ1-29aa); 5'-GAG AAGCTT ATG TTG ACG ACG AGT GG-3' (EcR-A-YFP). Reverse primers (*Bam*HI restriction site underlined): 5'-GAG GGA TCC AAC GTC CCA GAT C-3' (all constructs except CFP-EcR-A); 5'-GAG GGATCC TTA AAC GTC CCA GAT CTC-3' (CFP-EcR-A, termination codon italicized).

After double digestion, the PCR products were ligated into the *Hind*III and *Bam*HI restriction sites of pECFP-C1 using T4 ligase (Promega, Madison, USA). The double-tagged constructs were obtained by cloning the YFP sequence (taken from the pEYFP-N1 vector; Clontech, Mountain View, USA) into the *Bam*HI and *Xba*I restriction sites. For EcR-A-YFP, the PCR product was cloned into the *Hind*III and *Bam*HI sites of the pEYFP-N1 vector. Additional base pairs between the *Bam*HI

restriction site and ATG of the YFP sequence were deleted with QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) according to the manufacturer's instructions using a forward primer 5'-GGG ACG TT GGATCC ATG GTG AGC AAG GGC-3' and its reverse complement.

Point mutations K497E and E648K were introduced using QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's instructions. The following primers and their reverse complements were used (nucleotides introducing the mutation are underlined):

EcR-K497E: 5'-CAG ATC ACG TTA CTA GAG GCC TGC TCG TCG G-3'

EcR-E648K: 5'-G CCC AAG TTC CTC GAG AAG ATC TGG GAC GTT C-3'

In addition, the following plasmids were used for transfection studies: wild type Usp, Usp Δ DBD (deletion of amino acids 104–169 encoding DBD of Usp), pVP16-Usp I (encoding aa 98–507 of Usp, which includes the last six amino acids of the N-terminal AB-domain of wild type Usp), and pVP16-Usp III (aa 170–507 of wild type Usp encoding the DE-domains (Beatty et al., 2006). The integrity of all plasmids was confirmed by DNA sequencing (GATC, Konstanz, Germany).

Transcriptional activity was evaluated with the reporter plasmid pEcRE-tk-Luc carrying tandemly repeated copies of the hsp27 ecdysone response element attached to the minimal constitutive thymidine kinase promoter and a luciferase reporter gene as described previously in detail (Beatty et al., 2006). A cotransfected pGL4.74 [hRluc/TK] plasmid (Promega, Madison, USA) containing the *Renilla luciferase* gene was used for normalization of transcriptional activity on transfection efficiency.

2.2. Cell culture and transfection:

CHO-K1 cells were grown in DMEM/F12 medium (Gibco / Invitrogen, Carlsbad, USA) supplemented with 5% of fetal bovine serum (Sigma, Taufkirchen, Germany) under 5% CO₂. For luciferase reporter assay, cells were seeded in six-well plates (Nunc, Wiesbaden, Germany) with 4×10^5 cells per well. After 24 hours cells were transfected with nanofectin (PAA, Pasching, Austria) according to the manufacturer's instructions. Each well received 3 µg of plasmid DNA (0.75 µg of pEcRE-tk-Luc, 0.25 µg of pGL4.74 [hRluc/TK], and 1 µg of plasmids expressing EcR and Usp each; in case of experiments without Usp 2 µg of the plasmid expressing EcR). After 4 hours of incubation the medium containing the transfection mix was replaced by fresh culture medium. One hour later muristerone A (Sigma, Steinheim, Germany) was added (final concentration 1 µM). 24 hours after transfection cells were lysed by shaking in 1x passive lysis buffer (PLB 5x, Promega, Madison, USA; 100 µl per well). To improve homogenization the suspension was sucked several times through a thin needle (0.4 × 20 mm, Terumo, Leuven, Belgium).

For ligand binding assay, about 1.5×10^6 cells were seeded in 10-cm cell culture dishes (Greiner Bio-One, Frickenhausen, Germany) and transfected with nanofectin after 24 hours. Each 10-cm plate received 7 µg of total plasmid DNA. 24 hours after transfection, proteins were extracted from the cells as described previously (Azoitei and Spindler-Barth, 2009).

Human embryonic kidney (HEK) 293 cells (ATCC, Manassas, VA, USA) were maintained at 37°C under 5% CO₂ in minimal essential medium with Earle's salts supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin. Drosophila S2 cells were grown at 25 °C in Schneiders Drosophila medium (Gibco/Invitrogen, Carlsbad, USA) supplemented with 10 % fetal bovine serum. Cells

were transiently transfected using Fugene HD transfection reagent (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's instructions. For FRET analysis, cells were seeded on glass coverslips and transfected with 2 µg of total plasmid DNA (1 µg of dually tagged EcR CFP/YFP-tagged EcR and 1 µg Usp construct, In case of experiments without Usp 2 µg of dually tagged EcR construct). The ratio of DNA coding for CFP-EcR-A and EcR-A-YFP used for transfection was 1:3.

Luciferase reporter activities were assayed with the Dual Luciferase Reporter Assay System (Promega, Madison, USA) according to the manufacturer's instructions to compensate differences in transfection efficiency. Transcriptional activities were additionally normalized on receptor concentration determined by quantification of specific Western blot signals to eliminate variations in receptor protein content of EcR isoforms due to differences in the stability of receptor proteins, which is mainly determined by the AB-domain of nuclear receptors.

2.3. Western blotting and quantification of receptor content:

Cell extracts (25 µg protein/lane) were separated on SDS gels (Lämmli et al., 1970). Gels were electroblotted on nitrocellulose membranes (BA 85, 45 µm pore size, Schleicher and Schuell, Dassel, Germany). Membranes were stained with Ponceau to check transblotting efficiency. Membranes were soaked in blocking buffer (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween 20, pH 7.6, 0.02% Thimerosal) containing 3% milk powder (low fat, < 1 %) and 1 % bovine serum albumine (Sigma, Steinheim, Germany) for 1 hour and then probed over night with a monoclonal first antibody diluted in the same buffer (anti-GFP, Clontech, Heidelberg, Germany, diluted 1:500, or anti-EcR, DDA2.7, diluted 1:1000, respectively). For detection, a peroxidase-

conjugated secondary antibody diluted in TBS-T (20 mM Tris-HCl, 137 mM NaCl, 0.1 % Tween 20, pH 7.6) was used (anti-rabbit IgG, # A-6667, Sigma, Deisenhofen, Germany, diluted 1:500, or anti-mouse IgG, # A-5906, Sigma, Deisenhofen, Germany, diluted 1:3000, respectively). Signals were visualized with SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, USA). Specific bands were detected by the Chemi-Smart 5000 photo documentation system (Vilber Lourmat, Eberhardzell, Germany) and quantified relative to a standard probe using software Bio-1D (Vilber Lourmat, Eberhardzell, Germany). The linearity of the intensity of specific receptor bands was confirmed with a calibration curve as described previously (Braun et al., 2009).

2.4. Ligand binding assay:

CHO cells were transfected as described above and cell extracts prepared as described previously (Azoitei and Spindler-Barth, 2009). Ligand-binding was determined with $^3\text{[H]}$ -Ponasterone A (specific activity 7.9 TBq/mmol) using a filter assay (Turberg and Spindler, 1992). Briefly, cell extracts were incubated routinely with $^3\text{[H]}$ -Ponasterone for 5 hrs at 4°C. Free ligand was removed by filtration using NC45 nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and washed with 3 x 4 ml ice-cold washing buffer (20 mM HEPES, 20 mM NaCl, 10 % glycerol (v/v), 1 mM EDTA, 1 mM β -mercaptoethanol, pH = 7.9). Bound radioactivity was measured with a liquid scintillation counter (Tri - Carb 1500, Perkin Elmer, Rodgau-Jügesheim, Germany) and the non-specific binding, determined by competition with 1 μM 20-OH-Ecdysone, subtracted. As additional control extracts from non - transfected cells were used (< 100 cpm). The purity of $^3\text{[H]}$ -Ponasterone A was routinely checked by HPLC analysis prior to use.

2.5. FRET measurements:

HEK 293 cells were grown on glass coverslips for 48 h. Coverslips were then mounted in a custom-made chamber and covered with incubation buffer (138 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5.5 mM glucose, 2 mg/ml bovine serum albumin, and 10 mM Hepes, pH 7.5). FRET analysis was performed with an inverted microscope Axiovert 100 equipped with a Plan-Apochromat x63/1.4 objective (Carl Zeiss, Göttingen, Germany). CFP and YFP were alternately excited at 410 and 515 nm with a monochromator (Polychrome II; TILL Photonics, Gräfelfing, Germany) in combination with a dual reflectivity dichroic mirror (< 460 and 500–520 nm; Chroma Technology, Rockingham, VT, USA). Emitted light was filtered through 460–500 (CFP) or 535–580 nm (YFP) band pass filters changed by a motorized filter wheel (Lambda 10-2; Sutter Instruments, Novato, USA), and detected with a cooled CCD camera (Imago; TILL Photonics, Gräfelfing, Germany). For FRET analysis, CFP (40 ms) and YFP (8 ms) emission without acceptor bleach were recorded during 20 cycles. Then, 60 cycles were recorded with an additional 2-s illumination per cycle at 512 nm to bleach YFP. FRET was assessed as recovery of CFP (donor) fluorescence during YFP (acceptor) bleach. FRET efficiencies were obtained as described by Voigt et al. (2005), which allows to calculate the distance between donor and acceptor according to Förster's theory (Evers et al., 2006; Patterson et al., 2000). In brief, the FRET efficiency E is expressed as $E = 1 - (F_{DA}/F_D)$, where F_{DA} and F_D are intensities of the donor fluorescence in the presence and absence of the FRET acceptor, respectively. The increase in CFP fluorescence during acceptor bleach was plotted against the remaining YFP fluorescence, yielding a regression line that served to extrapolate F_{DA} and F_D . The data of each bleaching experiment were averaged over two to six single cells.

Means and SD were computed from three to five independent transfections each comprising at least three bleaching experiments.

2.6. Statistical evaluation:

Statistical significance of differences among groups was determined on the basis of a two-sided, matched pair Student's t-test. Förster's distances calculated from FRET efficiencies according to Evers et al. (2006) and Patterson et al., (2000) reveal that significant differences in FRET efficiency correspond to a differences in the distance between donor and acceptor of 4.5-14 Å.

3. Results

3.1. Functional properties of EcR isoforms labelled with CFP/YFP

The concentration of tagged receptor proteins is even increased especially for EcR - A and EcR - B2 compared with non-tagged receptors (Fig. 2). No degradation products of EcR isoforms tagged with fluorescent proteins CFP at the N-terminus and YFP at the C - terminus can be detected on Western blots, which rules out the possibility that interaction of receptor proteins with degraded fragments might cause increased FRET signals. The concentration of RXR, which may heterodimerize with EcR in the absence of Usp (Thomas et al., 1993)) in the CHO-K1 subline and HEK cells used for our experiments is below detection limit and does not cause any measurable physiological effect (Nieva et al., 2008). This implies that the proximity of N – and C - terminus of EcR isoforms can be studied in the absence of a heterodimerization partner. Basal transcriptional activity of wild type (Table 1) and mutated EcR isoforms (data not shown) is still observed, although considerably reduced compared with non-tagged receptors, which is in accordance with data reported from vertebrate receptors (Schaufele et al., 2005). Induction of transcriptional activity by muristerone A (Table 1) is in the same range as previously observed for EcR isoforms (Ruff et al., 2009), although the affinity of the ligand ponasterone A is considerably impaired ($K_D = 48.8$ nM for CFP-EcR-A-YFP/wt - Usp compared to $K_D = 7.0$ nM for EcR-A/wt – Usp) (Azoitei and Spindler-Barth., 2009).

3.2. FRET efficiency of dually tagged EcR varies for different isoforms.

FRET efficiencies of dually tagged EcR isoforms vary depending on the AB-domain of EcR (Table 2). Basal FRET efficiency is low for EcR-A and EcR-B1. In the presence of the ligand muristerone A FRET signalling increases with EcR - A, but not

EcR – B1. The hormone - induced increase depends on the presence of aa 15-29 of EcR - A, a peptide with the sequence STLP SHILLQQQLAA, as was shown by deletion experiments. In the presence of the hormone, FRET signals do not increase following deletion of aa 1-29 (Table 2) or truncation of the complete isoform- specific AB-domain of EcR-A (data not shown), whereas deletion of aa 1-14 results in FRET signals comparable to full length EcR- A. In contrast, FRET efficiency of EcR-B2 is already high in the absence of the hormone and is not further stimulated in the presence of muristerone A.

Control experiments with cotransfected CFP-EcR-A and EcR-A-YFP resulted in rather low FRET signals (< 2 %), indicating that the FRET signals in dually tagged EcR isoforms predominantly represent intramolecular and not intermolecular interaction of CFP- and YFP-tagged receptor proteins. Time course experiments revealed that heterodimerization and ligand binding take about 10-15 min and maximal FRET signals can be observed after this incubation time (data not shown).

3.3. Influence of Usp on FRET efficiencies of EcR isoforms

The heterodimerization partner Usp slightly increases basal and significantly stimulates hormone-induced FRET efficiency of EcR-A (Table 3). Enhanced constitutive FRET efficiency is observed only with wild type Usp. Deletion of the DNA-binding domain (DBD) of Usp diminishes the hormone-induced increase in FRET efficiency compared with the wild type heterodimer, but does not alter basal FRET. In contrast to EcR - A, FRET efficiencies of both EcR - B isoforms are not increased by Usp (EcR - B1/wt - Usp: basal FRET efficiency = 5.4 % ± 1.2 %, with hormone = 6.1 % ± 1.7 %; EcR - B2/wt - Usp: basal = 9.0 % ± 0.4 %, with hormone = 6.8 % ± 0.4 %).

To rule out, that cell- type specific conditions may influence FRET efficiencies of EcR, FRET studies were also performed with transfected *Drosophila* S2 cells, which abundantly express Usp, but no EcR (Spindler et al., 2009). Neither in the absence nor in the presence of muristerone A were significant differences in FRET efficiencies for EcR-A/wt-Usp detected between vertebrate cells (Table 3) and insect S2 cells (basal FRET efficiency: 7.0 % $\pm$ 1.2 %, with hormone: 11.1 % $\pm$ 1.5 %).

3.4. Influence of the salt bridge in the ligand-binding domain of EcR on FRET efficiency

Ligand binding of nuclear receptors is often accompanied by a conformational change of the ligand-binding domain (Bourget et al., 2000; Renaud and Moras, 2000). The altered position of helix 12 in the holo - receptor is stabilized by formation of a salt bridge between helix 4 and helix 12 (Fig. 3). If the salt bridge is disrupted either by mutation of lysine (K) at position 497 in helix 4 to glutaminic acid (E) or E at position 648 in helix 12 to K, basal FRET efficiency is not altered in EcR - A as expected, but the hormone -induced increase is abolished (Table 4). The affinity of the ligand itself is not reduced by disruption of the salt bridge (EcR - A^{K497E}/wt - Usp: K_D = 23.4 nM; EcR - A^{E648K}/wt - Usp: K_D = 19.5 nM) compared with the non-mutated construct (K_D = 48.8 nM, see above).

Heterodimers of EcR - A^{K497E} and wt - Usp exhibit increased FRET signals in the presence of the hormone, comparable with the corresponding wild type receptor complex (Table 4). In case of EcR^{E648K}, Usp is unable compensate the influence of the mutation in helix 12. Formation of a salt bridge with reversed polarity using

liganded EcR^{K497E/E648K}/wt - Usp (Table 4) only slightly increased FRET efficiency. With both EcR-B isoforms, neither point mutation showed an effect on FRET efficiency (data not shown).

3.5. Mutation of lysine in helix 4 enhances transcriptional activity of EcR - A and EcR-B1.

Besides participation in the salt bridge between helix 4 and helix 12 the amino acid, which corresponds to K497 in EcR is known to be a conserved corepressor binding site of nuclear receptors (Lempiäinen et al., 2005). In fact, basal transcriptional activity of EcR-A^{K497E} (36-fold) and EcR-B1^{K497E} (4-fold) is enhanced compared with the corresponding wild type EcR isoforms, whereas no further increase is observed with EcR-B2^{K497E} (Fig. 4). As shown for mutated EcR-A, Usp modulates the extent of basal transcriptional activity, but does not prevent the hormone-induced increase in transcriptional activity of the mutated receptor complexes (Fig. 5). No gain-of-function is observed in the presence of the hormone (data not shown). The AB - domain of Usp enhances basal activity of receptor complexes with wild type EcR-A, but reduces the increase of transcriptional activity caused by the exchange of K497 to E (5-fold). Exchange of the original AB - domain of Usp by the activation domain of VP16 (Usp I) increases basal activity in wild type and mutated receptor complexes (7-fold), whereas deletion of the DBD (Usp III) impairs basal transcriptional activity already in receptor complexes with wild type EcR-A, but has no major influence on fold increase (5-fold) of constitutive transcriptional activity of the mutated EcR.

4. Discussion

4.1. The AB-domain of EcR modulates constitutive and hormone - induced N/C interaction in an isoform - specific manner.

Full transcriptional activation by steroid hormone receptors requires functional synergy between the ligand-independent transcriptional activation domain AF-1 located in the amino-terminal region and the ligand-dependent transcriptional activation domain AF-2 at the C - terminus. As a mechanism for achieving this functional synergy, physical intramolecular association between N- and C-terminal domains of the receptor has been demonstrated in vertebrate nuclear receptors (Tetel et al., 1999).

According to analysis of the crystal structure, ligand binding induces a conformational change of the ligand-binding domain upon hormone binding (Renaud and Moras, 2000). The most obvious change is the repositioning of the C - terminal helix 12, which protrudes from the LBD core in the apo - receptor and folds back towards the LBD in the liganded holo - receptor. This conformation is stabilized by the formation of a salt bridge between helix 4 and helix 12 (Renaud et al., 1995), which is of functional relevance for transcriptional activity of EcR as shown by mutational analysis (Grebe et al., 2003; Bergman et al., 2004; Grebe et al., 2004).

The movement of helix 12 results in an increased FRET signal of the liganded holo - receptor, indicating a stabilized interaction of N - and C - terminus as described for vertebrate nuclear receptors like AR (Schaufele et al., 2005) and ER (Cvoro et al., 2007; Paruthiyil et al., 2009). This hormone - dependent increase in N/C interaction

was functionally confirmed by mammalian two - hybrid assays and by direct protein-protein interaction assays *in vitro* (Tetel et al., 1999).

The results obtained with EcR-A are in agreement with these previous reports and demonstrate that the repositioning of helix 12 in the liganded EcR-A also occurs without Usp, despite the considerably reduced hormone-binding capability in the absence of a heterodimerization partner (Azoitei and Spindler-Barth, 2009). However, in case of EcR-B1, FRET signals remained low even in the presence of the ligand. We cannot exclude that in EcR-B1 the distance between N- and C-terminus and/or the 3D-conformation may prevent an increase in FRET coupling efficiency (Koterba and Rowan, 2006; Paruthiyil et al., 2009). It is also possible that the enhanced intramolecular FRET efficiency of EcR - B2, which is already observed in the absence of the ligand may simply reflect the reduced distance between the shorter NTD and the LBD of EcR-B2 compared with EcR-A and EcR-B1. Conversely, these different FRET levels may represent a distinct conformation of the apo - receptor, where the position of helix 12 is more similar to the liganded holo - receptor as described for RAR β and RAR γ in contrast to RAR α (Farboud et al., 2003). We have to keep in mind that there exists a considerable degree of dynamic variability in the position of helix 12 among receptor isoforms. Receptor activation may not necessarily require repositioning of helix 12 as shown for PPAR γ (Uppenberg et al., 1998). Tanenbaum et al. (1998) report that helix 12, which is not essential for ligand binding and dimerization of ER α , is still in the apo-position even in the liganded holo - receptor.

Isoform-specific variations of interactions between the LBD and the NTD contribute to altered functionality of EcR, as reported previously for vertebrate receptors, e.g. for

PR (Tetel et al., 1999). Different basal transcriptional activities of EcR isoforms were reported repeatedly (Dela Cruz et al., 2000; Hu et al., 2003, Mouillet et al., 2001, Beatty et al., 2006; Ruff et al., 2009). The low basal transcriptional activity of EcR-A into transfected in mammalian cells (Mouillet et al., 2001) and insect cells (Hu et al., 2003) was consequently attributed to a repressive function in the AB domain of EcR-A in contrast to the activating functions of EcR-B isoforms, although the molecular mechanism for the selective repression by EcR-A was still unknown.

4.2. FRET efficiency and transcriptional activity are not correlated

Transcriptional activity and N/C interaction of EcR isoforms as determined by FRET do not change in parallel as was described previously for several vertebrate receptors, e.g. AR (Schaufele et al., 2005). This is most obvious for EcR - A, which exhibits rather low constitutive and hormone-induced transcriptional activity. In contrast, basal transcriptional activity of EcR-B1 is considerably higher (Ruff et al., 2009) despite low FRET efficiency, which remains low even in the presence of the ligand. In addition to the conformational change of helix 12 associated with ligand binding (Bourget et al., 2000), N/C interaction and transcriptional activities of nuclear receptors are modulated by many factors e.g. by heterodimerization and interaction with comodulators. Apart from direct association of N- and C-terminus, N/C interaction also can be mediated indirectly by comodulator interaction as suggested previously (Ikonen et al., 1997; Need et al., 2009). Isoform - specific comodulator recruitment is reported for vertebrate receptors like ER indicating that comodulator binding is modified by the AB - domain of nuclear receptors (Bai and Giguère, 2003). Other receptor functions like DNA binding may be also not correlated with transcriptional activity (Beatty et al., 2006; Braun et al., 2009) underlining the complex interplay of various factors regulating transcriptional activity.

4.3. Influence of the heterodimerization partner on FRET efficiency and transcriptional activity

The heterodimerization partner Usp modifies FRET efficiency and transcriptional activity. Whereas FRET signalling is mainly influenced by the ligand-binding domain of Usp, transcriptional activity of the heterodimer is modulated not only by the AB-domain of EcR, as outlined already by Dela Cruz et al., (2000), but also by the N-terminus of Usp (Ruff et al., 2009). Cross-talk of nuclear receptors mediated by the N-terminal AF-1 domain has been reported for vertebrate receptors like ER α and RAR β ; e.g., the N-terminal region of ER α modulates the transcriptional activity of RAR β . In addition, the AB - domain of the heterodimerization partner RAR modifies N/C interaction of ER (Rousseau et al., 2003). N- and C-terminus of both dimerization partners may also be involved in comodulator binding. According to Zamir et al. (1997) receptor dimers interact with comodulators and the C - termini of both receptors are required; e.g. heterodimerization with RXR provides the interaction surface necessary for corepressor recruitment of RAR/RXR heterodimers in the non-liganded state.

4.4. Impact of the salt bridge between helix 4 and helix 12 on FRET efficiency

No significant change in basal N/C interaction was observed for EcR-A^{K497E}. This was expected as the formation of the salt bridge between helix 4 (K497) and helix 12 (E648) is associated with ligand binding. The hormone-induced increase in FRET efficiency is abolished thereby confirming that the conformational change associated with formation of the salt bridge between helix 4 and helix 12 is responsible for the increased FRET signal of wild type EcR-A. In contrast, FRET coupling efficiency of EcR-A^{K497E} in the presence of Usp is comparable with wild type

receptor complexes, and confirms the hypothesis of Grebe et al. (2003) that the ligand-binding domain of the heterodimerization partner can compensate for the disruption of the salt bridge. According to (Uppenberg et al., (1998), hydrophobic interactions between helix 12 and the remaining part of the ligand-binding pocket additionally contribute to the stabilization of helix 12. As expected, Usp has no influence on EcR^{E648K} mutated in helix 12, which protrudes away from the ligand-binding domain as a result of the mutation. Formation of a salt bridge with opposite polarity restores N/C interaction at least partially, although no increase is obtained in transcriptional activity, which affords the integrity of AF-2 in helix 12.

4.5. The AB - domains of EcR - A and EcR - B1 inhibit basal transcriptional activity by interaction with K497 in helix 4 of the ligand binding domain.

Basal, but not hormone-induced transcriptional activity is considerably enhanced , if lysine at position of 497 is mutated to glutaminic acid as previously reported (Beatty et al., 2006). K497 may be involved in two different receptor functions. Besides formation of a salt bridge between helix 4 and helix 12 in the liganded holoreceptor, K497 is part of a conserved corepressor binding site of nuclear receptors (Lempiäinen et al., 2005). Therefore, it is reasonable to assume that mutation of K497 activates transcription by disruption of corepressor interaction with the non - liganded receptor.

This interpretation is further supported by experiments with N-terminally truncated EcR-A variants. According to deletion experiments, the inhibitory action of the NTD of EcR-A depends on aa 15-29. The sequence LPSTI(LLQQQL) within this peptide shows high similarity with the consensus sequence of the nuclear receptor interaction motif CoRNR box known from vertebrate corepressors SMRT and N-CoR: I/L-X-X-

I/V-I (Hu and Lazar, 1999; Cohen et al., 2001) and L-X-X-I/H-I-X-X-X-I/L (Perissi et al., 1999). Therefore, the simplest explanation for the gain-of-function in basal transcriptional activity observed after exchange of K at position 497 to E is an isoform - specific direct intermolecular interaction of the AB-domain of EcR-A and a weaker interaction of the AB-domain of EcR-B1, which lacks the typical CoRNR motif, with the ligand-binding domain via K497 resulting in reduced transcriptional activity.

The data presented here demonstrate that basal and hormone-induced transcriptional activities of EcR are regulated in an isoform-specific manner by interaction of the NTD with the ligand-binding domain and by variation of the position of helix 12. It is also shown that K497 in helix 4 is involved in two different functions: interaction with the AB- domain in the absence of the ligand and formation of the salt bridge between helix 4 and 12 in the liganded state depending on the isoform. This means that K497 acts as a molecular switch between basal and hormone-induced activity.

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Tables

Table 1: Influence of fluorescent tags on transcriptional activity of heterodimers of EcR isoforms and wild type Usp.

	basal transcriptional activity	fold induction by
	[% of non-tagged receptors]	1 μ M muristerone A
CFP-EcR-A-YFP	20.7 % $\pm$ 8.6 %	1.53 $\pm$ 0.34
CFP-EcR-B1-YFP	44.4 % $\pm$ 15.2 %	1.36 $\pm$ 0.38
CFP-EcR-B2-YFP	42.4 % $\pm$ 18.3 %	1.13 $\pm$ 0.38

Table 2: FRET efficiencies of EcR isoforms. Means $\pm$ SD of at least three independent transfection experiments are shown. The differences in FRET efficiencies as indicated by *, ° and + are significant (p < 0.01).

	constitutive	hormone-induced
	FRET efficiency [%]	FRET efficiency [%]
EcR-A	5.0 $\pm$ 1.0 *,°	8.7 $\pm$ 0.8 °,+
EcR-B1	4.7 $\pm$ 1.0	5.9 $\pm$ 1.0
EcR-B2	9.9 $\pm$ 1.0 *	10.4 $\pm$ 1.0
EcR-A Δ aa1-14	5.8 $\pm$ 1.6	9.8 $\pm$ 1.3
EcR-A Δ aa1-29	5.7 $\pm$ 0.4	5.6 $\pm$ 0.8 +
EcR Δ AB-domain	7.7 $\pm$ 1.3	7.3 $\pm$ 1.7

Table 3: Influence of Usp on FRET efficiency of EcR - A. Means $\pm$ SD of at least three independent transfection experiments are shown. The differences in FRET efficiencies as indicated by $^{\circ}$ ($p < 0.03$) and * ($p < 0.05$) and the differences between constitutive and hormone-induced FRET efficiencies of all receptor complexes ($p < 0.01$) are significant.

	constitutive	hormone-induced
	FRET efficiency [%]	FRET efficiency [%]
EcR-A	5.0 ± 1.0	$8.7 \pm 0.8^{\circ}$
EcR-A / wt-Usp	6.8 ± 1.1	$12.4 \pm 1.3^{\circ,*}$
EcR-A / Usp Δ DBD	4.8 ± 0.1	$9.5 \pm 0.5^*$

Table 4: Influence of point mutations of EcR - A, which affect the salt bridge between helices 4 and 12, on FRET efficiencies. Means $\pm$ SD of at least three independent transfection experiments are shown. The differences in FRET efficiencies indicated by * ($p < 0.02$) and $^{\circ}$ ($p < 0.05$) are significant.

	constitutive	hormone-induced
	FRET efficiency [%]	FRET efficiency [%]
EcR-A ^{K497E}	4.9 ± 0.1	4.4 ± 0.4
EcR-A ^{K497E} / wt-Usp	$5.5 \pm 0.2^*$	$16.2 \pm 2.3^*$
EcR-A ^{E648K}	4.8 ± 0.8	4.6 ± 0.4
EcR-A ^{E648K} / wt-Usp	5.3 ± 0.6	5.3 ± 1.0
EcR-A ^{K497E/E648K}	5.5 ± 0.6	6.2 ± 0.4
EcR-A ^{K497E/E648K} / wt-Usp	$6.5 \pm 0.7^{\circ}$	$8.4 \pm 0.2^{\circ}$

Legends

Fig. 1: EcR fusions with fluorescent proteins CFP and YFP used for FRET analyses.

Fig. 2: Specific Western blot signals showing the influence of fluorescent tags on expression of EcR isoforms. Experiments were performed with extracts from transfected HEK293 cells. 40 µg of protein were loaded per lane.

Fig. 3: Schematic view of the ligand binding domain of EcR with helix 12 (H12) in the open (A) or closed (B) conformation. The closed conformation of helix 12 is often observed in ligand-bound nuclear receptors and is stabilized by a salt bridge between helix 4 and helix 12. In case of EcR, amino acids K497 (helix 4) and E648 (helix 12) are involved in formation of the salt bridge. Scheme modified according to Vivat et al., 1997.

Fig. 4: Comparison of basal transcriptional activity of receptor complexes with wild type EcR and EcR^{K497E}. White bars: wild type EcR - A; black bars: EcR - A^{K497E}. Means + SD of three independent transfection experiments are shown.

Fig. 5: Influence of Usp on basal transcriptional activity of wild type EcR - A and EcR - A^{K497E}. White bars: wild type EcR - A; black bars: EcR - A^{K497E}. Usp I: VP16 - Usp_{CDE}; Usp III: VP16-Usp_{DE}. Means + SD of three independent transfection experiments are shown.

Fig. 1

Fig. 2

closed conformation

Fig. 3