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

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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.

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 (Gianní 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

Abbreviations: 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.

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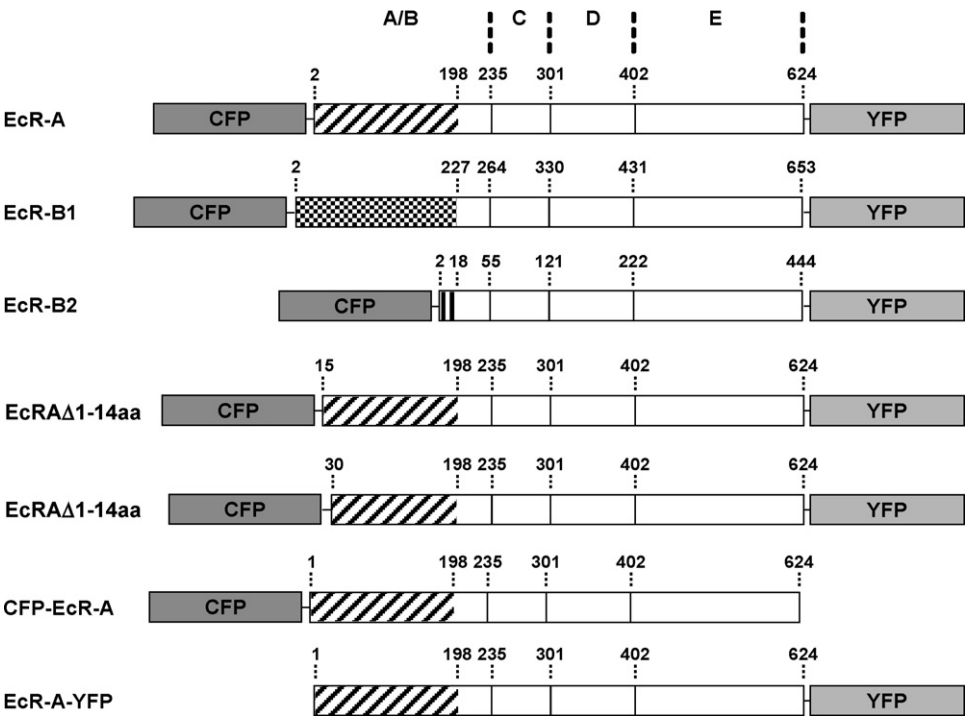

Fig. 1. EcR fusions with fluorescent proteins CFP and YFP used for FRET analyses.

bridge between helix 4 and helix 12 (Bourguet 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 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 XbaI 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, UspΔDBD (deletion of amino acids