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EcR interacts with corepressors and harbours an autonomous silencing domain functional in both *Drosophila* and vertebrate cells.

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Abstract

The ecdysone receptor (EcR) is a member of the large family of nuclear hormone receptors, which are ligand regulated transcription factors. In general, ligand converts these receptors into a transcriptional activator. Some vertebrate nuclear hormone receptors, such as the thyroid hormone and retinoic acid receptors, silence gene expression in the absence of ligand. EcR is involved in fly metarmorphosis and is used in vertebrates as an inducible system for expression of transgenes. Here, we show that a *Drosophila* receptor, the EcR, harbours an autonomous silencing function in its carboxy-terminus. Interestingly, EcR mediates also silencing in vertebrate cells. In concordance with this EcR interacts with the corepressors SMRT and N-CoR, while addition of ligand reduces this interaction. Conversely, the v-erbA oncogene product, a thyroid hormone receptor derivative, mediates silencing in *Drosophila* cells. Thus, our data suggest the involvement of an evolutionarily conserved mechanism by which nuclear hormone receptors mediate gene silencing in multicellular organisms.

Keywords: Ecdysone receptor; Nuclear hormone receptor; Silencing; Corepressor

1. Introduction

Nuclear hormone receptors, including the ecdysone receptor (EcR), represent a superfamily of transcription factors. They are involved in the regulation of various cellular processes such as development, differentiation, homoeostasis and metamorphosis [1–4]. Members of this superfamily are evolutionarily conserved and are found in vertebrates, flies, sea urchin, *Schistosoma* and hydra [5,6]. They have a highly conserved DNA binding domain (C-region) consisting of two Zn-fingers, a variable amino-terminus (A/B-region) and a conserved carboxy-(C) terminus (regions D–F). The latter harbours multiple functions including hormone binding, transactivation, dimerization, and nuclear localization [1–3].

The EcR regulates the morphogenic events in the *Drosophila* puparia formation and metamorphosis through the steroid hormone 20-hydroxy-ecdysone [7]. Thereby, the EcR is heterodimerized with Ultraspiracle (USP) which enhances the DNA binding, hormone binding and transactivation by EcR [8,9]. Interestingly, there are different isoforms of the EcR which are expressed in a tissue- and developmentally specific manner [10]. These isoforms have a common DNA- and hormone-binding domain but different N-termini. Sequence comparisons with nuclear hormone receptors show that the EcR has an unusual large extension of the F-region.

The EcR is used in vertebrate cells and for transgenic mice as an hormone-inducible system [11,12].

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Thereby, fusion proteins are used with the C-terminus of the EcR which allow the transfected genes to be under control of the ecdysteroid Muristerone A. Using an inducible gene expression system studies can be performed to analyze developmental or metabolic processes. For that purpose it would be important to know how EcR is regulating gene expression in vertebrate cells, which cellular factors are required and whether cell specific factors are involved in EcR mediated gene regulation.

A few members of the nuclear receptor family, such as TR (c-erbA) and RAR, contain an additional function to mediate active gene silencing in their C-termini [13]. So far, a C-terminal silencing domain has been identified only for vertebrate receptors.

Gene silencing is achieved by a receptor–corepressor complex [14–17]. In analogy to that, gene activation is achieved by a complex of liganded receptor and coactivators [16,18,19]. Thus, the role of hormone is to switch these receptors from a transcriptional silencer to an activator.

Here we show that the EcR harbours an autonomous silencing function in the C-terminus. This silencing domain is functional in both *Drosophila* and vertebrate cells. This is important for both tight control of gene expression and reduced background of promoter activity. Furthermore, we provide evidence that EcR interacts with the corepressors SMRT and N-CoR. Conversely, the V-ErbA oncoprotein mediates silencing in *Drosophila* cells. Our data also show that *Drosophila* nuclear hormone receptors have a transferable silencing domain and indicate the involvement of conserved mechanisms of gene silencing by nuclear hormone receptors.

2. Materials and methods

2.1. Plasmids

The plasmids pVP-SMRT [14]; pJG-N-CoR [15]; pEG202; pJG4–5, pSH18–34 [20], pABgal-v-erbA [13] and pABgal94-linker [21] have been described earlier. pABgal94-EcR 330–878 was generated by insertion of the filled-in-Eco52I-HindIII fragment (partial digestion) into the blunted AccI and HindIII sites of pABgal94-linker. The plasmid pAB-EcR 330–817-gal94 was created by insertion the EcR coding sequences from aa 330 (Eco25I) to aa 817 (NheI) 5́ to the coding sequence of Gal4-DBD and 3́ of the ATG (BglII-site) of pABgal94-linker. pEG-EcR 330–878 was constructed by insertion of the HindIII-Klenow/EcoRI fragment from pABgal94-EcR 330–878 into the XhoI-Klenow/EcoRI site of pEG202. The yeast two-hybrid assay was performed with the EGY strain (MATa his3 trp1 ura3–52 leu2: pLEU2-LexAop6) as described previously [22].

Constructs for in vitro translation were cloned as follows: EcR aa330–878 was excised from pABgal94-EcR 330–878 with blunted EcoRI-HincII, and ligated to the [23] HincII site. pT7-EcR aa330–878 was in vitro translated using the Promega TNT kit with ^{35}S-methionine and T7 polymerase according to manufacturers protocol.

2.2. GST-pull-down

GST-pull-down experiments were performed as described earlier [24]. Preparation of E. coli expressed GST-SMRT was according to [14] and Baniahmad et al. [22]. Interaction assays using hormone were done as follows: four microliters of in vitro-translated receptor were incubated with 10^{-4} M Muristerone A (Sigma) dissolved in interaction buffer for 30 min in a total volume of 60 μl and then added to the beads. Binding was allowed to proceed for 30 min and the beads were subsequently washed 5 times with NETN + 0.5% nonfat dry milk. The bound proteins were eluted by boiling in 10 μl of sodium dodecyl sulfate ($2 \times$ SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer and resolved by electrophoresis. The indicated input lane shows 10% of the added in vitro-translated material to each pull-down for immobilized GST or GST-SMRT. The GST fusion proteins were stained with Coomassie blue to ensure that equal amounts of fusion protein were used in this assay. The bound, labelled protein was visualized by autoradiography.

2.3. Yeast 2 hybrid assay

The yeast two-hybrid assay was performed according [20] and as described earlier [22].

2.4. Cell culture

Drosophila Schneider S2/L3 cells were grown in Schneiders' medium (Gibco/BRL) with 10% FBS (insect qualified, Gibco/BRL) and 1% glutamine at 25°C. Cotransfections were carried out by the $CaPO_4$ method [25] at room temperature. In general, a total of 4 to 7.5 μg of expression vector and 1 μg of the 2xUAS-Adh86-CAT reporter plasmid were added to 5×10^5 cells in 5 ml of medium. In studies involving hormone, Muristerone A, dissolved in ethanol, was added to 1 μM final concentration 24 h after DNA-transfection. The cells were cultured for an additional 24 h at 25°C before harvest. In experiments without hormone ethanol was added. DNA-transfection of Ltk- and CV1 cells were performed in hormone-depleted serum as described earlier [22]. The cotrans-

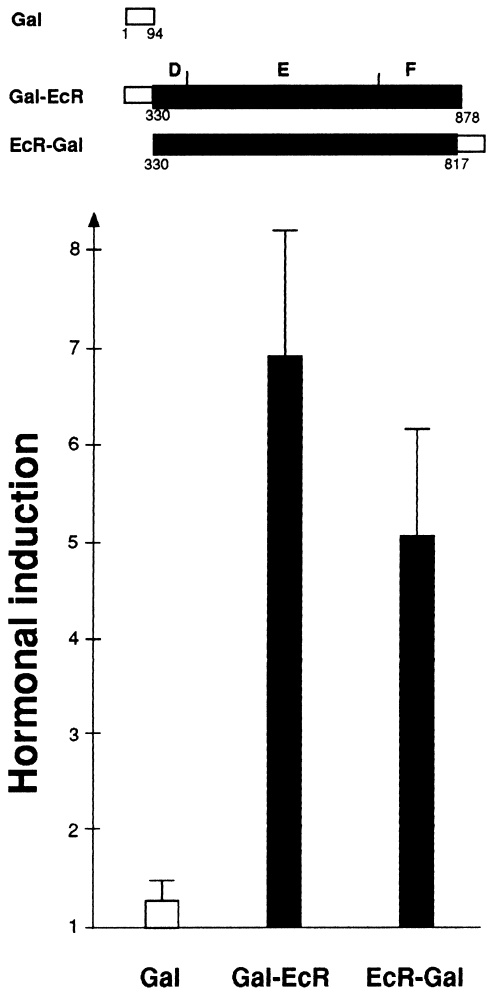

Fig. 1. The C-terminus of the ecdysone receptor (EcR) mediates hormonal response in *Drosophila* cells. *Drosophila* Schneider S2/L3 cells were cotransfected with the indicated expression plasmids for Gal4-DBD (aa 1–94) alone, Gal-EcR-(330–878) or EcR-(330–817)-Gal and with the reporter pUAS2x-Adh-CAT (1 μg). The ligand, Muristerone A (10^{-5} M), was added and incubated for 24 h. Fold induction was calculated by division of the values obtained in the presence of hormone with the values obtained in the absence of ligand.

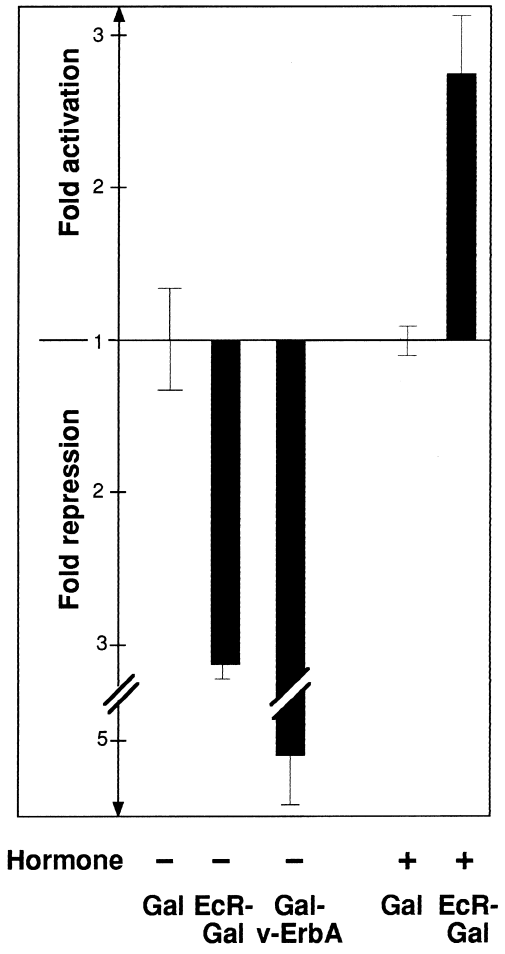

Fig. 2. The C-terminus of the EcR mediates silencing in the absence of hormone in *Drosophila* cells. Cotransfection experiments were done as in Fig. 1 with the reporter pUAS2x-Adh86-CAT and the indicated expression plasmids. Muristerone A was added at a concentration of 10^{-5} M. Values are normalized to that obtained with Gal4-DBD alone. Gal-v-ErbA is a fusion of the C-terminus of the v-erbA oncogene to the Gal4-DBD.

fected vector RSV-lacZ (3 μg), as internal control, yielded similar expression levels (in the range of about 15% variation) independent of the transcriptional effects of the Gal-fusions. The level of CAT-expression were in the range of 5%–15% CAT-conversion.

3. Results

3.1. The Drosophila EcR harbours a transferable silencing domain in its C-terminus, which is functional in both Drosophila and vertebrate cells

Based on the sequence homologies of EcR with TR [26] we characterized the C-terminal domain of the EcR, by replacing first the EcR DNA-binding domain (DBD) with that of Gal4 (amino acids 1–94). Hormone inducible gene expression was tested by cotransfection of the expression vector together with a UAS (Gal4-DBD binding site) containing reporter in *Drosophila* Schneider S2-cells. Fold induction was calculated by dividing the values obtained in the presence with those obtained in the absence of hormone. As seen in Fig. 1, amino acids 330 to the C-terminal end (aa 878) of the EcR are sufficient to mediate hormonal response. Similarly, fusion of the Gal-DBD to the F-region of the EcR also yielded a functional ecdysone responsive protein Fig. 1. This suggests that at least part of the F-region is not required for hormonal response and shows that the EcR-C-terminus represents a functional and transferable domain.

Interestingly, in the absence of ligand, the EcR med-

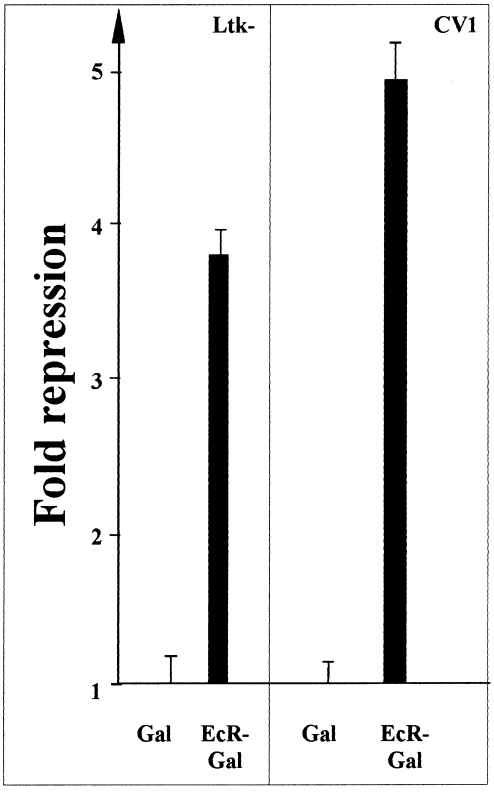

Fig. 3. The EcR mediates transcriptional silencing in vertebrate cells. Cotransfection experiments as described in Fig. 1 were performed with mammalian CV1 or Ltk-cells. The plasmid 17mer6x-tkCAT was used as a reporter. Values obtained with Gal4-DBD were set as one. RSV-lacZ was used as internal control.

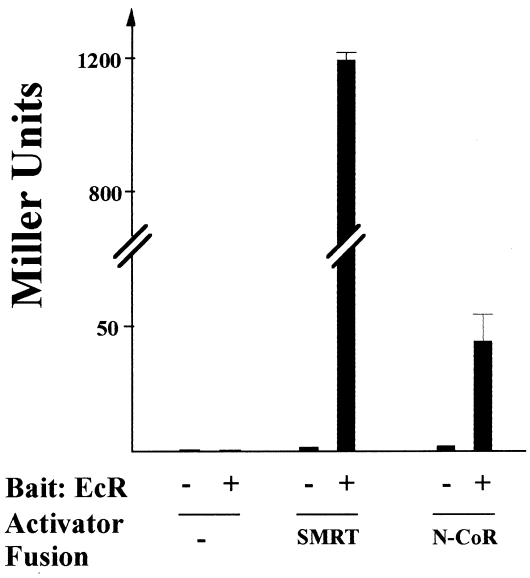

Fig. 4. The EcR interacts with the corepressors SMRT and N-CoR. Yeast 2-hybrid experiments were performed using the C-terminus of EcR (aa330-878) as a bait and SMRT or N-CoR as the activator fusion according to Gyuris et al. [20]. Specific interaction is seen with EcR and both corepressors. Controls represent the empty expression vectors.

iates repression of promoter activity in *Drosophila* cells. As seen in Fig. 2, the EcR-Gal fusion protein silenced promoter activity, while addition of hormone leads to promoter activation. This shows that in addition to ligand binding and transactivation the C-terminus of EcR also harbours a silencing function. The Gal-DBD aa 1–94 itself did not show any significant transcriptional activity in Schneider cells (not shown). Thus, EcR functions in a similar manner to the vertebrate receptors TR and RAR. Therefore, we wondered whether a vertebrate nuclear receptor with a silencing function would also be able to mediate repression in *Drosophila* cells. For this purpose we used the thyroid hormone receptor derivative, v-ErbA. Compared to TR (c-erbA), the v-erbA oncogene product differs by several amino acids and lacks the very C-terminal domain AF2-AD/τ4/τc [2–4,27–29] which is essential for hormone-dependent transactivation. This renders v-ErbA to a constitutive silencer. Indeed, expression of Gal-v-ErbA, a fusion of the silencing domain of v-ErbA to the Gal4-DBD [13] in *Drosophila* cells, results in promoter repression, to a similar extent as EcR-Gal Fig. 2.

Since a vertebrate nuclear receptor mediates silencing in *Drosophila* cells we examined whether *Drosophila* EcR was capable of silencing in vertebrate cells. We cotransfected the expression plasmid EcR-Gal and the UAS containing reporter 17mer6x-tkCAT [13] into two cell lines, monkey CV1- and mouse Ltk-cells. As seen in Fig. 3, EcR mediates the repression of promoter activity in both cell types. Thus, the C-terminus of EcR is also able to silence gene expression in vertebrate cells.

Taken together, in addition to the hormone-dependent transactivation domain, the *Drosophila* EcR has a transferable silencing domain in its C-terminus, which is functional in both *Drosophila* and vertebrate cells. Conversely, the vertebrate nuclear receptor v-ErbA is able to mediate silencing in *Drosophila* cells. This indicates that the mechanism of gene repression by nuclear hormone receptors is conserved between *Drosophila* and vertebrates.

3.2. EcR interacts with the corepressors SMRT and N-CoR

Since the silencing by nuclear hormone receptors in vertebrate cells is mediated by a receptor–corepressor complex, we analyzed whether EcR is interacting with the two known vertebrate corepressors SMRT and N-CoR [14,15]. We used the yeast-2-hybrid assay according to Gyuris et al. [20], as an established method for protein–protein interaction. As bait we fused the EcR

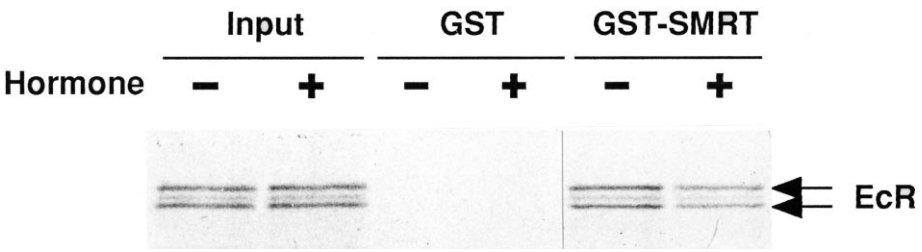

Fig. 5. Hormone leads to a reduced binding of EcR to the corepressor SMRT. GST pull down experiments were performed using bacterially expressed GST or GST-SMRT and in vitro translated, ^{35}S labeled EcR C-terminus (aa 330–878). The radioactive labeled protein was incubated with Muristerone A (10^{-4} M) dissolved in ethanol or ethanol alone for 30 min before incubation with GST or GST-SMRT. The input lane shows 10% of the labeled material used in the interaction assays.

C-terminus to the DBD of LexA and cotransformed yeast cells with a LacZ containing reporter and either the reported activator fusions SMRT [14,22] or N-CoR [15]. Interaction is determined by expression of the lacZ reporter. As seen in Fig. 4 both SMRT and N-CoR interact specifically with EcR. The combination of SMRT with EcR yielded a much higher lacZ expression compared to N-CoR with EcR. This is of special interest since no significant homology to the CoR-box of TR and RAR is found in EcR. We did not observe a hormone-sensitive interaction using this assay (data not shown). One possibility is that the penetration of the yeast cell wall by Muristerone A or 20-hydroxy-ecdysone is impaired or the ligands are rapidly degraded. Controls used in the yeast 2-hybrid assay are the empty expression vectors in each combination with either the bait or the activator fusion, as indicated in Fig. 4.

To verify the interaction and to analyze the effect of EcR ligand we used the GST-pull down system. Bacterially expressed GST or GST-SMRT were immobilized on glutathione beads and were incubated with in vitro translated, ^{35}S-labeled EcR (aa 330–878). The in vitro translation of EcR C-terminus gave rise to two bands, as seen in Fig. 5. We found strong binding of EcR to the corepressor SMRT, while we did not observe an interaction with GST alone. Addition of Muristerone A led to a significant and reproducible decrease in interaction Fig. 5. Due to the fact that the affinity of EcR to its ligand is greatly impaired when not heterodimerized with Ultraspiracle (USP) [30], we see only a partial release of bound EcR from GST-SMRT.

Thus, EcR interacts with the corepressors SMRT and N-CoR. Furthermore, we show that addition of the cognate hormone leads to a decrease in corepressor–EcR interaction.

4. Discussion

We have identified for the first time a transferable silencing domain required for an active repression mechanism in a *Drosophila* nuclear hormone receptor. Interestingly, the EcR silencing domain is functional in vertebrate cells as well. By analogy, a vertebrate member of the nuclear receptor superfamily is capable of mediating silencing in *Drosophila* cells. Taking this into account EcR has multiple similarities to the TR/RAR family of nuclear hormone receptors: EcR is localized in the cell nucleus in the absence of hormone [26], heterodimerizes with USP [30,31], which is functionally related to RXR [9,32], binds to both palindromic and direct repeats [33,34] and harbours a silencing function. Thus, although EcR binds steroids, it is less related to the mammalian receptors for steroids. Rather, EcR is functionally homologous to the non-steroid-receptors, such as TR and RAR. It would be of interest if there are further functional homologies between EcR and TR/RAR. There are reports which show that not only a hormone-dependent dissociation, but also a DNA-binding-site-dependent association of corepressors with RAR and TR is found [35,36]. Also, there are negative T_3REs and RAREs which exhibit activation by unliganded TR or RAR [37–39], which are reported to include corepressor association [40]. This observation may also be important for the Ashburner model, by which ecdysone removal leads to premature appearance of late puffs in the polytene chromosome of *Drosophila* [41,42].

Since the N-terminus of vertebrate nuclear hormone receptors also contributes to the overall transcriptional activity, it may be possible that the different isoforms of EcR have different influences on cofactor binding.

In *Drosophila* a corepressor has been characterized, Groucho, which acts in concert with transcriptional silencer proteins, such as Hairy and Engrailed [43]. Groucho is required for *Drosophila* neurogenesis, segmentation, and sex determination [44]. So far there is no known vertebrate homologue of Groucho. Conversely, *Drosophila* homologues of the vertebrate corepressors SMRT or N-CoR are not published.

EcR is used in mammalian cells as part of a hormone-inducible gene expression system. For tight control of gene expression, a strongly reduced background expression is desired. This is presumably possible since

unliganded EcR represses target genes. For proper hormone-inducible gene expression by nuclear hormone receptors cofactors are required. Thus, the levels of endogenous cofactors are important regulators involved in this aspect. Tissues lacking a specific cofactor are therefore likely to exhibit a different level of hormonal response of gene expression. A correlation between corepressor level and hormone induction has been shown for RAR [45], whereby low amounts of endogenous corepressor result in a weak hormone response. Such a cofactor dependency may limit these inducible systems to a certain extent. However, since cofactors seem to be present in a redundant manner, the overall effect may be less dramatic.

In *Drosophila* development EcR is expressed in a very early stage of the embryogenesis [26] and reaches several peaks during the larvea, prepupa and pupae stages. In situations where the intracellular concentrations of ecdysone are low, EcR may be complexed with *Drosophila* corepressors, which would mean that EcR responsive genes are silenced.

A repression function by natural ecdysone response elements of endogenous *Drosophila* genes has been shown [46,47], while gene activation depends on addition of ecdysone. In this manner EcRE functions as both repressor and activator elements, which is in accordance with our findings.

The fact that nuclear hormone receptors are able to silence gene expression in both *Drosophila* and vertebrate cells suggest an evolutionarily conserved mechanism. We postulate that in *Drosophila* either SMRT or N-CoR homologues exist or as yet unpublished corepressors are present, which complex with selected members of nuclear hormone receptors and mediate gene repression.

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