Ligand-induced heterodimerization between the ligand binding domains of the *Drosophila* ecdysteroid receptor and ultraspiracle

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Lezzi, M., , T. Bergman, V.C. Henrich, M. Vogtli, C. Fromei, M. Grebe, S. Przibilla, and M. Spindler-Barth (2002). Ligand-induced heterodimerization between ligand binding domains of Drosophila ecdysteroid receptor (EcR) and Ultraspiracle (USP). *Eur. J. Biochem*., 269: 3237-3246. DOI:10.1046/j.1432-1033.2002.03001.x

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Abstract:
The insect ecdysteroid receptor consists of a heterodimer between EcR and the RXR-orthologue, USP. We addressed the question of whether this heterodimer, like all other RXR heterodimers, may be formed in the absence of ligand and whether ligand promotes dimerization. We found that C-terminal protein fragments that comprised the ligand binding, but not the DNA binding domain of EcR and USP and which were equipped with the activation or DNA binding region of GAL4, respectively, exhibit a weak ability to interact spontaneously with each other. Moreover, the heterodimer formation is greatly enhanced upon administration of active ecdysteroids in a dose-dependent manner. This was shown in vivo by a yeast two-hybrid system and in vitro by a modified electromobility shift assay. Furthermore, the EcR fragment expressed in yeast was functional and bound radioactively labelled ecdysteroid specifically. Ligand binding was greatly enhanced by the presence of a USP ligand binding domain. Therefore, ecdysteroids are capable of inducing heterodimer formation between EcR and USP, even when the binding of these receptor proteins to cognate DNA response elements does not occur. This capability may be a regulated aspect of ecdysteroid action during insect development.

Keywords: Drosophila melanogaster; yeast; two-hybrid; ecdysone receptor; dimerization; ultraspiracle.

Abbreviations: EcR, ecdysone receptor protein; USP, ultraspiracle protein; RXR, 9-cis retinoic acid receptor; EcRE, ecdysone receptor protein receptor response element; EMSA, electromobility shift assay; ST-EMSA, supershift-type electromobility shift assay; LBD, ligand binding domain; GBD, DNA binding domain of GAL4; GAD, activation domain of GAL4; DBD, DNA binding domain of EcR.

Definition: in the present work the terms `monomer', `dimer', `homodimer', and `heterodimer' are being used although the protein complexes in question may in fact constitute multimers. Our nomenclature follows the common use and describes the state of interaction of the respective nuclear receptor in a protein complex with respect to another nuclear receptor.

Article:

Ecdysteroids are widespread steroid hormones found in invertebrates [1] and plants [2,3] that regulate a variety of developmental, physiological, and reproductive processes [1,3]. Among insects, these hormones regulate the expression of genes through a highly orchestrated and coordinated transcriptional network [4–6]. The widespread and diverse effects of ecdysteroids on transcriptional regulation have served as a powerful model for investigating the diverse mechanisms by which steroid hormones, acting via nuclear receptors, exert their effects on a variety of life processes [4,7].

The ecdysone receptor (EcR) [8], responsible for mediating these responses, occupies a special position among nuclear hormone receptors because it shows a unique combination of characteristics [9]. Unlike the vertebrate steroid receptors [10–12], EcR heterodimerizes with the insect RXR orthologue, ultraspiracle (USP) [13–15]. Nevertheless, while other nuclear receptors that dimerize with RXR normally are bound to DNA response elements already in their nonliganded state [11,16], this apparently is not true for the EcR/USP heterodimer (see however, [17]). Immunostaining has shown that the polytene chromosomes of a Chironomid or Sciarid are devoid of EcR/USP signals when prepared from developmental stages associated with low ecdysteroid titers [18,19]. A short *in vitro* incubation of the tissues with 20-hydroxyecdysone, however, is followed by the appearance of immunostaining signals at known ecdysteroid-responsive gene loci [18,19]. The affinity of EcR/USP dimers for ecdysone response elements (EcREs) clearly increases in the presence of the ecdysteroid muristerone A as demonstrated by electromobility shift assays (EMSAs) [20–23].

Neither immunostaining assay nor EMSA studies can distinguish between three possibilities concerning how ecdysteroids influence EcR/USP binding to DNA: (a) ecdysteroids promote dimerizing of EcR and USP, and enhanced DNA affinity arises as a spontaneous consequence of this partnering, as indicated for the glucocorticoid receptor [24]; (b) ecdysteroids induce binding of EcR to its cognate EcRE half-site, analagous to the effect noted for the thyroid hormone receptor [17]; or (c) EcR and USP dimerize spontaneously and ecdysteroids promote the binding of the dimer to the DNA response element [20].

The primary purpose of this work is to address the possibility that EcR and USP are capable of dimerization in the absence of a bipartite EcRE and to monitor the potential influence of ecdysteroid on dimerization. These studies were carried out by expressing the ligand-binding domain (LBD) of EcR and USP on two-hybrid vectors, and examining their ability to dimerize in the absence and presence of ecdysteroids. The experiments presented demonstrate that the EcR and USP LBD are capable of dimerization in the absence of a bipartite EcRE, and that this protein –protein interaction is dramatically enhanced by the presence of ecdysteroids.

MATERIALS AND METHODS

Plasmids

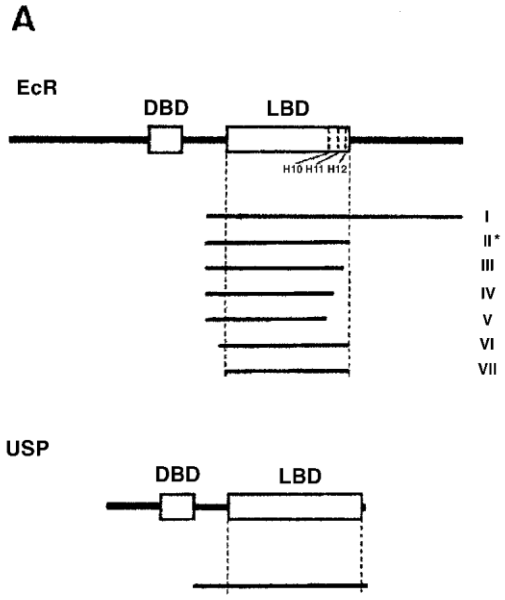
All plasmids used in the present work were purchased from Clontech (Palo Alto). For a description of the plasmids, see the supplier's protocol and the references therein. Test plasmid pCL1 encodes for full-length wild-type GAL4 and served to monitor nonspecific effects on the reporter enzyme, whereas test plasmids pTD1-1 and pVA3-1 were used to test the general conditions for two-hybrid formation and reporter gene activation. The other plasmids used are mentioned below.

Plasmids coding for fusion proteins with various C-terminal fragments of EcR

Schematic representations of the fragments are shown in Fig. 1A.

Construct encoding fragment I

A 1.5-kb cDNA fragment of the Drosophila EcR cDNA sequence [8] was produced by PCR using the forward primer 5'-CGACATATGGGCCAAGACTTTGTTAAG AAGG-3' and the reverse primer 5'-TCCCCCGGGTC TAGACTATGCAGTCGTCGAGTGCTC-3'. Thereby, an *Nde*I site was introduced at the 5' end, and *Xba*I and *Sma*I sites were introduced at the 3' end. The fragment was digested with *Nde*I/*Sma*I and cloned in-frame into pAS2 giving rise to clone pAS2-EcR(375–878), coding for fusion protein GBD–EcR(375–878) which consists of the GAL4 DNA binding domain fused to the N-terminal end of a fragment comprising a portion of hinge region, LBD, and the entire C-terminal domain (also called F domain) of EcR. A 679-nucleotide portion of the *EcR* fragment between *Aat*II (nucleotide 2441) and *Nar*I (nucleotide 3120) was exchanged with the corresponding fragment of the original cDNA clone [8].



B

Fusion protein	Fragment	2-H basal	2-H rI	ST. EMSA	LB EcR	LB EcR + USP
GBD-EcR(375-878)	I	+	+			
GBD-EcR(375-652)	II	+	+			
GAD-EcR(375-652)	II*)	+	+	+	+	+
GAD-EcR(375-642)	III	+	-	-	-	-
GBD-EcR(375-642)	III	+				
GAD-EcR(375-627)	IV	+	-	-		
GBD-EcR(375-627)	IV	+				
GAD-EcR(375-609)	V	-	-	-		
GBD-EcR(375-609)	V	-				
GAD-EcR(403-652)	VI	-	-	-	-	-
GBD-EcR(403-652)	VI	+				
GAD-EcR(421-652)	VII	-	-	-	-	-
GBD-EcR(421-652)	VII	+				

Fig. 1. Heterodimerization and ligand binding abilities of the EcR fragments investigated in the present studies as fusion proteins with GAL4 DNA binding domain (GBD) or GAL4 activation domain (GAD). (A) Delineation of EcR and USP fragments tested. *EcR* stands for the B1 isoform [46] of the *Drosophila melanogaster* ecdysone receptor [8]. It spans amino acids 1–878. DBD: DNA binding domain (amino acids 264–329). LBD: ligand binding domain (amino acids 417–651) as defined by [37]. Helices 10, 11 and 12 (H10, H11, H12) start at amino acids 610, 628, and 644, respectively [37]. Fragments are numbered I–VII and correspond to those listed in (B). The dash in fragment I indicates the location of the spontaneous mutation L442H. The asterisk designates the fragment which was used most frequently in the present studies. *USP* designates Ultraspiracle as characterized by [14]. Delineation of its DBD and LBD as proposed by [34]. (B) Results obtained with EcR fragments described in (A). Explanation of column heads and signs in columns: *2-H basal*, spontaneous heterodimerization with USP fragment *in vivo* as determined by the two-hybrid assay and indicated by the appearance of basal (noninduced) β-galactosidase activity; + or – signs mean this activity to be significantly or unsignificantly higher than background, respectively; *2-H rI*, relative induction of heterodimerization by muristerone A (10^{-6} to 5×10^{-5} M) as assayed by the two-hybrid assay (*rI*: induced level divided by basal level of β-galactosidase activity): + or – signs mean rI to be significantly or unsignificantly higher than 1, respectively; *ST-EMSA*, heterodimerization induced *in vitro* by 10^{-5} M muristerone A and assayed by ST-EMSA; + or – signs mean supershifted band could or could not be detected, respectively; *LB EcR*, ligand binding to EcR fragment alone; + or – signs mean specific [^{3}H] ponasterone A binding to be significantly or nonsignificantly higher than background, respectively. *LB EcR ± USP*: ligand binding to EcR fragment in presence of USP fragment; GBD- and GAD-EcR fusion proteins were combined with GAD- or GBD-USP(172–508), respectively; meaning of + and – signs as in LB EcR column.

Constructs encoding fragment II

Clone pAS2-EcR(375–652) encoding the fusion protein GBD–EcR(375–652) between the GAL4 DNA binding domain and a fragment comprising a large portion of hinge plus the entire LBD of EcR was constructed in an analogous manner to pAS2-EcR(375–878). The differences concern the reverse primer 5'-CGTCCCGGGTCTA GACT AAACGTCCCAGATCTCCTCG-3', the length of PCR fragment (800 nucleotides), and the exchanged fragment (566 nucleotides from *Aat*II at nucleotide 2441 to *Bgl*II at nucleotide 3008). The related clone, pAS2-1-EcR(375–652), was constructed by cutting out the *Nde*I–*Sma*I fragment from pAS2-EcR(375–652) and recloning it into the *Nde*I– *Sma*I site of the pAS2-1 vector. pAS2-EcR(375–652) and pAS2-1-EcR(375–652) will not be distinguished in the following. Clone pACT2-*Ec*R(375–652) encodes a fusion protein, GAD–EcR(375–652), consisting of the same EcR fragment as pAS2-1-EcR(375–652) but combined with GAL4 activation rather than DNA binding domain. It was constructed by use of the forward PCR primer: 5'-CATGCCATGGGCCAAGACTTTGTTAAGAAGG-3' and the reverse primer employed for constructing pAS2-1-EcR(375–652). The *Nco*I–*Sma*I fragment encompassing the EcR cDNA sequence nucleotide 2191–3024 was cloned into the *Nco*I–*Sma*I site of vector pACT2.

Constructs encoding fragment III

Clone pAS2-1-EcR(375–642) encodes the same EcR-containing fusion proteins as pAS2-1 -EcR(375–652) except that the C-terminal ochelix 12 of the LBD in EcR is missing. It was produced by inserting an *Nco*I – *Eco*RI restriction fragment of a PCR product into the pAS2-1 cloning site. For producing the PCR fragment, the same forward primer was used as for pACT2-EcR(375–652). The reverse primer was 5'-CGGAATTCTCAC

AGTTTGCGGTTTTTGAG CTTTAG-3' which generated a stop codon at nucleotide position 2995. Clone pACT2-EcR(375–642) is analogous to pAS2-1 -EcR(375–642) and was produced by exchanging the NcoI–EcoRI restriction fragment of pACT2-EcR(375–652) by that of pAS2-1-EcR(375–642).

Constructs encoding fragments IV and V
The fusion proteins encoded by clones pACT2-1-EcR(375– 627) and pAS2-1-EcR(375–627) or by pACT2-1-EcR (375–609) and pAS2-1-EcR(375–609) lack helices 11–12 or 10–12 of EcR LBD, respectively. They were produced in an analogous manner as pAS2-1- and pACT2-EcR(375–642) by using, however, as reverse primers 5'-CGGAATTCTCACTGGTTGCCCAGCGTACGCAG-3' and 5'-CGGAATTCTCAGACGAGGCTCATTGA GTCGCC-3', which introduced stop codons at nucleotide positions 2943 and 2896, respectively.

Constructs encoding fragments VI and VII
Clones pACT2-1-EcR(403–652) and pAS2-1-EcR(403–652) encode fusion proteins that contain a smaller piece of the EcR hinge region than clones pACT2-1-EcR(375–652) and pAS2-1-EcR(375–652). They were produced as described above by introducing into the respective vectors the *Nco*I/*Eco*RI-cut PCR fragment by use of the forward and reverse primers 5'-CATGCCATGGAAATAT TGGCCAAGTGTCAAGC-3' and 5'-CGGAATTCT CAAACGTCCCAGATCTCCTCGAG-3', respectively.

Clones pACT2-1-EcR(421–652) and pAS2-1-EcR (421–652) lack the entire hinge region of EcR. They were produced as described above using the forward primer 5'-CATGCCATGGAGTTGGCCGTTATATACAAGTT AATTTG-3'.

Plasmids coding for fusion proteins with the C-terminal fragment of USP
Clone pGAD424-USP(172–508) encodes a fusion protein consisting of the activation domain of GAL4 and the C-terminal part of USP including a portion of the hinge region and the LBD. It was produced by cloning an EcoRI/ StuI-cut PCR fragment into the *Eco*RI/*Sma*I-cut pGAD424 vector. To generate the PCR fragment, the following primers were used on the original USP cDNA clone of Oro and coworkers [14] as a template: 5'-AGGAATTCGA AGCGGTCCAGGAGGAG-3' and 5'-AAGGCCTTCTAGACTACTCCAGTTTCATCGCCA GGCC-3'.

Although the pGAD424 construct yielded less fusion protein than the corresponding pACT2 construct (see below), the same relative effects were obtained when comparing different EcR fragments or ligands in the two-hybrid assay.

pAS2-1-USP(172–508) was produced by cutting pGAD424-USP(172–508) with *Eco*RI and *Sal*I and the resulting fragment was recloned in to the respective sites of pAS2-1. From this plasmid, the clone pACT2-USP(172– 508) was constructed by producing a *Nco*I–*Sal*I fragment that was blunted at its *Sal*I side (filling-in reaction) and then cloned into the *Nco*I–*Sma*I site of pACT2.

PCR reaction, sequence verification
For PCR amplification, standard PCR conditions were employed. All PCR fragments and the resulting inserts were verified by commercial sequencing (Microsynth and GENterprise; Gachnang, Switzerland and Mainz, Germany, respectively).

Yeast strains
All yeast strains (*Saccharomyces cerevisiae*) were purchased from Clontech. For routine two-hybrid work, strain Y187 was used; this strain harbors the reporter gene *lacZ* under the control of a GAL1UAS-GAL1TATA element. In preliminary two-hybrid studies or experiments with `cell-toxic' fusion proteins, the low expressing strains Y153 and Y157 were also employed, which carry the same reporter gene. For ST-EMSA and ligand binding tests, strain Y190 was employed, which is favorable for fusion protein expression. Although these four strains differ in their overall fusion protein expression and/or strength of reporter gene activity (*lacZ*),

comparative experiments showed that the relative effects of different EcR/USP fragment combinations or ligand types are not influenced by the yeast strain used.

Preparation of yeast extracts for two-hybrid studies

Yeast cells were grown in YPD (1% yeast extract, 2% Bacto-peptone, 2% glucose) at 30 °C, and then transformed or cotransformed by the plasmids mentioned above. The lithium acetate procedure was used, following the manufacturer's protocol (Clontech). Yeast cells were plated on synthetic dextrose (SD) minimal medium [0.67% yeast nitrogen base (DIFCO) and 2% glucose] lacking leucine and tryptophan to select for cells bearing the plasmids. Colonies were grown at 30 °C for 3 days and three colonies were then selected for subsequent liquid culture. With colonies that did not grow well, a colour reaction was performed on filter lifts to monitor lacZ activity, according to the manufacturer's instructions, as a means to select colonies for two-hybrid experiments and as an auxiliary method to evaluate two-hybrid experiments. The selected colonies were inoculated in 3 mL of an SD liquid medium, and the culture was grown overnight with shaking. When the D_{600} of the culture reached ≈1.0, two 100-µL aliquots were removed and transferred to a second tube with 1.9 mL of SD liquid medium. One of the tubes was supplemented with ligand (for type and final concentration, see Results) and the other was supplemented with ethanol solvent of the same final concentration, normally 0.25%. Triplicate samples from each liquid culture were processed according to manufacturer's instructions (freeze-thawing) and measured for *lacZ* activity by assessing the colour change of Gal-ONp as measured by absorbance spectrophotometry (D_{420}). The mean reading was then used to calculate the *lacZ* activity in `Miller Units', according to the manufacture's protocol (Clontech).

Ligands

Muristerone A (Sigma, Invitrogen or Alexis Biochemicals), ponasterone A (Sigma), poststerone (a kind gift of R. Lafont, ENS, Paris), 20-hydroxyecdysone (Sigma), and the nonsteroidal ecdysone agonist RH 5992 (a kind gift of Rohm and Haas company, Spring House, PA, USA) were prepared as ethanol solutions (10 mg.mL-1) and then diluted to the final concentration indicated in the text for use in the two-hybrid experiments (for use in biochemical analyses, see below).

Preparation of yeast extracts for biochemical analyses

For supershift-type EMSA and ligand binding studies, growth and transformation of yeast cells were carried out as for two-hybrid studies. Single colonies less than 4 days old of yeast transformants expressing GAD–EcR(375–652) or GBD–USP(172–508) were picked and cultured with shaking (150–200 r.p.m) at 30 °C overnight in 5 mL of SD medium. The overnight cultures were diluted in 50 mL YPD and grown under the same conditions until D_{600} = 0.6–0.8. The cells were then prepared on ice. Cells were harvested by centrifugation (1500 ***g***, 5 min, 4 °C) in prechilled tubes. Pellets were washed with 50 mL ice-cold wash buffer (20 mM Hepes, 20 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.9), transferred into plastic tubes, and frozen in liquid nitrogen. The frozen pellets were disrupted for 2 min at 2000 r.p.m. using a Mikro-Dismembrator S (B. Braun Biotech International; Melsungen, Germany). After thawing, the homogenates were diluted with binding buffer [wash buffer with 1 mM phenylmethanesulfonyl fluoride, pH 7.9] supplemented with a mixture of protease inhibitors (aprotinin, leupeptin, pepstatin, benzamidine, antipapain, and cymostatin; final concentration: 2 µg·mL-1 each) just before use. After a short ultrasonication the samples were centrifuged (100 000 ***g***, 1 h, 4 °C). Phenylmethanesulfonyl fluoride (final concentration: 1 mM) was added to the supernatants. The extracts were frozen in aliquots at -80 °C until tested.

Supershift-type electrophoretic mobility shift assays (ST-EMSAs)

A double-stranded probe of the GAL4 binding motif was prepared and labelled with [α-^{32}P]dCTP, as described previously [25]. The reaction mix contained binding buffer [20 mM Hepes, pH 7.4, 100 mM KCl, 5% (v/v) glycerol, 2 mM dithiothreitol, 0.1% NP-40] and yeast cell extracts with the EcR or USP fusion proteins, 1 µg of the nonspecific competitor poly(dIdC), ≈10 fmol labeled oligonucleotide, and muristerone A at a final concentration of 10^{-5} M or as indicated in the Results. The reaction mix was incubated at room temperature for 30 min and separated at 10 V·cm^{-1} on a 5% nondenaturing polyacrylamide gel in Tris/borate/EDTA (45 mM

Tris, 45 mM boric acid, 0.5 mM EDTA pH 8.0) for 2 h. The gel was analyzed by a PHOSPHORIMAGER system (Molecular Dynamics, Sunnyvale, CA, USA).

Ligand-binding assays

Yeast cell extracts were diluted with binding buffer and supplemented with protease inhibitors immediately before use. Ligand-binding was determined with [^{3}H]ponasterone A (specific activity 7.9 TBq·mmol^{-1}; a kind gift from. H. Kayser, Novartis, Switzerland) using a filter assay, as described previously [26]. Yeast extracts expressing C-terminal EcR or USP fusion proteins were incubated with 4–5 x 10^{-9} M [3H]ponasterone A for 1 h at room temperature, either separately or after mixing. For each sample, the nonspecific binding, determined by addition of 10-4 M nonlabelled 20-hydroxyecdysone, was subtracted. The purity of the [^{3}H]ponasterone A was checked routinely by HPLC analysis before use.

RESULTS

Spontaneous heterodimerization *in vivo*

The results listed in Table 1 (see also Figs 1 and 2) indicate that EcR and USP fragments lacking their own DNA binding domain can form heterodimers *in vivo*. Yeast cells cotransfected with plasmids expressing these fragment types in the form of fusion proteins with GAL4 activation and DNA binding domains, respectively, exhibited 0-galactosidase activity above background. Neither empty vector pairs nor combinations of empty vector with a matching vector coding for a fusion protein were able to bring about β-galactosidase activity above background levels (not shown), whether or not muristerone A was included as an inducer. This indicates that heterodimerization between the EcR–LBD and the USP–LBD containing fusion proteins is not the result of an interaction between the GAL4 activation and DNA binding domains. Coexpression of GAD-/GBD-fusion protein pairs containing only EcR or USP fragments did not lead to induced β-galactosidase activity (Table 1), even when muristerone A or the juvenile hormone analogue methoprene (10^{-5} M) was added to the culture (Table 1; T. Bergman, unpublished observation, respectively). This suggests, first, that these fragments do not homodimerize and that the reported homodimerization of full length EcR and USP [22,27] is coordinated and established by the respective multimeric binding motifs in DNA. Second, together with experiments in which the GBD-fusion proteins were expressed alone (results not shown), the EcR/EcR and USP/USP combinations indicate that the (putative) AF-2 functions within the LBD of EcR and USP are inactive in yeast cells, unlike in insect cells [28], presumably because the essential coactivators are missing [29]. As expected for a true mutual protein–protein interaction, reciprocal exchange of the GAL4 DNA binding and activation domain had no impact on the reporter gene regulation. However, the growth rate was always drastically reduced when the yeast cells were transformed with GBD–EcR fusion peptides encoding constructs. The phenomenon of `cell-toxicity' has been encountered previously with C-terminal progesterone receptor fragments fused to a ubiquitin peptide [30]. It thus cannot be attributed to the GAL4 moiety.

Table 1. Controls to two-hybrid experiments. Betagalactosidase activity is measured as averaged 'Miller Units' ± 95% confidence limit (number of experiments). ND, not determined.

DNA binding construct	Activation construct	Basal activity[a]	Induced betagalactosidase activity (10^{-6} M)[a]	Induced betagalactosidase activity ($1–5 \times 10^{-5}$ M)[a]
pCL1	pCL1 [b]	1244.00 ± 480.00 (5)	1021.00 ± 353.00 (3)	1508.00 (2)
pTD1-1	pVA3-1	55.10 ± 3.00 (8)	54.90 ± 2.40 (6)	59.20 ± 37.00 (3)
pAS2-1-*USP(172-508)*	pACT2-*EcR(375-652)*	0.41 ± 0.07 (29)	0.71 ± 0.21 (8)	7.90 ± 1.80 (28)
pAS2-1-*USP(172-508)*	pACT2-*USP(172-508)*	0.02 ± 0.02 (3)	0.06 ± 0.00 (1)	0.02 ± 0.00 (3)
pAS2-1-*EcR(375-652)*	pACT2-*USP(172-508)*	0.68 ± 0.10 (12)	1.04 ± 0.19 (13)	1.77 ± 1.37 (4)
pAS2-1-*EcR(375-652)*	pACT2-*EcR(375-652)*	0.03 ± 0.01 (5)	0.03 ± 0.01 (4)	ND

[a] Muristerone A concentration in yeast culture medium. [b] DNA binding and activation function in one protein.

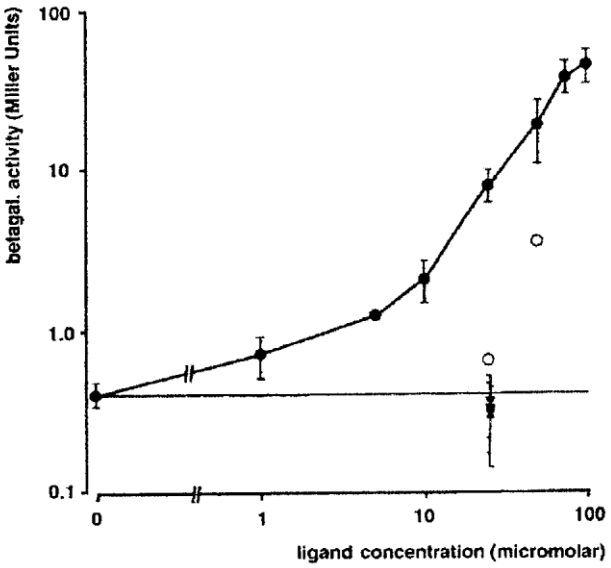


Fig. 2. Dose–response curve. Effect of increasing concentrations of muristerone A (●, solid line) on two–hybrid interaction between GBD-USP(172–508) and GAD-EcR(375–652). The effectof 20-hydroxyecdysone (▼), poststerone (■), RH5992 (▲), and of ponasterone A (○) is also shown at a concentration of 2.5 or 5×10^{-5} M. Ligand concentration refers to concentration of ecdysteroids or agonist in the yeast culture medium. Where error bars (spanning 95% confidence limits) are given, the points represent averages of at least three experiments, each.

The EcR and USP fragments normally employed for our studies comprised the whole ligand binding domains plus the carboxy-terminal portion of the hinge region. In the routine experiments, the F domain of EcR that lies on the C-terminal side of the LBD was removed as its presence did not affect heterodimerization appreciably (Fig. 1). Truncating helix 12 alone or helices 11 and 12 did not abolish heterodimerization (Fig. 1). It was only after the additional deletion of helix 10 that the EcR–LBD fragment became incapable of interacting with USP–LBD. At the N-terminal end, a gradual shortening of the EcR fragment resulted in a peculiar dichotomy: GBD fusion proteins tolerated a removal of the whole hinge region while GAD fusion proteins failed to heterodimerize when their hinge portion was further reduced by only 28 amino-acid residues (Fig. 1). We interpret this negative effect as a steric hindrance of the EcR–LBD functions through GAL4-AD when the intervening region was too small or missing.

Ligand-induced heterodimerization *in vivo*

Presenceofmuristerone Ain thecultivation medium caused a further increase in f~.galactosidase activity of cotransfected yeast cells (Table 1, Figs 1 and 2). As this effect was observed neither with a plasmid (pCL1) constitutively expressing GAL4 nor with a test two-hybrid pair (pTD1-1 and pAV3-1), it was concluded that the ligand promotes interaction between the EcR- and USP-LBDs rather than affecting reporter enzyme or fusion protein stabilities. Theinduction by ligand fully depends on the presence ofhelix 11 and 12 but not the EcR F domain. TheN-terminal portion ofthehingeregion is dispensible for ligand-induced heterodimerization. The question of whether the same holds true for the C-terminal regioncouldnot be assessed because ofthe technical problems mentioned above: fusion proteins with EcR fragments deprived of their hinge were either toxic to the yeast cells or did not heterodimerize, possibly because of steric hindrance.

The effect of muristerone A on heterodimerization between EcR–LBD and USP–LBD containing fusion proteins was clearly dose-dependent (Fig. 2). A small increase was evident at a ligand concentration of 10^{-6} M, and the highest concentration tested (10^{-4} M muristerone A) caused an ≈100-fold stimulation of reporter gene expression compared to the noninduced state. Higher muristerone A concentrations could not be tested because of solubility limitations. The effect of muristerone A on heterodimerization was ligand-specific and clearly related to the biological activity of the compounds tested. Besides muristerone A, only ponasterone A (for a

description of ecdysteroids; [2]) was able to promote heterodimerization. Expectedly, the inactive ecdysteroid poststerone [2] was ineffective. The natural ecdysteroid 20-hydroxyecdysone and the nonsteroidal ecdysone agonist RH5992 [31] also failed to promote heterodimerization at the concentration tested (2.5 x 10^{-5} M) probably because of lesser activity.

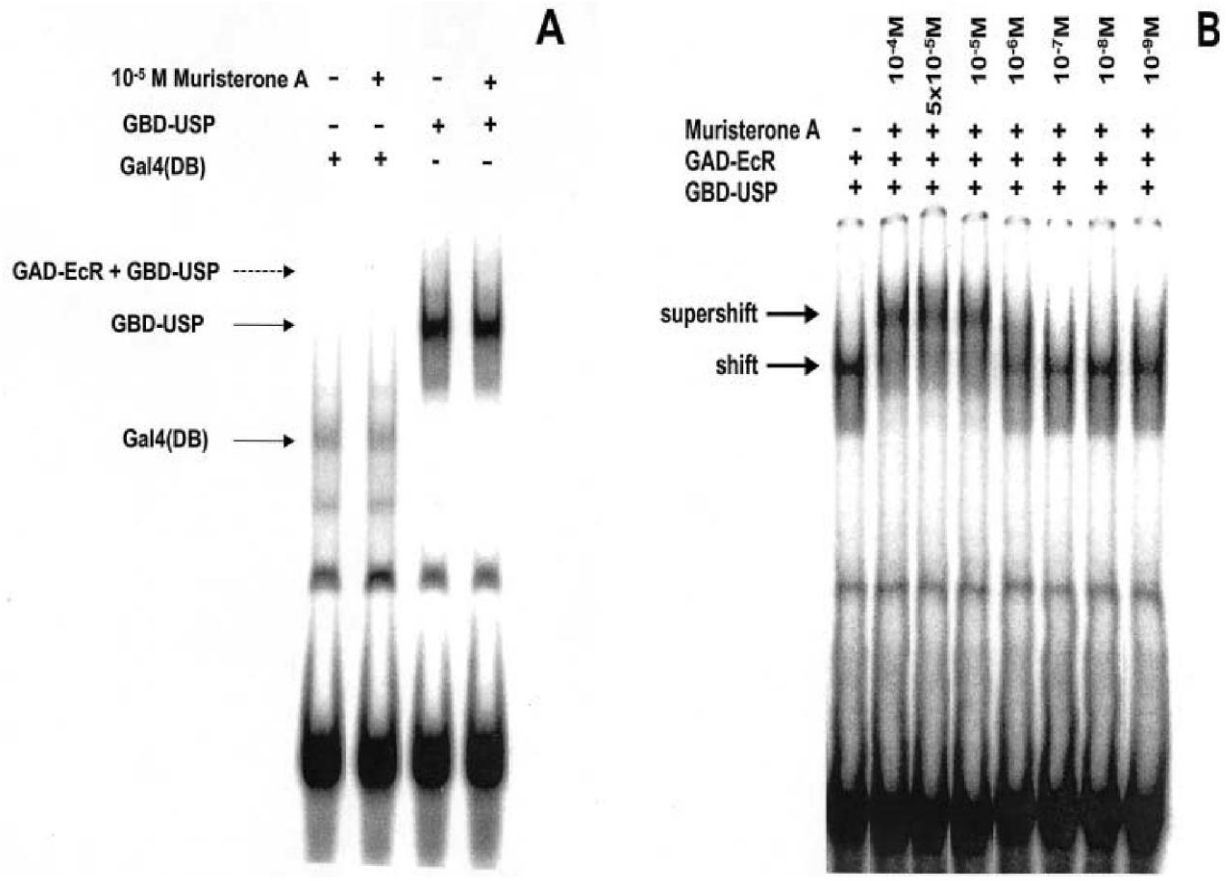


Fig. 3. Heterodimer formation *in vitro* between GBD–USP(172–508) (GBD–USP) and GAD–EcR(375–652) (GAD–EcR) as assayed by supershift-type (ST)-EMSA. (A) Controls. Arrows: Position of shifted bands. GAL4 (BD), DNA binding domain of GAL4. Dashed arrow: expected position of band if supershifted. (B) Influence of various muristerone A concentrations on heterodimerization between GBD–USP(172–508) and GAD–EcR(375–652). Arrows: position of shifted or supershifted bands, respectively.

Ligand-induced heterodimerization *in vitro*

To demonstrate protein –protein interaction directly, a modification of the electrophoretic mobility shift assay was devised, the supershift-type (ST)-EMSA, using extracts of transfected yeast cells. Thereby, the migration of a radioactively labeled DNA probe was further retarded when complexed with a second protein that in turn binds to the DNA binding protein. A GAL4 binding motif was used as a probe to test for the GBD–USP/GAD–EcR interaction. The investigated proteins corresponded to the fusion proteins constructed for two-hybrid assays. The DNA binding domain of GAL4 alone shifted the probe to some extent (Fig. 3A), whereas its fusion with USP(172–508) caused a further shift, which, however, was not supershifted by the addition of muristerone A (Fig. 3A, lanes 4 and 5). This excludes the possibility that muristerone A would produce the supershifts seen in Fig. 3B (lanes 2–4) by causing, for example, endogenous yeast proteins to interact with USP or by inducing multimerization of USP. In the absence of muristerone A, addition of the EcR fusion protein also did not cause supershifting (Fig. 3B). Supershifting caused by muristerone A was noticeable at 10^{-6} M (Fig. 3B) and reached saturation at 10^{-5} M (Fig. 3B). The effect of muristerone A is not due to a general property of ecdysteroids as the inactive ecdysteroid poststerone was unable to induce supershifting (results not shown). As far as tested, all those EcR fragments which did not react towards muristerone A by heterodimer formation *in vivo* also failed to react toward this ecdysteroid *in vitro* when investigated by ST-EMSA (Fig. 1B).

Ligand binding to EcR or USP fragments containing fusion proteins

As heterodimerization between C-terminal fragments of EcR and USP could be induced by the potent ecdysteroids muristerone A or ponasterone A, we wanted to investigate whether these fragments can bind ponasterone A in a ligandspecific manner. Therefore we conducted ligand binding experiments with the same yeast-expressed fusion proteins as used for the two-hybrid experiments, employing [^{3}H] ponasterone A as a probe. Extract of yeast cells expressing only GBD–USP(172–508) did not bind [^{3}H]ponasterone A as its binding level did not differ significantly from that of nonexpressing yeast extract (background). In contrast, extract containing only expressed GAD–EcR(375–652) showed a low level of [^{3}H]ponasterone A binding that was significantly higher than background. Binding to GAD– EcR(375–652) expressing extract was specific as it could be competed out by the active hormone 20-hydroxyecdysone. Supplementation of this extract with GBD-USP(172–508) increased specific [^{3}H]ponasterone A binding 10-fold. Ligand binding to the EcR fusion protein both in the presence or absence of the USP fusion protein was dependent on the existence of an intact helix 12. As far as tested, those EcR fusion protein types that could not be induced by ligand to heterodimerize also failed to bind [^{3}H]ponasterone A, in either the presence or absence of the USP partner (Fig. 1). This suggests that ligand-induced heterodimerization of the EcR and USP fragments is related to the ligand binding to EcR in presence but also in absence of USP.

DISCUSSION

The present work shows that fragments of EcR and USP containing a complete LBD but lacking their DNA binding domains are able to heterodimerize *in vivo* as well as *in vitro*. This heterodimerization obviously did not depend on a coordinated binding to a bipartite motif in DNA. Thus, heterodimer formation may take place in their free, nonchromosomally bound state. Moreover, the D- and T-boxes [32,33] that are part of a weaker dimerization interface in other nuclear receptors are not required for EcR and USP to heterodimerize. However, binding to specific EcREs and the presence of these boxes or other N-terminal regions may play an important role in modulating the EcR/ USP interaction, *in vivo* as well as *in vitro* [25]. Moreover, spontaneous heterodimerization in the absence of ligand between EcR and USP fragments requires neither the hinge region nor the F domain of EcR, and even helices 11 and 12 of its LBD are dispensible for a protein –protein interaction to occur. However, helix 10 which contributes substantially to the dimerization interface in nuclear receptors [34] must be present (Fig. 1).

There is little doubt that the induction of β-galactosidase activity in yeast cells cotransfected by GBD–USP(172–508) and GAD–EcR(375–652) results from an interaction of the two nuclear receptor fragment types rather than recruitment of endogenous activator proteins (see controls in Table 1; [29]). However, indirect interactions between the EcR and USP fragments being mediated by, for example, SRC-1-like proteins [35] cannot be excluded. Strikingly, such indirect interactions would still depend on the presence of EcRs helix 10. In any event, the frequency of EcR–USP heterodimers in untreated cotransformants is very low (i.e. less than 1% as inferred from the maximal reporter enzyme activity being inducible by muristerone A), (Fig. 2).

In contrast to spontaneous heterodimerization, ligandinduced heterodimerization requires the presence of helices 11 and 12. *In vitro* analyses like ST-EMSA and ligand binding experiments substantiate this notion. This interpretation conforms with the role ascribed to these helices in the `mouse-trap' mechanism that connects ligand binding to conformational changes of EcR–LBD [34]. The requirement of the C-terminal half of the hinge region for ligand-induced heterodimerization could not be assessed because of technical problems. Thus, the role of the hinge region for liganddependent effects and ligand binding remains a matter of conjecture (compare [36] with the alignment in [37]). The induction by ligand of heterodimerization between C-terminal EcR and USP fragments is not a general effect of steroidal or small lipophilic molecules (Fig. 2).

The concentration of muristerone A needed in the yeast culture medium to induce heterodimerization is relatively high. We estimated the half maximal muristerone A concentration (EC_{50}) to be about four orders of magnitude higher than determined with other assays [28,38]. However, it is a general experience with yeast two-hybrid assays that high steroid hormone concentrations must be applied to induce dimerization [39,40]. This is because yeast cells are equipped with efficient extrusion mechanisms to exclude small molecules such as

steroids [41] and, as recently shown, the ecdysone agonist RH5992 [42]. The muristerone A concentration required to induce heterodimerization as revealed by supershifting in ST-EMSAs was about the same (Fig. 3) as needed for demonstrating heterdimerization when using full-length EcR and USP molecules and natural EcREs [20–23,28]. The ponasterone A concentration used in ligand binding experiments was about 5 x 10^{-9} M and high enough to induce heterodimerization. This was concluded from the fact that ligand binding was enhanced approximately 10-fold if GAD-EcR was assayed in presence of GBD–USP which by itself does not bind [^{3}H]ponasterone A. Thus, the two LBDs must cooperatively interact by heterodimerization leading to an enhancement of ligand binding to the EcR partner. A concentration of 5 x 10^{-9} M is well within the range of biological and binding activity of ponasterone A as determined with full-length *Drosophila* receptors [20,28,37,43].

Even though ligand binding to EcR–LBD was low, it was significant and has been observed before with full-length EcR [20,43–45]. There exists a clear positive correlation between ligand binding and ligand inducibility of heterodimerization. All those EcR fragments that did not bind [3H]ponasterone A also failed to show an enhancement of heterodimerization by muristerone A (Fig. 1). This correlation is further noted in a series of point mutations in EcR– LBD (T. Bergman, M. Lezzi, M. Grebe, W. W. Hitchcock & V. C. Henrich, unpublished results). Furthermore, it is striking that EcR forms that do not bind ligand in the absence of USP cannot be induced to heterodimerize by ligand when expressed by *in vitro* translation (compare EcR of *Chironomus tentans* [23,45]).

The ability of *Drosophila* EcR to bind ligand as a `monomer' opens a new possibility of how ligand may act. In a schematic model, Yao and coworkers [20] proposed ecdysteroids to interact only with the EcR/USP heterodimer, either in its free or DNA-bound state. We suggest it may be possible that ecdysteroids interact with the EcR monomer, rendering it more competent to heterodimerize with USP. With such a possibility, ligand-induced dimerization could be an early step, clearly preceding the DNA binding step of the receptor complex.

Acknowledgements:
We thank Mrs Himmelberg for excellent techmcal assistance and W. Schaffner and A. Barberis (University of Zurich) as well as C. Elke, F. Thoma and J.-F. Mouillet (E.T.H Zurich) for technical advice and intellectual inputs. We are grateful to R. Palli (Rohm and Haas Company) and J.-M. Wurtz (University of Strasbourg) for providing us with preprints. M. G. and S. P. were supported by fellowships of DFG (Graduiertenkolleg: Oekotoxikologie und Umwelthygiene) and the `Düsseldorf Entrepreneur Foundation', respectively. This work was financially supported by grants from ETH Zurich to M. L. and T. B. as well as to M. V. and C. F., from the US Department of Agriculture to V. C. H., and from DFG (Sp 350/3–1) to M. S.-B. Finally, V. C. H. was a visiting professor at ETH Zurich during a research assignment from UNC-Greensboro.

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