

Functional studies on the ligand-binding domain of Ultraspiracle from *Drosophila melanogaster*

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Abstract:

The functional insect ecdysteroid receptor is comprised of the ecdysone receptor (EcR) and Ultraspiracle (USP). The ligand-binding domain (LBD) of USP was fused to the GAL4 DNA-binding domain (GAL4-DBD) and characterized by analyzing the effect of site-directed mutations in the LBD. Normal and mutant proteins were tested for ligand and DNA binding, dimerization, and their ability to induce gene expression. The presence of helix 12 proved to be essential for DNA binding and was necessary to confer efficient ecdysteroid binding to the hetero-dimer with the EcR (LBD), but did not influence dimerization. The antagonistic position of helix 12 is indispensable for interaction between the fusion protein and DNA, whereas hormone binding to the EcR (LBD) was only partially reduced if fixation of helix 12 was disturbed. The mutation of amino acids, which presumably bind to a fatty acid evoked a profound negative influence on transactivation ability, although enhanced transactivation potency and ligand binding to the ecdysteroid receptor was impaired to varying degrees by mutation of these residues. Mutations of one fatty acid-binding residue within the ligand-binding pocket, I323, however, evoked enhanced transactivation. The results confirmed that the LBD of Ultraspiracle modifies ecdysteroid receptor function through intermolecular interactions and demonstrated that the ligand-binding pocket of USP modifies the DNA-binding and transactivation abilities of the fusion protein.

Keywords: dimerization; DNA binding; ecdysteroid; nuclear receptor; transactivation; two-hybrid assay.

Article:

Introduction

The ligand-binding domain (LBD) of nuclear receptors participates in several functions: ligand binding, dimerization, hormonal regulation of transactivation and inter-action with comodulators. Among insects, two nuclear receptors, the ecdysone receptor (EcR) and Ultraspiracle (USP), an ortholog of the vertebrate RXR, form the functional ecdysteroid receptor. The interaction of 20-hydroxy ecdysone with this heterodimer sets off the transcriptional changes associated with insect larval and metamorphic development. Previous studies have conclusively demonstrated that ecdysteroids bind only to EcR, and there is less certainty about what ligand, if any, interacts with the LBD of USP. Other studies have shown that USP physically interacts with juvenile hormone III (JHIII) and can be induced by JHIII with specific promoters (Xu et al., 2002). Moreover, protein models suggest that JHIII can bind via specific residues in the USP (LBD) (Sasorith et al., 2003).

The combination of LBDs including the C-terminal part of the hinge region of EcR and USP show the same ligand-binding properties as reported for full-length receptors of *Drosophila melanogaster* (Grebe et al., unpublished; Yao et al., 1993) and tags used for purification of receptor proteins or other fused protein moieties have no effect (Grebe and Spindler-Barth, 2002). For the ecdysteroid receptor from *Chironomus tentans* it has been shown that purified receptor proteins and crude extracts possess the same K_d values (Grebe and Spindler-Barth, 2002). Therefore we conclude that hormone-binding capability is an autonomous function of the LBD and is influenced only by the dimerization partner.

In previous papers we characterized dimerization and hormone-binding properties of the LBD of the ecdysone receptor EcR (Lezzi et al., 2002; Grebe et al., 2003) using mutated receptor LBDs created by site-directed mutagenesis. We now investigate the impact of USP LBD mutations on ecdysteroid receptor function.

Although *Drosophila* EcR is able to bind hormone in the absence of USP to a small but significant degree, a tenfold increase in ecdysteroid binding is observed after addition of USP (Lezzi et al., 2002; Grebe et al., 2003) which is accompanied by an allosteric change of the ligand-binding pocket of EcR (Grebe et al., 2003). Whereas the three-dimensional structure of the EcR LBD is unknown, some relevant insights have been obtained from the structure of the USP(LBD) of *D. melanogaster* (Clayton et al., 2001) and *Heliothis virescens* (Billas et al., 2001). Their structures show that helix 12 of the USP LBD is fixed in an antagonistic position even in the absence of a specific ligand because of a hydrophobic interaction between helix 12 residues and other amino acids located in the loop between helix 1 and helix 3. The loop is highly conserved in Diptera and Lepidoptera but not in other arthropods. According to Billas et al. (2001), the non-specific binding of a phospholipid further stabilizes this apposition of helix 12, involving a different subset of amino acid residues than those associated with possible JHIII binding.

Using yeast two-hybrid analysis, previous studies have demonstrated that the LBDs of EcR and USP, fused to GAL4 activation (AD) and DNA-binding (DBD) domains, respectively, promote expression of a GAL4-inducible promoter. This response is enhanced significantly in a dose-dependent fashion by the addition of muristerone A (Lezzi et al., 2002). This system has been used previously in conjunction with biochemical methods to analyze the effects of several site-directed mutations on the functionality of the EcR LBD (Grebe et al., 2003). In this study, mutations in helix 12 of the USP LBD were studied for their effects on the ability of helix 12 to maintain its antagonistic position. Mutations in the USP ligand-binding pocket associated with fatty acid-binding were also evaluated for their effects on function.

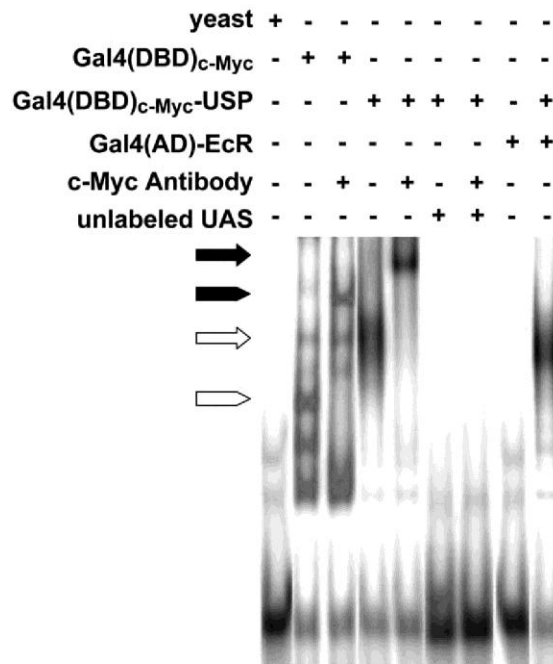


Figure 1 Gel mobility shift assays of yeast extracts with and without Gal4(DBD) fusion proteins using [³²P]-labeled oligonucleotides (UAS). The Gal4(DBD)-USP(LBD) band was shifted by a c-Myc specific antibody. Addition of unlabeled UAS (100-fold excess) demonstrates the specificity of DNA binding. *Yeast* indicates extracts prepared from non-transfected Y190 yeast cells. Gal4(DBD), Gal4(DBD) supershift with c-Myc-specific antibody, Gal4(DBD)-USP(LBD), Gal4(DBD)-USP(LBD) supershift with c-Myc antibody.

Results and discussion

Fusion proteins of USP(LBD) with Gal4(DBD) and Gal4(AD)-EcR(LBD) were used to study the influence of mutations in the LBD of USP on EcR(LBD)/USP(LBD) heterodimer function. First the induction of reporter

gene activity by the heterodimer was measured by two-hybrid assay and the influence of hormone determined. DNA binding, a prerequisite for reporter gene induction, was tested by gel mobility shift assays and ligand-binding studies were performed to determine the influence of the USP(LBD) on the binding of ecdysteroid with USP's dimerization partner, EcR. The results are summarized in Table 1.

Most of the mutated USP fusion proteins were highly reduced in their ability to induce reporter gene activity, although it was unclear from this result alone whether the effect reflected a loss of expression, a destruction of all activity, or a specific loss of transactivation capability. By contrast, ligand binding among EcR-mutated proteins ranges from nearly normal levels to no binding at all, thus demonstrating that general receptor function was not destroyed, but that specific functions were affected by each mutation (Grebe et al., 2003). Indeed, the mutated proteins included in this study were expressed and formed heterodimer complexes that showed at least some ligand-binding and DNA-binding activity. In other words, these USP mutations affected the ability of the GAL4 fusion protein to induce transcription, but did not eliminate other LBD functions.

DNA-binding properties were characterized by gel mobility shift assays using Gal4-specific UAS. The identity of the band representing Gal4(DBD)-USP(LBD)-complex with DNA was verified by a supershift in the presence of a c-Myc specific antibody recognizing the Gal4(DBD) fusion protein (Figure 1). This confirms that the retarded band was in fact due to USP(LBD) and not another transcription factor present in the yeast extract also interacting with the radiolabeled oligonucleotide. Competition with the corresponding non-labeled oligonucleotides also demonstrated the specificity of the DNA-receptor interaction. No corresponding band was observed in non-transformed yeast. No heterodimerization with GAL4(AD)-EcR(LBD) was visible in the absence of ecdysteroid (Figure 1; Grebe et al., 2003). A supershift indicating heterodimerization was observed only in the presence of muristerone A (Figure 3B, 5B, 6B).

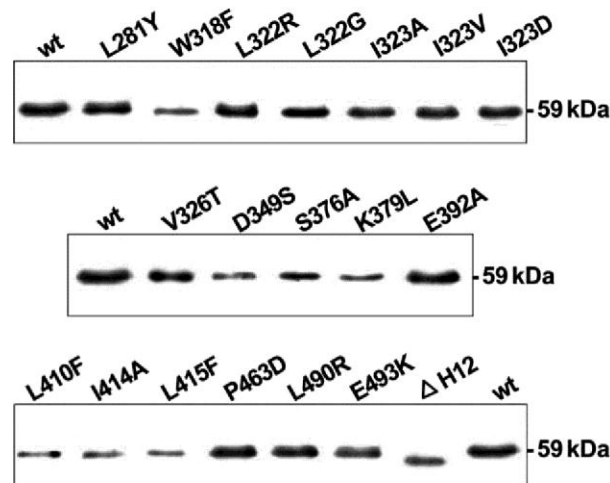


Figure 2 Western blots of wild-type and mutated Gal4(DBD)-USP(LBD): the expression level of individual USP fusion proteins varies considerably.

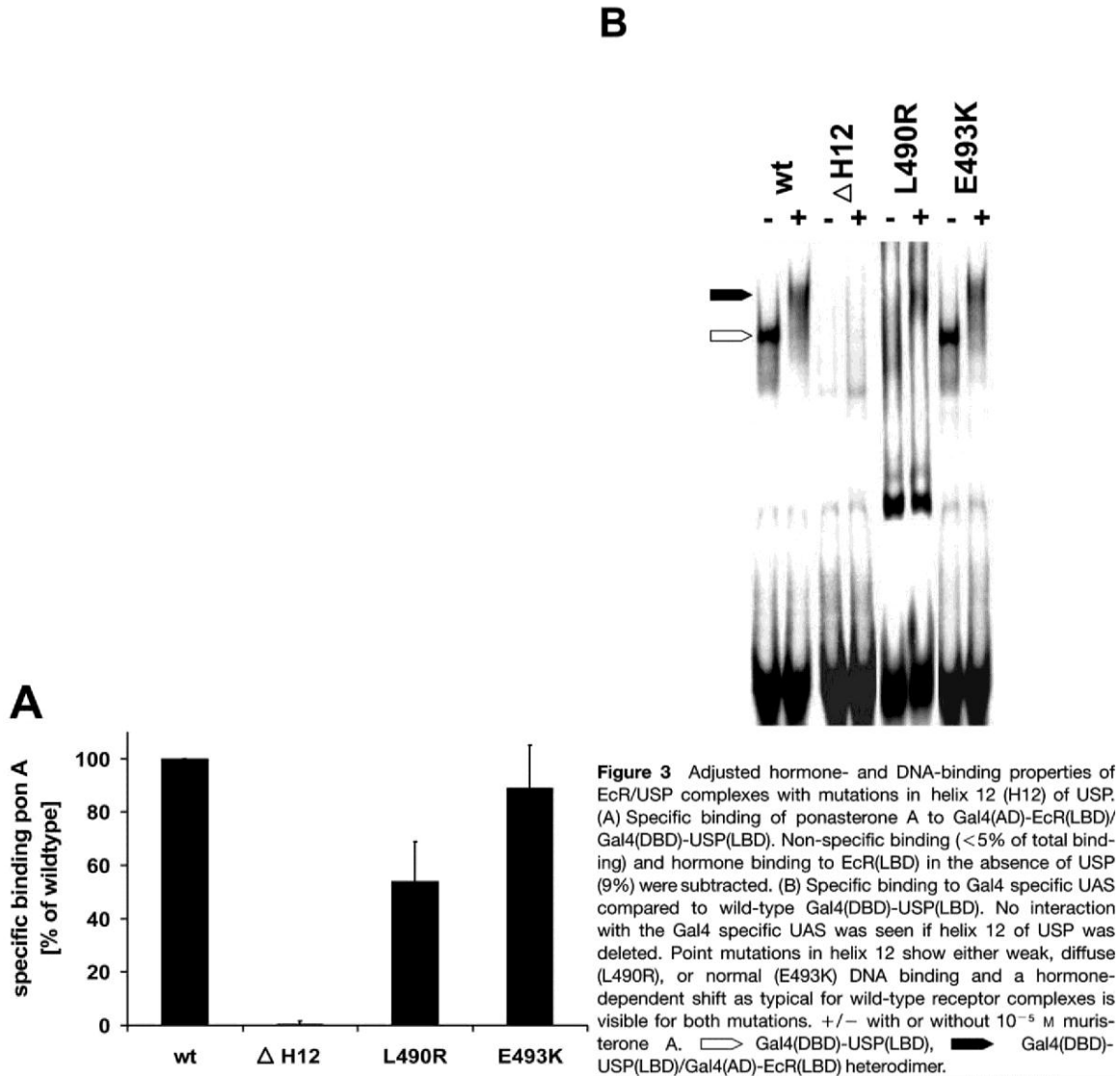
The intensities of the Western blot signals were quantified and compared with the signal of wild-type Gal4 (DBD)-USP(LBD) as described in the material and methods section.

The expression rates of various mutated USP fusion proteins varied approximately 6 fold for individual mutations (Figure 2). Therefore the receptor concentrations of the mutated USPs were determined by quantification of the Western blot signals using a standard curve derived from wild-type USP fusion protein to ensure that the same receptor concentration was used for subsequent biochemical studies.

Functional role of helix 12

Helix 12 is essential for hormone-dependent transactivation in most nuclear receptors and is also required for ligand binding in some receptors like EcR (Lezzi et al., 2002; Grebe et al., 2003). Helix 12 of USP is unique compared to all other nuclear receptors and is fixed to an antagonistic position even in the absence of a ligand (Billas et al., 2001; Clayton et al., 2001). Therefore we were especially interested in the functional role of helix

12. Truncation of USP's helix 12 abolished reporter gene induction on the two-hybrid assay (Table 1), interaction with DNA, and the hormone-binding capability of USP's dimerization partner, EcR (Figure 3). All of these receptor functions depend on the ability of the receptors to dimerize with each other. To measure dimerization directly, Gal4(DBD) fusion proteins with wild-type USP(LBD) and truncated USP(LBD) were further examined by gel filtration (Figure 4). Gal4 (DBD) eluted at a much lower apparent molecular weight (M_r) than calculated due to interactions with the gel in the absence of a Gal4 specific UAS. Dimerization mediated by the DBD was only observed in the presence of DNA. The elution pattern of the fusion protein with wild-type USP(LBD) showed a main peak even in the absence of DNA that corresponded to the predicted M_r of the homodimer. Since dimerization via the DBD is not possible without an UAS, dimerization in this case must be mediated by the USP(LBD). The truncated receptor protein eluted at an apparent molecular weight M_r that corresponds fairly well to the calculated value of the homodimer without UAS. This is in agreement with the EMSA which showed no DNA binding if helix 12 of USP(LBD) was deleted (Figure 3B).



X-Ray studies revealed that helix 12 in USP is fixed in an antagonistic position even in the absence of a specific ligand (Billas et al., 2001; Clayton et al., 2001). Two amino acids were tested: L490 which interacts with the L1-L3 loop and stabilizes the antagonistic position of helix 12 and E493 which is not engaged in fixation of helix 12. The results show that reporter gene induction was severely affected by both mutations, but that hormone binding, DNA binding and hormone-dependent hetero-dimerization still occurred at an observable level (Figure 3). Comparison of USP^{L490R} and USP^{E493K} showed that the antagonistic position of helix 12 was indispensable for reporter gene induction, but did not disrupt ligand binding. Since dimerization is a prerequisite for the observed level of ligand binding (Grebe et al., 2003), it was inferred that dimerization is nearly normal in

USP^{E493K} and only moderately reduced in USP^{L490K}. DNA binding as shown by EMSA was reduced for L490R but was normal for E493K. The data obtained with E493K demonstrate clearly that, besides dimerization and DNA binding, an additional factor is important for reporter gene induction.

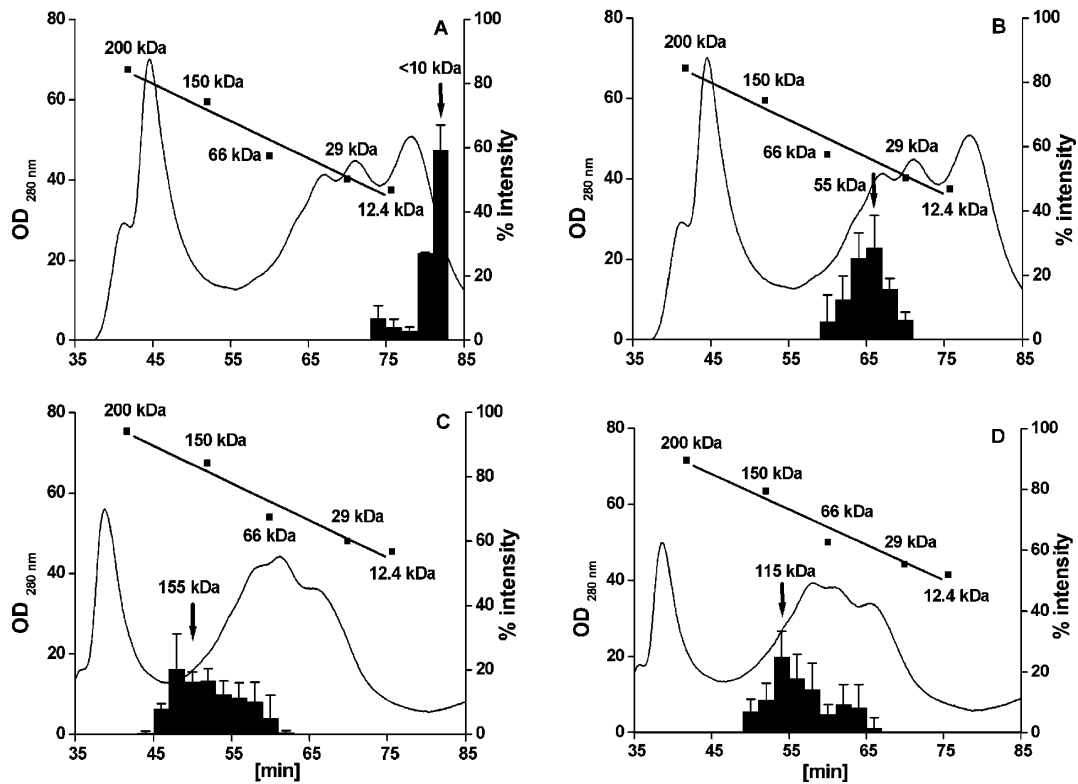


Figure 4 Gel filtration of Gal4(DBD) and fusions with wild-type and Δ H12-USP(LBD) based on protocols described in the materials and methods section.

(A) A 0.4 M NaCl extract of yeast cell expressing Gal4(DBD) was treated with DNase to prevent DNA-mediated dimerization. Gal4(DBD) eluted at an apparent M_r below 10000, which is considerably lower than the calculated M_r = 21500. (B) In the presence of Gal4-specific UAS GAL4(DBD) dimerizes and elutes at an apparent M_r = 55000. (Calculated M_r of the receptor dimer/DNA-complex: 69600.) (C) Gal4(DBD)-USP(LBD) wild type elutes at an apparent M_r = 155000, which is consistent with the formation of dimers bound to UAS (calculated M_r = 142000). (D) Deletion of helix 12 causes a shift to an apparent M_r = 115000, which corresponds to the calculated M_r = 113000 of the dimer of Gal4(DBD)-USP(LBD) Δ H12 in the absence of DNA binding.

A functional role of helix 12 according to the model proposed by Westin et al. (1998) requiring a flexible helix seems questionable. Based on homology models (Sasorith et al., 2003), an agonistic position preferred after docking of a putative ligand such as juvenile hormone seems possible, although this ligand does not contact amino acid residues of helix 12 directly (Sasorith et al., 2003).

Helix 12 of USP is centrally important not only for DNA binding and transactivation but also for hormone binding with the heterodimerization partner, EcR. The retained ability for dimerization in the absence of helix 12 in USP rules out the possibility that the observed effects are caused by a generally altered three-dimensional structure of the ligand-binding pocket that affects all receptor functions simultaneously. Rather, the influence of helix 12 in USP(LBD) on ligand binding of the heterodimerization partner EcR and on DNA-binding ability of the Gal4 (DBD) demonstrates both intra- and intermolecular allosteric effects of the USP(LBD). Interdomain interaction as well as intermolecular signaling are common in nuclear receptors as described previously (Scheller et al., 1998; Kumar et al., 1999) and may help to couple and coordinate different receptor functions. Gel filtration experiments confirmed that dimerization mediated by the Gal4(DBD) moiety depends on the presence of DNA and is required for reporter gene induction. By contrast, dimerization via the USP(LBD) is DNA-independent and is not necessarily linked with the transactivation potency of the receptor complex.

Mutations of amino acids that contact the non-specifically bound phospholipid

Another set of mutations is characterized by substituted residues that form a salt bridge with the hydrophilic end of a phospholipid such as the one that copurified with USP extracted from *E. coli* (Clayton et al., 2001). While

the phospholipid has not generally been viewed as a natural ligand for USP, it apparently is important for stabilizing USP structure during purification. Among the putative phospholipid-binding sites, S376 is universally conserved among known USP proteins, but K379 is not. In both cases, mutational changes severely reduced the activity in two-hybrid experiments, and hormone inducibility was also eliminated at a hormone concentration of 25 mM (Table 1). The reduced hormone binding (Figure 5A) in combination with the weak DNA binding (Figure 5B) may be partially responsible for this effect, although weak heterodimerization in the presence of hormone was still observed in EMSAs. However, S376A did not completely eliminate hormone-induced two-hybrid activity. In fact, USP^{S376A} evoked a higher level of induction at an elevated dosage of muristerone A (80 μM=13.94-fold induction, 150 μM=51.60-fold induction) further revealing a residual level of ligand-binding activity in the heterodimer.

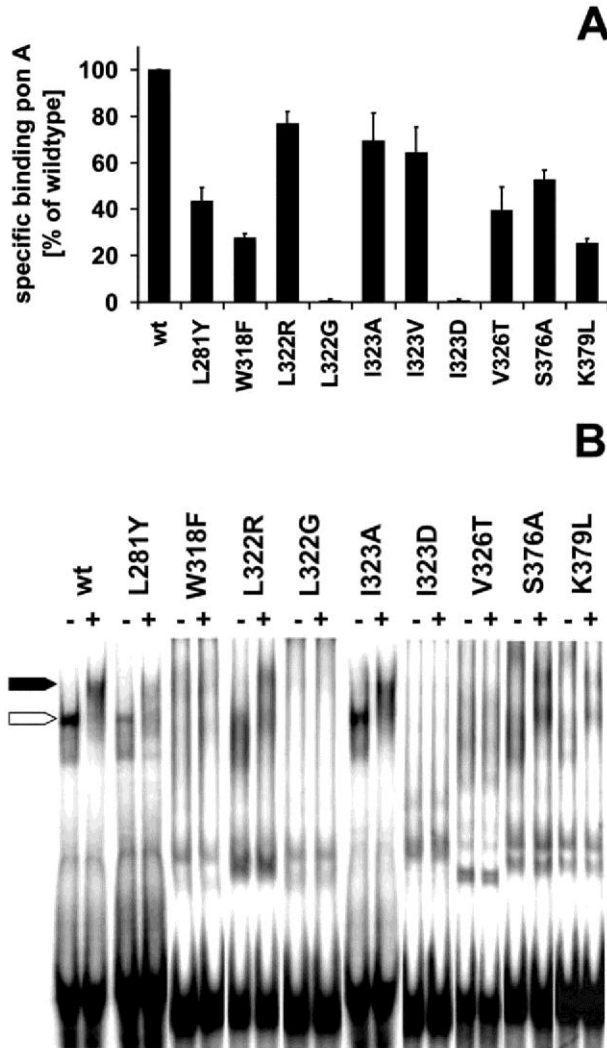




Figure 5 Hormone- and DNA-binding properties of EcR/USP complexes with mutations of putative contact sites in USP for the non-specifically bound phospholipid. (A) Specific binding of ponasterone A to Gal4(AD)-EcR(LBD)/Gal4(DBD)-USP(LBD). Non-specific binding (<5% of total binding) and hormone binding to EcR/LBD in the absence of USP (9%) were subtracted. (B) DNA binding of Gal4(DBD)-USP(LBD) varies considerably among mutated USP fusion proteins. The hormone-dependent shift, indicative of hormone-induced heterodimerization is visible with USP fusion proteins, which show sufficient DNA binding of the mutated Gal4(DBD)-USP(LBD) in the absence of hormone. EMSAs were performed in the presence of Gal4(AD)-EcR(LBD). +/- designates with or without 10⁻⁵ M muristerone A.  Gal4(DBD)-USP(LBD),  Gal4(DBD)-USP(LBD)/Gal4(AD)-EcR(LBD) heterodimer.

A third set of mutations was created for hydrophobic residues that lie within the ligand-binding pocket of USP (L281, W318, L322, I323, V326) and apparently contact the phospholipid via hydrophobic interactions. Each of these residues is partially to highly conserved among USP proteins in insects. Four of these mutations eliminated both basal and ligand-induced activity on the two-hybrid assay, whereas two different substitutions of a residue that lies at the base of the USP ligand-binding pocket, I323A and I323V, behaved as superinducers on the two-hybrid assay (Table 1, Henrich et al., 2000). Position 323 is occupied by an amino acid that encodes a leucine or isoleucine in all known insect USPs. Ligand-induced activity is much higher at both low (Table 1) and high dosages of muristerone A (unpublished results). This is especially remarkable because ligand binding was slightly reduced by these same mutations. The opposite effects of these mutations compared to the other amino acids involved in phospholipid binding suggests an additional or different role for I323. In fact, both the alanine and valine substitutions of I323 remove the large side chains that protrude into the ligand-binding pocket. Other substitutions, notably I323D, which introduces a negative charge, and I323F (data not shown)

with a rather bulky side chain are deleterious and eliminate yeast two-hybrid activity. Hormone binding is reduced (Table 1, Figure 5A) in heterodimers carrying a mutation of amino acids engaged in phospholipid binding (Clayton et al., 2001; Billas et al., 2001).

The size of the side chain seems to be very critical in L322, because reduction in L322G caused a complete loss of receptor activities. The length of the side chain was sufficient to allow DNA binding in USP^{L322R}, and seems to be more important than the introduction of an additional charge.

Table 1 Effect of mutations in the ligand-binding domain of USP on reporter gene activity (two-hybrid assay), ecdysteroid- and DNA-binding properties of Gal4(AD)-EcR(LBD) and Gal4(DBD)-USP(LBD) fusion proteins.

Mutational site	Position	% wild type no hormone	Two-hybrid assay		Ligand binding ^a (% EcR/USP wild type ± SD)	EMSA
			% wild type 25 μm murA	Fold induction		
Wild type		100	100	8–25	100	(+ +/+ +) ^b
L281Y	H3	52	32.2	2.2	43 ± 6	(+ +/+ +)
W318F	H5	1.2	0.1	1	28 ± 2	(-/-)
L322G	H5	0.9	0.03	1	0 ± 2	(-/-)
L322R	H5	0.7	0.1	1.81	77 ± 5	(+ +/+ +)
I323A	H5	70.5	354.4	157.4	69 ± 12	(+ +/+ +)
I323V	H5	52.0	564.4	176.4	64 ± 11	n.d.
I323D	H5	1.42	0.2	1	0 ± 2	(-/-)
V326T	H5	0.7	0.1	1	40 ± 10	(+ /+)
D349S	L5–L6	1.5	0.4	14.66	36 ± 10	(+ /+)
S376A	H6	1.4	0.1	1	53 ± 4	(+ /+)
K379L	H6	1.2	0.2	1	25 ± 2	(+ /+)
E392A	H7	1.0	0.0	1	5 ± 3	(-/-)
L410F	H8	0.7	0.1	1	43 ± 6	(+ /+)
I414A	H8	1.0	0.1	1	65 ± 8	(+ /+)
L415F	H8	0.4	0	1	64 ± 10	(+ /+)
P463D	H10	0.6	0	1	0 ± 2	(-/-)
L490R	H12	1.45	0.3	3.03	54 ± 15	(+ /+)
E493K	H12	36.0	4.0	6.36	89 ± 16	(+ +/+ +)
ΔH12	H12	1.34	0	1	0 ± 2	(-/-)

^a Ligand binding to EcR in the absence of USP is 9 ± 3% and is subtracted.

^b DNA binding in the absence of hormone/supershift in the presence of hormone (+ +, strong signal; +, weak signal; -, no signal; n.d., not determined).

Amino acids of the ligand-binding pocket of USP with no contact to a putative ligand

In USP^{D349S}, hormone binding to EcR was reduced indicating that hormone-induced dimerization is still possible. DNA binding was reduced only partially, but two-hybrid activity was abolished completely. This example revealed that an additional condition for the receptor heterodimer is necessary for transactivation of reporter gene activity. Its failure also shows that specific functions are selectively destroyed by individual mutations.

The E392 residue is highly conserved among all USP receptors and we, therefore expected that this amino acid plays an essential role for USP function. Ligand binding of the heterodimer carrying the USP^{E392A} mutation was almost destroyed and two-hybrid activity was severely reduced, but not completely abolished (Figure 6A).

For L410F, I414A, L415F, a hormone-dependent super-shift indicating heterodimerization was still observed although the DNA-binding capacity was reduced in the absence of hormone. This was further confirmed by the observation that ponasterone A binding to EcR was reduced in the presence of these mutated USPs. Two-hybrid activity was completely abolished, thus underlining the general importance of amino acids in the ligand-binding pocket of USP for reporter gene induction and indicating once more that ligand and DNA binding alone is not sufficient for two-hybrid activity.

P463 is located in helix 10, which lies along a dimerization interface. It is reasonable to assume that exchange of proline by asparagine destroys the dimerization capability of USP(LBD). All receptor functions were

abolished except DNA binding, demonstrating that dimerization mediated by the LBD of USP is not required for DNA binding of the fusion protein, but that dimerization of the Gal4 moieties is sufficient.

Two-hybrid assay

The behavior of the USP mutations on the yeast two-hybrid assay revealed a different pattern of effects than previously noted for analogous EcR mutations (Lezzi et al., 2002; Grebe et al., 2003). In the former case, mutations of the EcR LBD evoked a range of effects. Many of the mutations impaired both basal and ligand-induced functions indicating that a basal process is disturbed.

Two-hybrid assays are generally considered to be dimerization assays. This may be justified, when the bait is the same and the interaction with different preys is examined as was shown with the EcR mutations which were coupled to the activation domain of Gal4 and probed with the wild-type Gal4(DBD)-USP(LBD). In experiments presented in this paper the bait itself was modified, meaning that mutated USP(LBD)s fused to the DNA-binding domain of Gal4 were used. Consequently, the Gal4(DBD) is not considered as a constitutive functional unit for monitoring reporter gene activity. Instead, its functionality, especially DNA binding, is modified by alterations of the USP(LBD) to which it is fused.

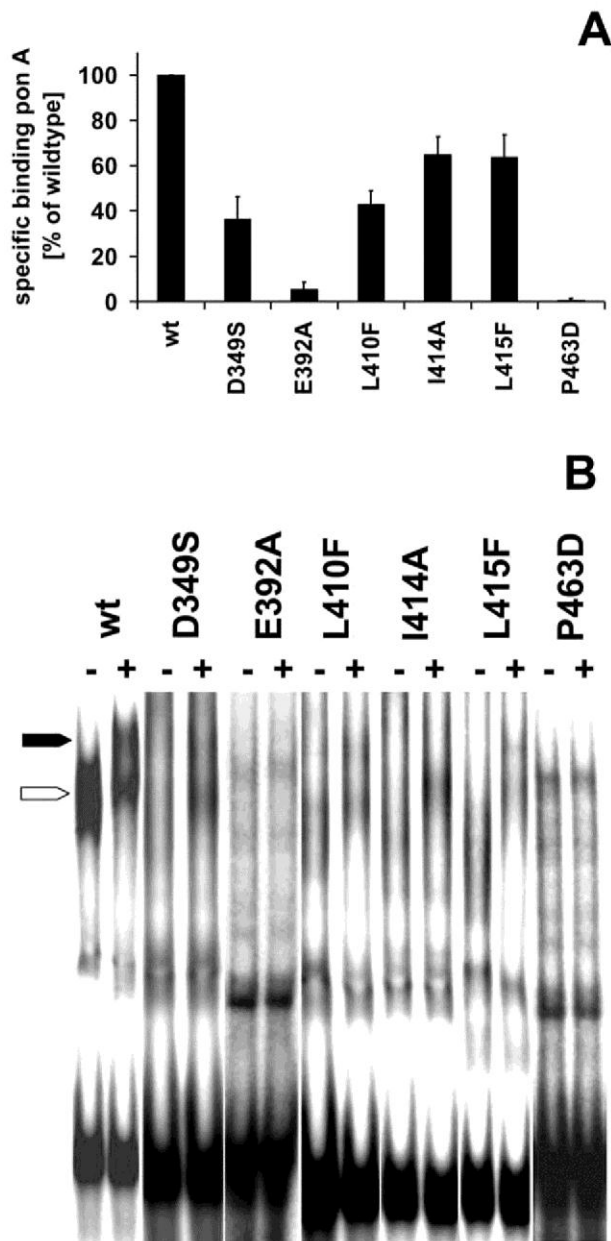


Figure 6 Hormone- and DNA-binding properties of EcR/USP complexes with mutated amino acids of the USP ligand-binding pocket which do not contact the phospholipid. (A) Specific binding of ponasterone A to Gal4(AD)-EcR(LBD)/Gal4(DBD)-USP(LBD). Non-specific binding (<5% of total binding) and hormone binding to EcR/LBD in the absence of USP (9%) were subtracted. (B) DNA binding of Gal4(DBD)-USP(LBD). EMSAs were performed in the presence of Gal4(AD)-EcR(LBD). +/- designates with or without 10^{-5} M muristerone A. \square Gal4(DBD)-USP(LBD), \blacksquare Gal4(DBD)-USP(LBD)/Gal4(AD)-EcR(LBD).

DNA binding of Gal4(DBD) fused to mutated USP(LBD)

DNA binding, a prerequisite for induction of reporter genes, is impaired or even abolished in several mutated USP fusion proteins. As shown by EMSA (Figure 6B), the positive impact of wild-type USP(LBD) on DNA binding of the Gal4 moiety depends not only on the presence but also on the unique antagonistic position of helix 12 in Ultraspiracle. These results may explain the failure to observe any effects of helix 12 USP mutations used in transfection studies with insect cells (Hu et al., 2003). Because the mutated USP proteins may have lost their ability to interact with DNA, only the positive DNA binding of endogenously expressed wild-type USP would be measured in the insect cells, thus masking the effect of the mutated protein.

Dimerization, ligand and DNA binding are not sufficient for transactivation

A comparison of the two-hybrid data and the results of DNA- and ligand-binding tests of some mutations (USP^{S376A}, USP^{I323V} and USP^{I323A}) are not always in parallel. The superinduced reporter gene levels evoked by USP^{I323V} and USP^{I323A} may be interpreted as the consequence of enhanced dimerization that offsets a reduction in ligand-binding capability by the EcR/USP dimer, although direct experimental proof is missing. Alternatively, the results may indicate an additional regulatory step necessary for transactivation besides ligand and DNA binding or dimerization partners such as a comodulator. This is illustrated by USP^{E493K} which clearly demonstrates that despite retaining its dimerization, hormone- and DNA-binding abilities, an additional factor or comodulator is required for transactivation as pro-posed already by Tran et al. (2001) and VomBaur et al. (1998) for vertebrate receptors. Mutations that allow a normal fold-induction but at a highly reduced quantitative level and the superinducer mutations indicate that USP plays an active role in transactivation of the reporter gene and that its function is not restricted to dimerization only.

Dimerization in nuclear receptors is mediated by several dimerization interfaces. Our aim is to study each dimerization site and its regulation separately to evaluate the impact of each site. In the present study we concentrated on the dimerization properties of the ligand-binding domains of EcR and USP. In the Gal4 fusion proteins the A/B domains of EcR and USP were absent. Therefore the dimerization properties of this receptor domain (Rymarczyk et al., 2003) did not influence our results. We are currently determining the influence of the C and D domain on dimerization properties.

Materials and methods

Yeast strain

Saccharomyces cerevisiae strain Y190 was cultured according to manufacturer instructions (Clontech Laboratories, Palo Alto, CA, USA). Cells were transformed with lithium acetate (Guthrie and Fink, 1991) and selected by auxotrophy for tryptophan (pAS2-1) and leucine (pACT2), respectively.

Yeast expression plasmids

DNA encoding the C-terminal part of the D domain and the E domain of the *Drosophila* ecdysone receptor EcR (aa 375–652) was cloned into the expression vector pACT2 (Li et al., 1994; Lezzi et al., 2002) resulting in a Gal4(AD)-EcR(LBD) fusion. For expression of Gal4(DBD)-USP(LBD) the corresponding domain of Ultraspiracle (aa 172–508) was cloned either into the vector pGBKT7 (Louvét et al., 1997) or into the vector pAS2-1 (Harper et al., 1993; Lezzi et al., 2002).

Construction of site-directed mutations

All site-directed mutations in Gal4(DBD)-USP(LBD) were carried out with a site-directed mutagenesis kit (Stratagene, Amsterdam, Netherlands) following the manufacturer's instructions. Briefly, the plasmid vector is amplified by polymerase chain reaction with a pair of oligonucleotide primers in which the site of mutagenesis is included, so that during replication cycles, the mutated plasmid is the predominant PCR product. The original template strands are methylated and destroyed by the restriction enzyme, DpnI, and the new plasmids were transformed into *E. coli* and recovered. All mutated products were verified by sequencing and also checked for possible second site mutations in both the USP(LBD) and the GAL4(DBD) portion of the plasmid. In Δ H12 aa 490–508 were deleted. The oligonucleotides used to produce the mutations described are available upon request.

Yeast two-hybrid conditions

All wild-type and mutated plasmids were tested according to the procedures described by Lezzi et al. (2002), except that yeast transformations were carried out with the Frozen EZ Transformation II kit (ZymoResearch; Orange, CA, USA) following manufacturer's protocols. In all cases, three colonies were tested for each replicate, and three replicates were performed for each mutation. Additionally, all mutational results were compared with simultaneously run wild type controls, since the absolute level of lacZ activity reported on the yeast two-hybrid assay varies even under controlled conditions, though general levels of relative performance were stable.

Preparation of yeast extracts

Single colonies (not older than 4 days) of yeast transformants carrying the expression plasmids were picked and cultured at 30°C overnight in 5 ml selective medium containing 2% glucose with vigorous shaking (150–200 rev./min) to disperse the cells thoroughly. They were then diluted in 50 ml YPD medium (20 g/l peptone, 10 g/l yeast extract, 2% glucose) and grown under the same conditions until the OD₆₀₀ reached 0.6–0.8. Cells were harvested by centrifugation (1500 g, 5 min, 4°C) in pre-chilled tubes and washed with 50 ml ice-cold binding buffer (20 mM HEPES, 20 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.9). The pellets were frozen in liquid nitrogen for approximately 20 s and disrupted for 2 min at 2000 rev./min using a Micro-dismembrator S (B. Braun Biotech International, Melsungen, Germany). After thawing, homogenates were diluted with binding buffer and supplemented with a mixture of protease inhibitors (aprotinin, leupeptin, pepstatin, benzamidine, antipain, chymostatin; final concentration 2 µg/ml each and 1 mM phenylmethyl-sulfonyl fluoride) immediately before use. After short treatment with ultrasonic power (microtip 2×2 s, 90 Watt, Branson Sonifier, B-12; Branson, Danbury, CT, USA) the samples were centrifuged (100 000 g, 1 h, 4°C) and frozen in aliquots at -80°C until use.

Western blot and quantitative determination of fusion proteins

Yeast extracts were diluted with sample buffer (final concentration: 100 mM Tris, 3% SDS, 2% 2-mercaptoethanol, 10% glycerol, 0.05% bromphenol blue, pH 8.8) and boiled for 3 min (Laemmli, 1970). 10–20 µg protein (Bradford, 1976) were applied on each lane of an acrylamide gel and subjected to electrophoresis using a Hoefer miniVe, (300 V, 15 mA; Amersham Biosciences, Freiburg, Germany). Gels were electroblotted on nitrocellulose membranes (BA 85, 45 µm pore size, Schleicher and Schuell, Dassel, Germany) according to Khyse-Andersen (1984). The membranes were soaked in blocking buffer (5% milk powder, 1 % fat in 20 mM Tris-HCl, 137 mM NaCl, 0.1 % Tween 20, pH 7.6, 0.02% Thimerosal). EcR(LBD) fusion protein was probed with a Gal4(AD)-specific antibody (5398-1, Clontech Laboratories) diluted in blocking buffer 1:5000. USP fusion proteins were probed either with Gal4(DBD) specific antibody (# sc-577, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:100 or with c-Myc specific antibody (3800-1, Clontech Laboratories) diluted 1:1000. Specific Western signals were detected with peroxidase conjugated secondary antibodies diluted 1:1000 (anti-mouse IgG, Sigma A-5906, Sigma-Aldrich, Taufkirchen, Germany) or 1:500 (anti-rabbit IgG, Sigma A-6667), in TBS-T (20 mM Tris-HCl, 137 mM NaCl, 0.1 % Tween 20, pH 7.6). Detection and quantification was done as described in detail by Rauch et al. (1998). Specific signals were imaged with Fluor-S MultiImager (Bio-Rad Laboratories, Hercules, CA, USA) and evaluated with the software Multi-Analyst/PC (Version 1.1, Bio-Rad Laboratories).

Ligand-binding assays

Yeast extracts were diluted with binding buffer and supplemented with protease inhibitors as described above immediately before use. Ligand binding was determined with [³H]-ponasterone A (specific activity 2.5 TBq/mmol; kind gift of Dr H. Kayser, Syngenta, Basel, Switzerland) using a filter assay described in detail previously (Turberg and Spindler, 1992). Radiolabeled ponasterone A was used, because the affinity to EcR is higher compared to 20-OH-ecdysone. Fusion proteins were quantified by Western blots as described above and normalized based on wild-type expression levels. Receptor proteins were mixed with 4–5 nM [³H]-ponasterone A and incubated for 1 h at room temperature. Non-specific binding determined in the presence of 0.1 mM non-labeled 20-OH-ecdysone was subtracted. Purity of [³H]-ponasterone A was checked routinely by HPLC analysis. Ligand-binding data of mutated receptors were expressed as % of wild-type hormone binding

(=100%).

Electrophoretic mobility shift assay (EMSA)

The oligonucleotides dgal 1: 5'-GATCGCACAGTGCCGGAGGACAGTCCTCCGGTTCGAT-3' and dgal: 5'-GATCATCGAACCGGAGGACTGTCCTCCGGCACTGTGC3' were formed by annealing 5' extensions using the sequence GATC labeled with [α^{32} P]-dCTP by fill-in reaction with Klenow polymerase.

The reaction mix contained EMSA buffer w20 mM HEPES, pH 7.4, 100 mM KCl, 5% (v/v) glycerol, 2 mM dithiothreitol, 0.1 % NP-40x, yeast cell extracts with EcR or USP fusion proteins, 1 μ g non-specific competitor poly[dIdC] and approximately 10 f mol labeled oligonucleotide. 10^{-5} M muristeroneA (final concentration) was used where indicated. Muristerone A was used instead of 20-OH-ecdysone, because the affinity to EcR is higher. After incubation for 30 min at room temperature, the samples were loaded on a 5% nondenaturing polyacrylamide gel in 0.5xTBE (45 mM Tris, 45 mM boric acid, 0.5 mM EDTA pH 8.0) and separated at 10 V/cm for 2 h. Gels were dried, scanned with a phosphorimager (Fluorescent Image Analyzer FLA-3000 series, FUJIFILM, Düsseldorf, Germany) and evaluated with software Aida Image Analyzer 3.25 (Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany).

Chromatography Samples were prepared exactly as described for gel shift experiments. 500 μ g protein (1 μ g/ μ l) were incubated for 30 min at room temperature and subjected to size exclusion chromatography (Superdex 200 HR 10/30, Amersham Pharmacia Biotech, Uppsala, Sweden) using an Äkta™ purifier (Amersham Pharmacia Biotech). After equilibration of the column with elution buffer (20 mM K-phosphate, pH 7.4, 50 mM KCl, 1 mM EDTA, 10% glycerol), the sample was loaded on the column and separated (flow rate of 0.25 ml/min, 4°C). Fractions were collected (500 μ l) and proteins precipitated with 7.5% TCA (final concentration). A molecular weight marker kit (Sigma-Aldrich) was used to calibrate the column. Fractions were subjected to Western blotting with Gal4(DBD) or c-Myc specific antibodies and quantified as described above.

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