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Ligand control of interaction *in vivo* between ecdysteroid receptor and ultraspiracle ligand-binding domain

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Ecdysteroids (Ecs) enhance the formation of Ec receptor–ultraspiracle protein (EcR–USP) heterodimers which regulate gene transcription. To study EcR–USP heterodimerization, fusion proteins were constructed from the LBDs (ligand-binding domains) of *Drosophila* EcR or USP and the activation or DNA-binding region of GAL4 respectively. Reporter gene (*lacZ*) activation was fully dependent on the co-expression of the two fusion proteins and thus constitutes a reliable measure for the interaction *in vivo* between the two LBDs in the yeast cell. To identify structures involved in heterodimerization, a total of 27 point mutations were generated in the EcR and USP LBDs at selected sites. Heterodimerization and its inducibility by ligand were mainly affected by mutations in the dimerization interface and in the ligand-binding pocket of EcR respectively. However, also mutations not located in these structures or even in the LBD of USP influenced ligand-dependent heterodimerization. Together with previously reported ligand-binding studies, the existence of such local, intra- and inter-molecular mutation effects suggest that ligand-induced dimerization results from a synergistic interaction between ligand-binding and heterodimerization functions in EcR LBD, and that it depends on global features of the LBDs of EcR and USP and on their mutual surface compatibility.

Key words: *Drosophila melanogaster*, ecdysone, heterodimerization, nuclear hormone receptor, site-directed mutagenesis, yeast two-hybrid analysis.

INTRODUCTION

Ecdysteroids (Ecs) govern insect development [1,2], mainly by means of the so-called EcR (Ec receptor) complex, a heterodimer [3] between EcR [4] and USP (Ultraspiracle protein) [5–7], which acts through regulation of gene activity. It has been shown by experiments *in vivo* and *in vitro* that heterodimerization of EcR with USP is ligand-controlled ([8,9], and references therein). Ligand-induced dimerization between EcR and USP may thus constitute an important step in the Ec-controlled process, leading to the functional EcR complex [9].

The present study shows for the first time some general principles underlying Ec-controlled interactions between the LBDs (ligand-binding domains) of EcR and USP at a molecular level and under conditions occurring in a living cell. Twenty-seven site-directed mutations of selected usually conserved amino acid residues located in various functionally different structures of the EcR and USP LBDs were tested for their effects on spontaneous and ligand-induced heterodimerization by the yeast two-hybrid system. This *in vivo* system is devoid of interfering nuclear receptors, co-activators and co-repressors [10], and monitors even weak and ephemeral interactions under conditions which exclude artefacts *in vitro* (see e.g. [11,14], for critical role of buffer composition) and ensure optimal control of EcR–USP interaction. Our results demonstrate that the mutations mainly affected the targeted function, but also influenced the behaviour of distant structures, even of the non-mutagenized heterodimerization partner. These intra- and inter-molecular effects suggest that the various regions of the LBDs are functionally linked and regulated across the entire trinary complex. They imply that ligand binding causes not only local, but also global transitions of the EcR LBD to control the interaction with the USP LBD.

EXPERIMENTAL

Yeast strains and two-hybrid assay

Yeast strain, growth and hormone treatment were the same as described previously [9]. For induction experiments, the cultures were split; one half received the potent Ec muristerone A, and the other half solvent only. The muristerone A concentration in the culture medium was always 25 μM, which is much higher than the effective intracellular concentrations, but still far from saturating the heterodimerization reaction (see [9]).

Expression plasmids and site-directed mutagenesis of EcR and USP LBDs

The wild-type two-hybrid components are as described previously [9]. Selected amino acid residues in the LBD of EcR were mutated using the QuikChange® Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A.), with plasmids pACT2-EcR(375-652) or pST-EcR(2-878) [12] as a template. The *Stu*I–*BSi*WI fragment of wild-type EcR in pACT2-EcR(375-652) was replaced by the corresponding mutated fragment generated by PCR and the primers listed in the Supplementary Table 1 (http://www.BiochemJ.org/bj/378/bj3780779add.htm). Similarly, site-directed mutations were introduced into the LBD of USP using pSCT-dUSP [13] as a template and by replacing the *Cla*I–*Bss*HII fragment in pAS2-1-USP(172-508). The mutations at the given

Abbreviations used: Ec, ecdysteroid; EcR, Ec receptor; USP, ultraspiracle protein; LBD, ligand-binding domain; DIF, dimerization interface; LBP, ligand-binding pocket; MT, 'mouse-trap'; RXR, 9-*cis*-retinoic acid receptor; RAR, retinoic acid receptor; Ef_{lig}, ligand effect; M.U., Miller units.

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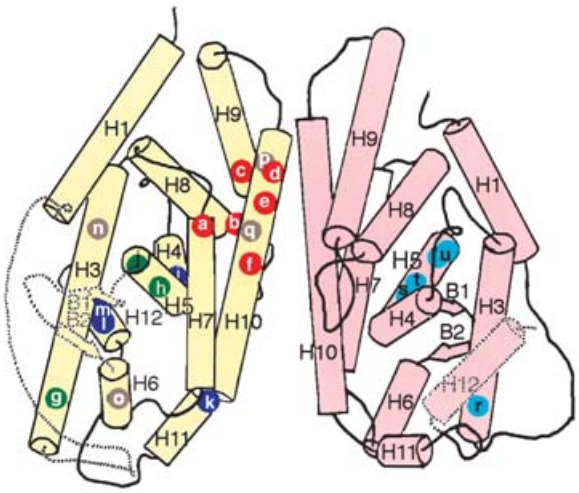
A



B

mutant	two-hybrid partners [1] (mutated partner in colour)	heterodimerization			main impact [4]	
		spontaneous M.U.[2]	lig.-induced M.U.[2]	ligand effect Ef_{lig} [3]	spontan. heterodimer.	ligand effect Ef_{lig}
wt	EcR+ x USP+	0.41*	7.9*	20.6*	-	-
	EcR+ [5]	Bkgd. [6]	Bkgd. [6]	-	-	-
	USP+ [5]	Bkgd. [6]	Bkgd. [6]	-	-	-
a	EcR S553A x USP+	0.93*#	16.0*	17	●	
b	EcR D572A x USP+	0.13#	0.77*#	6.5*#	●	●
c	EcR E583L x USP+	0.70*	23.6*#	35*	●	●
d	EcR K613A x USP+	0.10#	1.0#	10	●	
e	EcR L615A x USP+	0.07#	0.32*#	5	●	
f	EcR T619A x USP+	0.90#	25.8*#	28*	●	
g	EcR I463T x USP+	0.33	3.5	12.7*		●
h	EcR M504R x USP+	0.50*	0.56*#	1.2#		●
j	EcR R511Q x USP+	0.09*#	0.13*#	1.7*#		●
i	EcR K497A x USP+	5.60*#	9.0*	1.7*#	●	●
k	EcR N626A x USP+	0.40*	15.0*	37.5*#		●
l	EcR E647R x USP+	0.40*	2.5*#	5.9*#		●
m	EcR E648K x USP+	0.24*#	0.73*#	2.8*#		●
n	EcR E476A x USP+	0.13*#	0.41*#	3.1*#		●
o	EcR S531T x USP+	0.06#	0.13*#	2.0*#		●
p	EcR A612V x USP+	0.06#	0.76*#	11*	●	
q	EcR I617A x USP+	1.03*#	4.0*	4.8*#		●
r	EcR+ x USP L281Y	0.34*	1.6*#	4.6*#		●
s	EcR+ x USP L322G	0.50*	20.0*#	40*		●
t	EcR+ x USP I323A	0.30*	28.2*#	105*#		●
u	EcR+ x USPC329A+S330N	0.30*	1.6*#	6.5*#		●

Figure 1 For legend see facing page

sites of the EcR [4] and USP sequence [6], the designation of the respective plasmid, and the oligonucleotide employed for each are listed in Supplementary Table 1 (http://www.BiochemJ.org/bj/378/bj3780779add.htm). Sequencing of inserts (by Microsynth, Gachnang, Switzerland or GENterprise, Mainz, Germany) to check for the absence of random mutations revealed a $Ser^{330} \rightarrow$ Asn (S330N) mutation in the USP C329A construct [therefore named USP(C329A + S330N)]. With constructs exhibiting very low expression, the entire plasmid was sequenced. Mutated EcR and USP LBD-fusion proteins were expressed individually in yeast cells and checked for expression level, expected size, ligand binding and DNA binding by Western blot analysis, [^{3}H]ponasterone A-binding assay and electrophoretic mobility-shift assay respectively (see [9,14,15]). Correlation analyses showed that the upto 2-fold variation in expression levels observed with the different mutants [14] cannot be responsible for the effects described in the present work.

Choice of mutations

LBDs of mammalian RAR (retinoic acid receptor) and RXR (9-*cis*-retinoic acid receptor) are the only EcR-/USP-related nuclear receptor LBDs [16,17] for which a heterodimeric crystallographic structure is available to date [18]. This three-dimensional information, together with sequence alignments (Supplementary Figure, http://www.BiochemJ.org/bj/378/bj3780779add.htm), gave a general idea of the overall structure of the EcR–USP heterodimer and allowed the identification of respective regions as the DIF (dimerization interface), LBP (ligand-binding pocket) or MT ('mouse-trap') (the term mouse-trap has been coined by [19] and designates those LBD structures which ensure the entrapping of the ligand in the LBP; these structures comprise the movable 'lid' composed of helices 11 and 12 and their flexible interhelical 'joints', as wells as the 'lock' in helix 4 which holds the 'lid' closed). Figure 1(A) shows the approximate spacial relationship of DIF, LBP and MT. To produce DIF, LBP or MT mutations, sites in EcR LBD were selected that correspond to sites in RAR and/or RXR LBD which were shown to contact with either the dimerization partner or the ligand, or relate to the 'mouse-trap' mechanism [see Supplementary material (http://www.BiochemJ.org/bj/378/bj3780779add.htm) and references therein], unless stated otherwise. Two mutations were subsequently reclassified because of inconsistencies with or among the various published homology models for the EcR LBD [see Supplementary material (http://www.BiochemJ.org/bj/378/bj3780779add.htm)]. USP mutations were made in the cavity that corresponds to the ligand binding pocket in other receptors (Figure 1A). The current debates regarding possible ligands for and the natural conformation of USP LBD (compare with [17]) are not addressed in the present work nor do they affect its basic outcome and overall interpretation. Many selected residues were mutated to the moderately hydrophobic, small amino acid, alanine, which has a minimal impact on the protein structure, in order to eliminate the function of the native residue. Additional mutations were produced to change hydrophobicity and/or charge of EcR LBD. Some mutations were patterned according to other specific criteria (see below).

RESULTS AND DISCUSSION

In order to identify structures involved in ligand-induced heterodimerization between the LBDs of EcR and USP, 27 individual site-directed mutations were generated and analysed by the yeast two-hybrid system for effects on β-galactosidase activity, used as a reporter for this interaction, in the absence or presence of muristerone A. Control experiments revealed that activation of the respective reporter gene (*lacZ*) was fully dependent on the co-expression of the two fusion proteins between EcR or USP LBD and GAL4 activation or DNA-binding domain respectively (Figure 1B, [9] and results not shown). This conforms with the previously obtained evidence that yeast cells lack co-activators, co-repressors or other general transcription factors which could interact with EcR or USP LBDs ([10], see also combination experiments below) and thereby jeopardize the yeast two-hybrid assay. The potent Ec muristerone A promoted heterodimerization between the fusion proteins of EcR and USP LBDs with all mutations investigated, albeit to different degrees varying over a large range from 0.13 (EcR S531T) to 67 (EcR T619K) Miller Units (M.U.; a measure for β-galactosidase activity; Figure 1B, column 4). The majority of mutations reduced the heterodimerization induced by muristerone A compared with the wild-type, although a remarkable minority enhanced it.

Mutations increased ligand-induced heterodimerization compared with the wild-type because they enhanced spontaneous heterodimerization (EcR mutations K497A, K497E, S553A and I617E; see Figure 1A and Table 1), because they enhanced the ligand effect (mutations: EcR, N626A and T619K; USP, L322G and I323A) or because of both (EcR mutations E583L and T619A). The first group are called 'hyperdimerizers', as they achieve a score for spontaneous heterodimerization significantly greater than wild-type (> 0.41 M.U.); the second group are called 'superinducers', as they confer to the two-hybrid assay an inducibility by 25 μM muristerone A that is significantly greater than wild-type ($Ef_{lig} > 20$, where Ef_{lig} is the ligand effect, quotient induced/spontaneous heterodimerization); and the third group will be called either one. Mutations reducing ligand-induced heterodimerization below the wild-type level may be grouped in an analogous manner. The ligand effect showed no consistent negative correlation with the extent of spontaneous heterodimerization, but a strong positive correlation with ligand binding to EcR LBD for all mutations analysed (Figure 2). These findings suggest a relation between ligand binding and dimerization, and indicate the existence of a functional interaction between the presumed sites of action, i.e., the LBP and DIF in EcR LBD. This interaction must be positive, since ligand

Figure 1 Site-directed mutations in the LBD of *Drosophila* EcR and USP and their effects

(**A**) Approximate location of mutations projected into the canonical three-dimensional model for nuclear receptor LBDs [20], which is based on crystallographic measurements of the all-*trans*-RAR (yellow) [19] and the RXR LBD (pink) [22,27] and of their heterodimeric complex [18]. H1–H12, α-helices 1 to 12. B1 and B2: β-strands 1 and 2. Stippled regions, most variable and most uncertain structures. Colour of dots: red, DIF mutations in EcR LBD; green, LBP mutations in EcR LBD; blue, MT mutations in EcR LBD; grey, controls to EcR mutations and mutations at sites of unknown or uncertain functions (Supplementary Figure, http://www.BiochemJ.org/bj/378/bj3780779add.htm); turquoise, USP mutations. Letters in dots correspond to mutations listed in (**B**). (**B**) Impact of mutations on spontaneous and ligand-induced heterodimerization between EcR and USP LBDs fused to GAL4-activation and DNA-binding region, respectively, unless stated otherwise. [1] Colour code as in (**A**); [2] M.U.: Miller Units, a measure for reporter β-galactosidase activity in the two-hybrid assay; [3] ligand effect (Ef_{lig}) defined as averaged ratios of induced/spontaneous heterodimerization; [4] main impact of mutation calculated from the fold increase or decrease of spontaneous heterodimerization and Ef_{lig} in comparison to wild-type. [5] Fusion protein with GAL4 DNA binding or activation domain combined with reciprocal GAL4 domain alone (for respective constructs, see [9]); [6] Bkgd.: Background $>$ 0.04 M.U.. *: $P_{=Bkgd.}$ or $P_{=1}$ for Ef_{lig})$_{-} \leqslant 0.05$. #: $P_{=wt} \leqslant 0.05$. For detailed statistical information, see Supplementary Table 2 (http://www.BiochemJ.org/bj/378/bj3780779add.htm).

Table 1 General enhancement of spontaneous and ligand-induced heterodimerization by a polar exchange

In the first step, the natural residue was replaced by alanine to exclude effects due to extinction of specific functions by natural residues. The alanine residues were then substituted with the following polar amino acids: Lys[497] and Ile[617], Glu; Ser[553], Asp; Asp[572], Ser; Thr[619] and Asn[626], Lys. For details of the statistics, see Supplementary Table 2 (http://www.BiochemJ.org/bj/378/bj3780779add.htm).

	Heterodimerization (M.U.)					
	Spontaneous			Ligand-induced		
Residue	WT	Alanine	Polar	WT	Alanine	Polar
Lys[497]	0.41	5.6	23	7.9	9	46
Ser[553]	0.41	0.93	3.7	7.9	18	51
Asp[572]	0.41	0.13	1.0	7.9	0.77	19
Ile[617]	0.41	1.0	4.0	7.9	4.0	19
Thr[619]	0.41	0.9	1.3	7.9	26	67
Asn[626]	0.41	0.4	0.9	n.d.		

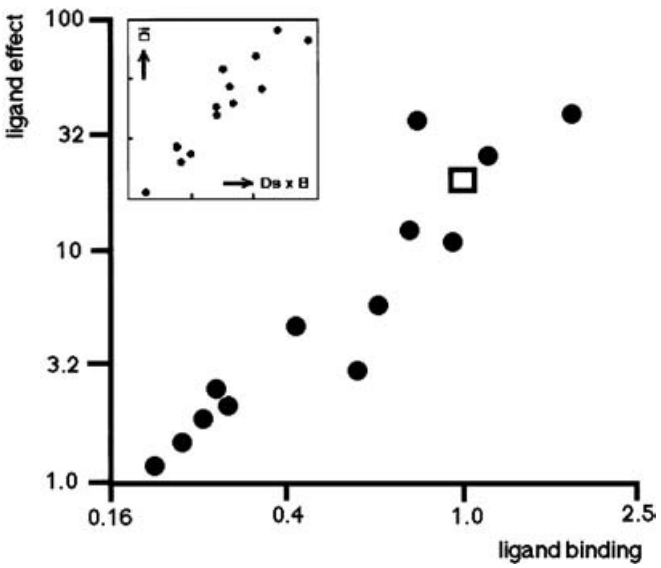


Figure 2 Correlation between ligand binding (*B*) and ligand effect (Ef_{lig})

Double logarithmic plot of ligand binding to mutated EcR in the absence of USP LBD (abscissa, data from [14]) and ligand effect (Ef_{lig}) on heterodimerization between mutated EcR and wild-type USP LBD (ordinate, this work, only values with 95 %-confidence limits $< \pm 50$ % of Ef_{lig} are shown). Ligand binding was determined by specific [^{3}H]ponasterone A binding [9,14]. □, Wild-type EcR LBD; its ligand binding value is set to unity. The EcR mutants are from left to right: M504R, K497A, E648K, R511Q and K497E (double dot), I617A, E476A, E647R, I463T, N626A, A612V, wild-type EcR, T619A and T619K. For localization and classification of mutations, see Figure 1. $r = 0.97$ ($P_{r=0} \ll 0.001$). For statistical information, see Supplementary Table 2 (http://www.BiochemJ.org/bj/378/bj3780779add.htm). Note that the positive correlation shows that $Ef_{lig} \sim B$. As $Ef_{lig} = D_i/D_s$ (D_i, ligand-induced dimerization; D_s, spontaneous dimerization), it follows that $B \sim D_i/D_s$, which can be transformed mathematically into $D_i \sim Ds \times B$. This theoretical consideration is supported by the positive correlation (inset, double logarithmic plot) between the measured Di values (Figure 1B and Table 1) and the products ($D_s \times B$) of measured Ds (Figure 1B and Table 1) and published B values [14]. $r = 0.72$ ($P_{r=0} < 0.01$). The multiplication function signifies cooperativity (synergism) between dimerization and ligand binding.

binding enhances heterodimerization and vice versa (present study and [9]). Biochemical studies performed under saturating conditions (see below) suggested the action of dimerization on ligand binding to be 'complementary' or 'compensatory' [14]. However, correlation analyses of ligand binding to EcR LBD and spontaneous or ligand-induced heterodimerization (Figure 2) indicate that the interaction between the dimerization and the ligand-binding function is in fact synergistic (Figure 2, inset).

Consideration of the effect of mutations targeted to the two presumably key players, the DIF and the LBP, shows that there is no doubt that both structures are involved in ligand-induced heterodimerization (Figure 1). The main impact of DIF mutations (red colour in Figure 1) was on spontaneous heterodimerization, whereas that of LBP mutations (green) was on the ligand effect (ligand binding, [14]). The so-called MT, (see [19] and the Experimental section for explanation) plays a pivotal role in ligand-controlled effects on LBD, since it is known to undergo a conspicuous conformational change upon ligand binding ([20,20a]). MT mutations (blue colour in Figure 1) dramatically affected spontaneous heterodimerization and/or the ligand effect. For example, E497A was the most efficient 'hyperdimerizer', whereas N626A was the second most potent 'superinducer' among the non-polar EcR mutations. These findings substantiate the postulated role of the MT as a master switch for allosteric effects induced by ligand. However, parts of the LBD other than the MT could also be crucial for allosteric transitions occurring during ligand-induced heterodimerization. The mutation I617A depressed ligand action as an exception among non-polar 'hyperdimerizers', whereas D572A and E583L had an equal impact on ligand effect and heterodimerization (Figure 1B). Although all three sites are located in important structures (helices 10, 7 and 9 respectively) of the DIF, these sites might also contribute to protein architecture.

The EcR mutation E476A was constructed to serve as a control to the DIF, LBP and MT mutations. Its impact on spontaneous heterodimerization, but particularly on the ligand effect (Figure 1B), is unexpected, because this site neither contacts USP LBD or the ligand nor is it involved in the MT or any other structure that undergoes a large ligand-induced conformational change [Figure 1A and Supplementary Figure (http://www.BiochemJ.org/bj/378/bj3780779add.htm)]. This led us to assume that the E476A mutation and possibly many other mutations cause global alterations of the LBD. Investigations on isolated and purified LBDs of the thyroid receptor or of RXR demonstrate that global alterations of the LBD also occur upon ligand binding, i.e., in addition to the triggering of the MT. These ligand-inducible global effects are: (i) reduction of surface hydrophobicity, (ii) compaction and (iii) stabilization of the overall structure of the LBD [21–23]. The ligand is obviously required for a dynamic stabilization of the intermolecular interaction between a helix 1-truncated thyroid receptor LBD and a helix 1-containing peptide [21]. Collectively, these observations gave rise to the idea that ligand-inducible global effects may positively affect the interaction of EcR with the USP LBD. To test the possible influence of surface hydrophobicity, sites were investigated which do not belong to LBD core structures. A first round of site-directed mutations indicated (Figure 1B) that a change in hydrophobicity may indeed correlate with heterodimerization in some cases (E476A, S531T, D572A and K613A), but not in others. Thus to reduce the possibility of an interference by effects other than surface hydrophobicity, we chose seven sites that had been already exchanged by alanine and introduced a subsequent substitution by a polar residue. This allowed a clear comparison between a hydrophobic and polar situation. The data presented in Table 1 demonstrate that in all the cases investigated the polar exchange caused an increase in spontaneous and ligand-induced heterodimerization when compared with the respective alanine residue. Although it is not yet known how a change in hydrophobicity affects EcR–USP dimerization, the possibility that Ecs, such as thyroids, might reduce their receptors' hydrophobicity and thereby promote intermolecular interactions is obvious and deserves further investigations.

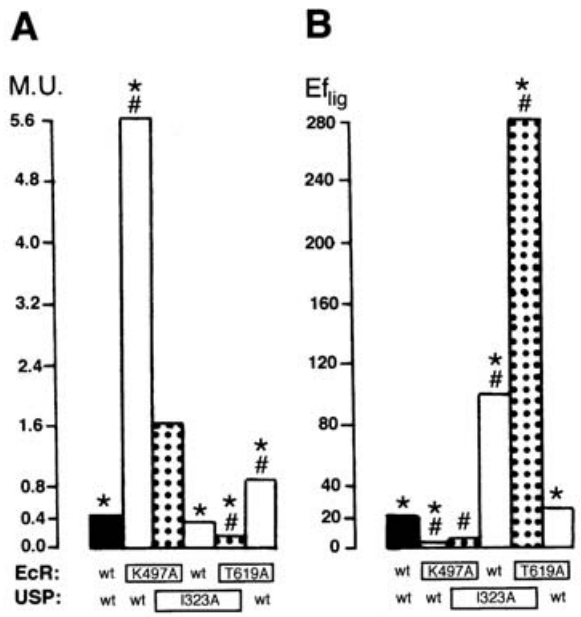


Figure 3 Effects of combining site-directed mutations in EcR and USP LBDs on spontaneous heterodimerization (A) and ligand effect Ef_{lig} (see Figure 1) on heterodimerization (B)

The wild-type (wt) or mutated (overlapping boxes) EcR LBD indicated in the top line was combined with the wild-type or mutated USP LBD indicated underneath respectively. Closed columns, wild-type × wild-type combinations; Dotted columns, mutated × mutated combinations. *, # and M.U., see Figure 1; For detailed statistical information, see Supplementary Table 2 (http://www.BiochemJ.org/bj/378/bj3780779add.htm).

Mutations inside the USP LBD either reduced (L281Y and C329A + S330N) or enhanced (I323A and L322G) Ec-induced heterodimerization (turquoise colour in Figure 1). These ligand effects are not due to a direct interaction between muristerone A and the LBP of USP, since Ecs neither bind to [9,14,15] nor stimulate the dimerization [9] or transactivation ability (Figure 1B and results not shown; [11]) of the USP LBD itself, whether or not the latter has been mutated. A 'transmolecular effect' [24] of the USP mutations on the ability of the EcR LBP to bind ligand can practically be ruled out as well. This would require a strong interaction of the USP LBD with that of EcR prior to ligand binding, which is not supported by our results (Figures 1B and 3). We prefer to interpret the effects of the USP mutations according to the so-called 'DIF compatibility model' [25]. This model postulates that the intermolecular effect of USP mutations is due to an indirect alteration of the USP DIF, thus decreasing or increasing the affinity of the USP LBD for a given, e.g. ligand-dependent, conformation of the EcR LBD. The combination of the 'superinducible' mutants EcR T619A and USP I323A potentiated the ligand effect on heterodimerization, suggesting that USP I323A has an exceptionally high affinity to the holoconformation of EcR T619A. In contrast, the combination of the 'hyperdimerizer' EcR K497A and 'superinducer' USP I323A not only curtailed the 'hyperdimerization' activity of EcR K497A, but also antagonized the 'superinducer' activity of USP I323A (Figure 3B), which is explained by a very low compatibility between these two mutants [25], rather than by an interaction with hypothetical yeast proteins or transcription factors (compare with [14]).

'Hyperdimerization' and 'superinduction', as observed in the present work, only occur under conditions which do not saturate the heterodimerization reaction. At saturation, each EcR LBD can bind no more than possibly one ligand molecule and one USP LBD. Therefore, differences in ligand-induced heterodimerization, e.g. between wild-type EcR or USP and USP I323A or EcR K497A, disappear or become reversed respectively at very high muristerone A concentrations (W.W. Hitchcok and V.C. Henrich, unpublished work). Most assays *in vitro* for heterodimerization are performed at saturating conditions and thus yield at best results corresponding to those obtained with wild-type EcR and USP LBDs (compare Figure 3B in [9] and Figure 4C in [14]). It is only ligand binding to EcR LBD alone (a very weak binding being far away from the point of saturation) which allows values that exceed those achieved by wild-type LBD at the same ligand concentration to be seen [14]. The determined ligand-binding values for the set of EcR LBD mutants analysed generally paralleled the ligand effect on heterodimerization in the yeast cell (Figure 2). In the living organism Ecs act under conditions which ensure optimal control rather than maximal effect [8]. Therefore experiments carried out at non-saturating conditions *in vivo*, as in the present study, are especially adequate to characterize the situation in the living insect cell.

From our mutational analyses, one may deduce the following general points regarding EcR–USP interaction. (i) The capability of a receptor to undergo ligand-induced heterodimerization relates to both its basic ability to bind ligand and to bind its heterodimerization partner, each independently of the other (present study and see also [9,14] for a naturally resistent EcR form). In the actual process of ligand-induced heterodimerization these two functions then interact synergistically. (ii) The LBP, DIF and MT play a crucial, although not exclusive, role in this interaction. (iii) Global effects, such as a decrease in the hydrophobicity of the LBD of a receptor, facilitate intermolecular interactions; it is known that ligands can elicit such global effects. (iv) Ligand binding to a receptor influences its compatibility with cognate dimerization partners which, conversely, 'sense' to a varying degree whether the receptor partner is liganded. The relevance of our findings is supported by other studies *in vivo*: the USP mutation I323A, for example, potentiates the effect of Ec in a transgenic mouse cell line (M.O. Imhof, personal communication), whereas the mutations S531T and A612V in EcR (which are reconstructions of published *in vivo* mutations [26]) impair survival of *Drosophila* larvae [26] at the same proportion as they do in our assay system. Thus our studies using site-directed mutagenesis, and subsequently analysis *in vivo*, yielded useful information to characterize the regulation of the EcR–USP interaction under physiological conditions. A detailed mechanistic interpretation of the effects observed is yet not feasible. It will have to await the solution of the molecular structure of the natural EcR–USP heterodimer in the liganded and non-liganded configuration. For both further investigations in physiological/developmental or biochemical/structural directions, this group of 27 mutations we have provided and characterized is undoubtedly of great pratical value (cf. [14]).

Note added in proof (received 19 November 2003)

After the acceptance of the present paper for publication, an article by Billas and co-workers [27a] was published on the crystal structure of the heterodimer between the LBDs of EcR and USP in their holoconformation from the moth *Heliothis virescens*. Although the measured EcR LBD structure, exhibiting the canonical folding, deviates from any homology model published previously, the three-dimensional data in the study by Billas et al. [27a] do not affect the rough classification of our mutations nor the principal conclusions drawn from our findings in the present paper. The high structural adaptability and flexibility discovered

by these authors [27a] support our postulation of extensive and strong intra- and inter-molecular interactions in ligand-controlled EcR–USP heterodimerization.

Note added in proof (received 5 December 2003)

Recent experiments (V. C. Henrich, unpublished work) indicate that mutation K497E conveys a high level of constitutive activity to a cellular mammalian gene induction system when introduced into a specific full-length EcR form. This is consistent with the high spontaneous heterodimerization activity of that mutation which we found in our yeast two-hybrid assay.

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REFERENCES

1 Henrich, V. C., Rybczynski, R. and Gilbert, L. I. (1999) Peptide hormones, steroid hormones and puffs: mechanisms and models in insect development. Vitam. Horm. **55**, 73–125
2 Lezzi, M. (1996) Chromosome puffing: supramolecular aspects of ecdysone action. In Metamorphosis, Postembryonic Reprogramming of Gene expression in amphibian and insect cells (Gilbert, L. I., Tata, J. R. and Atkinson, B. G., eds.), pp. 145–173, Academic Press, San Diego
3 Yao, T.-P., Forman, B. M., Jiang, Z., Cherbas, L., Chen, J. D., McKeown, M., Cherbas, P. and Evans, R. M. (1993) Functional ecdysone receptor is the product of EcR and Ultraspiracle genes. Nature (London) **366**, 476–479
4 Koelle, M. R., Talbot, W. S., Segraves, W. A., Bender, M. T., Cherbas, P. and Hogness, D. S. (1991) The *Drosophila* Ec*R* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell (Cambridge, Mass.) **67**, 59–77
5 Henrich, V. C., Sliter, T. J., Lubahn, D. B., MacIntyre, A. and Gilbert, L. I. (1990) A steroid/thyroid hormone receptor superfamily member in *Drosophila melanogaster* that shares extensive sequence similarity with a mammalian homologue. Nucleic Acid Res. **18**, 4143–4148
6 Oro, A. E., McKeown, M. and Evans, R. M. (1990) Relationship between the product of the *Drosophila* ultraspiracle locus and vertebrate retinoid X receptor. Nature (London) **347**, 298–301
7 Shea, M. J., King, D. L., Conboy, M. J., Mariani, B. D. and Kafatos, F. C. (1990) Proteins that bind to *Drosophila* chorion cis-regulatory elements: a new C2H2 zinc finger protein and a C2C2 steroid receptor-like component. Genes Dev. **4**, 1128–1140
8 Lezzi, M., Bergman, T., Mouillet, J.-F. and Henrich, V. C. (1999) The ecdysone receptor puzzle. Arch. Insect Biochem. Physiol. **41**, 99–106
9 Lezzi, M., Bergman, T., Henrich, V. C., Vögtli, M., Frömel, C., Grebe, M., Przibilla, S. and Spindler-Barth, M. (2002) Ligand-induced heterodimerization between ligand binding domains of *Drosophila* ecdysteroid receptor (EcR) and Ultraspiracle (USP). Eur. J. Biochem. **269**, 3237–3245
10 Tran, H. T., Shaaban, S., Askari, H. B., Walfish P. G., Raikhel, A. S. and Butt, T. R. (2001) Requirement of co-factors for the ligand-mediated activity of the insect ecdysteroid receptor in yeast. J. Mol. Endocrinol. **27**, 191–209
11 Hu, X., Cherbas, L. and Cherbas, P. (2003) Transcription activation by the ecdysone receptor (EcR/USP): identification of activation functions. Mol. Endocrinol. **17**, 716–731
12 Vögtli, M., Elke, C., Imhof, M. and Lezzi, M. (1998) High level transactivation by the ecdysone receptor complex at the core recognition motif. Nucleic Acids Res. **26**, 2407–2414
13 Vögtli, M., Imhof, M. O., Brown, N. E., Rauch, P., Spindler-Barth, M., Lezzi, M. and Henrich, V. C. (1999) Functional characterization of two Ultraspiracle forms (CtUSP-1 and CtUSP-2) from *Chironomus tentans*. Insect Biochem. Mol. Biol. **29**, 931–942
14 Grebe, M., Przibilla, S., Henrich, V. C. and Spindler-Barth, M. (2003) Characterization of the ligand-binding domain of the ecdysteroid receptor from *Drosophila melanogaster*. Biol. Chem. **384**, 105–116
15 Grebe, M. (2001) Charakterisierung des Ecdysteroidrezeptors als target für arthropodenspezifische Insektizide. PhD Thesis, University of Düsseldorf (FRG), pp. 1–122
16 Wurtz, J.-M., Guillot, B., Fagart, J., Moras, D., Tietjen, K. and Schindler, M. (2000) A new model for 20-hydroxyecdysone and dibenzylhydrazine binding: a homology and docking approach. Protein Sci. **9**, 1073–1084
17 Sasorith, S., Billas, I. M. L., Iwema, T., Moras, D. and Wurtz, J.-M. (2002) Structure-based analysis of the Ultraspiracle protein and docking studies with putative ligands (11 pp). J. Insect Sci. **2.25** (http://insectscience.org/2.25/)
18 Bourguet, W., Vivat, V., Wurtz, J.-M., Chambon, P., Gronemeyer, H. and Moras D. (2000) Crystal structure of a heterodimeric complex of RAR and RXR ligand-binding domains. Mol. Cell **5**, 289–298
19 Renaud, J.-P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H. and Moras, D. (1995) Crystal structure of the RAR-γ ligand-binding domain bound to all-*trans* retinoid acid. Nature (London) **378**, 681–689
20 Wurtz, J.-M., Bourguet, W., Renaud, J.-P., Vivat, V., Chambon, P., Moras, D. and Gronemeyer, H. (1995) A canonical structure for the ligand-binding domain of nuclear receptors. Nat. Struct. Biol. **3**, 87–94
20a Wurtz, J.-M., Bourguet, W., Renaud, J.-P., Vivat, V., Chambon, P., Moras, D. and Gronemeyer, H. (1995) Erratum. Nat. Struct. Biol. **3**, 206
21 Pissios, P., Tzameli, I., Kushner, P. and Moore, D. D. (2000) Dynamic stabilization of nuclear receptor ligand binding domains by hormone or corepressor binding. Mol. Cell **6**, 245–263
22 Egea, P. F., Mitschler, A., Rochel, N., Ruff, M., Chambon, P. and Moras, D. (2000) Crystal structure of the human RXRα ligand-binding domain bound to its natural ligand: 9-*cis* retinoid acid. EMBO J.**19**, 2592–2601
23 Apriletti, J. W., Baxter, J. D., Lau, K. H. and West, B. L. (1995) Expression of the rat α1 thyroid hormone receptor ligand binding domain in *Escherichia coli* and the use of a ligand-induced conformation change as a method for its purification to homogeneity. Protein Expression Purif. **6**, 363–370
24 Vivat, V., Zechel, C., Wurtz, J.-M., Bourguet, W., Kagechika, H., Umemiya, H., Shudo, K., Moras, D., Gronemeyer, H. and Chambon, P. (1997) A mutation mimicking ligand-induced conformational change yields a constitutive RXR that senses allosteric effects in heterodimers. EMBO J. **16**, 5697–5709
25 Lezzi, M. (2002) Dimerization interface (DIF-) model for nuclear receptor interaction (Abstracts from the XVth International Ecdysone Workshop). J. Insect Sci. **2.16** (http://insectscience.org/2.16/)
26 Bender, M., Imam, F. B., Talbot, W. S., Ganetzky, B. and Hogness, D. S. (1997) *Drosophila* ecdysone receptor mutations reveal functional differences among receptor isoforms. Cell (Cambridge, Mass.) **91**, 777–788
27 Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H. and Moras D. (1995) Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-α. Nature (London) **375**, 377–382
27a Billas, I. M., Iwema, T., Garnier, J. M., Mitschler, A., Rochel, N. and Moras, D. (2003) Structural adaptability in the ligand-binding pocket of the ecdysone hormone receptor. Nature (London) **426**, 91–96

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