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

Thomas BERGMAN\textsuperscript{1,2}, Vincent C. HENRICH\textsuperscript{1,3}, Uwe SCHLATTNER and Markus LEZZI\textsuperscript{4}

Institute for Cell Biology, ETH-Hönggerberg, CH-8093 Zürich, Switzerland

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 (Ultraspireacle 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–BS/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–BspHI fragment in pAS2-1-USP(172-508). The mutations at the given sites were confirmed by DNA sequencing.

**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; E14, ligand effect; M.U., Miller units.

1 Both authors contributed equally to this work.
2 Present address: Aflowbody AB, P.O. Box 201 37, SE-161 02 Bromma, Sweden.
3 Permanent address: Institute for Health, Science and Society, University of North Carolina, Greensboro, NC 27402, U.S.A.
4 To whom correspondence should be addressed (e-mail lezzi@cell.biol.ethz.ch).
Figure 1  For legend see facing page

© 2004 Biochemical Society
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, Gaachnang, 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, [3H] ponasterone A-binding assay and electrophoretic mobility-shift assay respectively (see [9,14,15]). Correlation analyses showed that the uto 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 moveable 'lid' composed of helices 11 and 12 and their flexible interhelical 'joints', as well as the 'lock' in helix 4 which holds the 'lid' closed). Figure 1(A) shows the approximate spatial 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 confirms 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 'superinducers', as they achieve a score for spontaneous heterodimerization significantly greater than wild-type (>0.41 M.U.); the second group are called 'superinhibitors', as they confer to the two-hybrid assay an inducibility by 25 μM muristerone A that is significantly greater than wild-type ($E_{\text{ff}}^{\text{wt}} > 20$, where $E_{\text{ff}}^{\text{wt}}$ 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
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: Lys497 and Ile617, Glu; Ser553, Asp; Asp572, Ser; Thr619 and Asn626, Lys. For details of the statistics, see Supplementary Table 2 (http://www.BiochemJ.org/bj/378/bj3780779add.htm).

<table>
<thead>
<tr>
<th>Residue</th>
<th>Spontaneous</th>
<th>Ligand-induced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Alanine</td>
</tr>
<tr>
<td>Lys497</td>
<td>0.41</td>
<td>5.6</td>
</tr>
<tr>
<td>Ser553</td>
<td>0.41</td>
<td>0.93</td>
</tr>
<tr>
<td>Asp572</td>
<td>0.41</td>
<td>0.13</td>
</tr>
<tr>
<td>Ile617</td>
<td>0.41</td>
<td>1.0</td>
</tr>
<tr>
<td>Thr619</td>
<td>0.41</td>
<td>0.9</td>
</tr>
<tr>
<td>Asn626</td>
<td>0.41</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Figure 2  Correlation between ligand binding ($B$) and ligand effect ($E_{100}$)

Double logarithmic plot of ligand binding to mutated EcR in the absence of USP LBD (abscissa, data from [14]) and ligand effect ($E_{100}$) on heterodimerization between mutated EcR and wild-type USP LBD (ordinate, this work, only values with 95 % confidence limits $< \pm 50 \%$ of $E_{100}$ are shown). Ligand binding was determined by specific [3H]ponasterone A binding [9,14]. 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.
The heterodimerization reaction. At saturation, each EcR LBD present work, only occur under conditions which do not saturate [14]).

Figure 3 Effects of combining site-directed mutations in EcR and USP LBDs on spontaneous heterodimerization (A) and ligand effect $E_{l_{in}}$ (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 holocomformation 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. Hitchcock 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 resistant 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 practical 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 holocomformation 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
REFERENCES


5 Henrich, V. C., Sliter, T. J., Lubahn, D. B., Machityre, 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


© 2004 Biochemical Society