The Ecdysone Receptor Puzzle

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Abstract:
The present article reviews some recent findings on the functional ecdysone† receptor which is a heterodimer of two proteins: ecdysone receptor (EcR) and Ultraspiracle (USP). Emphasis is given to some unique aspects of this receptor, in particular to its dimerization, binding to DNA, and transactivation capabilities. The effects of ligands (ecdysone, juvenile hormone) on these functions are discussed. In addition, perspectives on future work on this receptor are outlined, which are shaped by recent progress in the nuclear receptor field in general. This preview part of the present article concerns mainly the 3-D structure of receptor domains, the formation of large supramolecular receptor complexes, their influence on chromatin remodelling, receptor phosphorylation, as well as inter- and intramolecular cross-talks of receptor domains.

Keywords: ecdysone receptor; Ultraspiracle; receptor dim erization; juvenile hormone; transactivation

Abbreviations Used: Ec = ecdysone; EcR = ecdysone receptor; EcRE = ecdysone response element; JH = juvenile hormone; RAR = retinoic acid receptor; RXR = retionid X receptor; USP = ultraspiracle.

Abstract:

INTRODUCTION

The present short review and preview article deals with that type of ecdysone† (Ec) receptor that is composed of the two proteins called EcR and USP (for "Ec receptor" and "Ultra spiracle," respectively) and whose principal site of action is the genome. This does not exclude other putative sites of action for this receptor nor does it rule out the likely existence of other Ec receptor types (Tomaschko, 1999). EcR and USP are members of the nuclear (hormone) receptor superfamily that typically exhibit a modular structure where ligand binding, dimerization, and transactivation are mainly assigned to the so-called E domain while DNA binding is localized in the C domain (Beato, 1989). On their way from the cytoplasm to the genome, EcR and USP are postulated to undertake three important actions as they, first, heterodimerize, second, bind to specific DNA, and, third, activate nearby target genes. All three actions are suggested to be controlled by Ec. The present article follows EcR and USP through these three events, reporting published results of the past few years and new yet unpublished observations, mostly from our group. At the beginning, however, recent findings obtained with vertebrate members of nuclear receptor superfamily will be outlined as they may give additional important perspectives on future work in the Ec receptor field.

† The term "ecdysone" (Ec) rather than "ecdysteroid" is used as a generic name for designating all Ec-active compounds including nonsteroidal Ec agonists. In the present article these compounds are: 20-hydroxyecdysone, ponasterone A, muristerone A, and tebufenozide (RH 5992).

LESSONS TAUGHT BY OTHER NUCLEAR RECEPTORS

3-D-Model of E Domain

X-ray crystallographic analyses of isolated E domains of RXR and RAR yielded a 3-D-model, which evidently is also applicable to other nuclear receptors including EcR and USP (Wurtz et al., 1996). This model predicts 12 a-helices, which includes the formation of a ligand binding pocket. Helix 10 is a major component of the dimerization interface. Upon ligand binding, the conformation of the E domain changes with a most conspicuous flipping-over of helix 12, thereby establishing a new interface to which the coactivator complex can bind (Glass et al., 1997; Westin et al., 1998).

Coactivator Complex

The coactivator complex consists of many proteins (Kamei et al., 1996; Torchia et al., 1997, 1998). However, its exact constitution at a given gene in a given cell remains to be investigated. Many proteins of the coactivator complex carry a so-called signature motif (LXXLL) that mediates their binding to nulcear receptors (Heery et al., 1997). Among these coactivator proteins, bHLH-PAS family members are of special interest since one of them (p160) is a dioxin receptor (Kamei et al., 1996). This opens the possibility of multiple regulation of a coactivator complex not only by 1 or 2 ligands to its intrinsic nuclear receptor dimer (see Westin et al., 1998) but in addition by ligands to other receptor types. It is intriguing that MET—a protein important for the binding and action of methoprene (a potent juvenile hormone analog)—also belongs to the bHLH-PAS family (Ashok et al., 1998; for a discussion of the JH receptor problem, see Feyereisen, 1998). Other components of the coactivator complex are endpoints of various signalling pathways involving different protein kinases (see Torchia et al., 1997, 1998). Collectively, the coactivator complex may, thus, be conceived as an integrator of a variety of input signals (Glass et al., 1997). Moreover, since several members of the complex have the ability to bind to specific sequences in DNA (beside nuclear receptors, e.g., CREB, STAT-1, JUN/FOS, see Torchia et al., 1997, 1998), one could imagine that the coactivator complex, as a functional unit, recognizes different sets of such sequences, depending on its actual protein complement. By such a combinatorial principle, a high specificity of target gene recognition is guaranteed. Not only the input but also the output signals of the coactivator complex are highly coordinated (Montminy, 1997; Torchia et al., 1998). In principle, they fulfill a dual role: (1) activation of the transcriptional machinery and (2) changing the structure of nearby nucleosomes. In fact, many of the constituents of the complex are both in one: transcription factors and specific histone acetyl transferases.

For nuclear receptors that are already bound to their cognate response element in the non-liganded state, their domains accommodate a yet different protein complex, the corepressor complex. This complex, again, has a dual function: repression of the transcriptional machinery and histone deacetylation (Wolffe, 1997; Torchia et al., 1998). Upon ligand binding, the corepressor complex is exchanged with the coactivator complex.

Nuclear Receptors and Chromatin Structure

At present, it is not yet known how acetylation of specific lysine residues in histones H3 and H4 changes the structure of nucleosomes and chromatin. The general notion is that it facilitates transcription (Wolffe, 1997). In the context of previous models on Ec action and local chromatin changes (puffing; Lezzi, 1996), two points have to be considered regarding the role of H3 and H4 acetylation: (1) enhanced transcription is a consequence of changed nucleosome structure rather than its cause; (2) the change in nucleosome structure is a consequence of the receptor's binding and activation rather than their prerequisite. It, thus, constitutes a *post-receptor-binding event.*

However, there are nucleosomal changes that must take place prior to receptor binding in order for the receptor to specifically recognize its response element (HRE) in nucleosomal DNA. The major groove of the portion of the DNA double helix that contains the crucial contact sites of an HRE must be accessible to the receptor. This is the case with HREs lying in internucleosomal linker DNA or with nucleosome-attached HREs exposing their contact sites to the outside. However, if an HRE faces towards the nucleosomal core, its contact sites are inaccessible and would have to be made accessible by a change in the DNA's rotational position on the

nucleosome (Wong et al., 1997). Such a change represents a *pre-receptor-binding event.* How it is brought about is still unclear and may vary with different response elements.

Based on cytological and immunohistochemical work with polytene chromosomes of *Chironomus tentans*, an accessibility model has been developed for the Ec receptor (Lezzi, 1996). In the case of the chromosome region I-18C, accessibility is suggested to vary during development and the diurnal cycle (Lezzi et al., 1991) whereas with chromosome region IV-5C, it is induced by heat shock (Lezzi, 1996). It remains to be seen whether these parameters act by histone acetylation of the nucleosomal core or at a supranucleosomal level.

DIMERIZATION OF ECR AND USP Effect of Ec

In the above-described model, the ligand's action would be restricted to an exchange of the corepressor by the coactivator complex. All previous steps, such as nuclear receptor dimerization, nuclear translocation, and binding to DNA, would occur in a ligan d-in dependent fashion. Numerous in vitro as well as in vivo experiments (e.g., with EcR/USP overexpressing cells) show that EcR and USP may in fact heterodimerize and bind to their response element in the absence of ligand (Thomas et al., 1993). However, it is still controversial whether ligand-independent dimerization also occurs with endogenous EcR and USP under physiological conditions in the living insect. Undoubtedly, Ec promotes heterodimer formation even in cell-free extracts, as evidenced by DNA binding assays (Thomas et al., 1993), as well as in intact tissue as suggested by immunological localization of endogenous EcR/USP on polytene chromosome loci after tissue culture in the presence or absence of Ec (Stocker et al., 1997; Lezzi, unpublished findings). Moreover, recent two-hybrid studies with isolated EcR and USP E domains in yeast (Bergman, unpublished findings) prove that the ability to heterodimerize resides in the E domain (plus portions of the D domain). Apparently, heterdimerization does not require concomitant binding of the C domain to a bipartite recognition site in DNA. The Ec analog, muristerone A, stimulates heterodimerization more than 150-fold, an effect that cannot be attributed to the transactivation functions (AF-1, AF-2) inherent to EcR and USP. In an attempt to assess the importance of predicted sites for dimerization of EcR and USP, several truncations and point mutations were introduced into the E domain of EcR. While omission of helices 11 and 12 had little effect on the constitutive level of dimerization, removal of helix 10 completely abolished heterodimerization capacity. Point mutations in helix 10, except for one case, diminished spontaneous dimerization. Much to our surprise, muristerone A-induced dimerization was affected by these mutations, independently of the extent of constitutive dimerization. A superinducible mutant was found in which the effect of muristerone A was doubled when compared to that observed with wild-type E domains. Since helix 10 is not thought to contact the ligand directly, an event lying "downstream" to ligand binding must have been altered by the respective helix 10 mutations.

Effect of Juvenile Hormone

It has been known for a long time that juvenile hormone (JH)[‡] quite often counteracts Ec in its effects on, e.g., cell proliferation and differentiation, enzyme induction, and puffing (Lezzi and Wyss, 1976; Shaaya and Spindler, 1990). Transactivation assays with reporter gene constructs carrying an EcRE (Berger et al., 1992; Lümmen, personal communication) suggest that the JH effect has its final target in the Ec-controlled machinery, i.e., in EcR and USP. Recent two-hybrid assays in plant cells (Crossland and Goff, 1996) and yeast (Bergman, unpublished results) strongly substantiate this notion as JH partially suppresses Ec-promoted dimerization of the E domains of EcR and USP (see above). Conversely, JH appears to promote the homodimerization of USP as indicated by similar types of assays (Crossland and Goff, 1996; Jones and Sharp, 1997). These two sets of findings can be combined into a model (Fig. 1) in which an equilibrium between EcR and USP monomers and their various dimers is driven to the USP/USP homodimer by JH whereas Ec pushes it to the EcR/USP heterodimer. In the case of Ec, an actual binding to the EcR/USP complex (Yao et al., 1993) has been shown and implicated to be the basis of its heterodimer stabilizing action while the claimed evidence for an actual binding of JH to USP (Jones and Sharp, 1997) awaits further substantiation. Interactions of EcR

[‡] The term "juvenile hormone" (JH) is used in an analogous manner as "ecdysone". In the present article, the JH-active compounds referred to are: JH III, JH bisepoxide, methoprene, methyl farnesoate, and fenoxycarb.

and USP with other nuclear receptors (SVP, DHR3, DHR38) are not shown in this model and are not discussed here (Zelhof et al., 1995; White et al., 1997; Sutherland et al., 1995).

An assessment of the claimed direct binding of JH to USP has been sought by mutating predicted ligand-contacting residues in the ligand binding pocket of the E domain of USP. No change of the counteracting potential of JH in Ec-stimulated dimerization was observed. However, it was found that some USP mutations potentiated the stimulatory effect of Ec on heterodimerization (Bergman and Henrich, unpublished findings). Since it is believed that groups in the ligand binding pocket of EcR rather than USP are contacted by Ec, these USP mutations cannot directly influence Ec binding to the heterodimer and ligand-controlled heterodimerization. These mutations obviously do so indirectly by an unknown mechanism that resembles effects named "phantom ligand effect," "transmolecular allosteric effect," or "intermolecular cross-talk" in the case of other receptors (see, e.g., Schulman et al., 1998; Westin et al., 1998; Vivat et al., 1997).

DNA BINDING OF ECR/USP

One of the most puzzling aspects of the Ec receptor is its heterodimeric nature, typical for nonsteroid receptors within the superfamily, and its preference for palindromic DNA sequences (PAL1, PAL0) for binding (Vögtli et al., 1998). Heterodimeric receptors usually prefer tandemly arranged motifs (direct repeats). However, this apparent paradox becomes alleviated by the fact that EcR/USP also recognizes direct repeats (DR1 up to DR12; Vögtli et al., 1998, and references therein). This, in turn, leaves us puzzled by the large variety of binding motifs, a situation that recently became accentuated by the finding of strong binding sequences that show no homology at all to the known consensus half site (AGGTCA) of PALs and DRs (Seibel et al., 1997; Seibel, unpublished data). This large array of recognized sequences indicates that the protein complex constituting the Ec receptor, i.e., EcR/USP, exhibits an extremely high degree of flexibility, a property that is also suggested by the lack of a preferred orientation of DRs in a gene's promoter (Vögtli et al., 1998). Even though the synthetic PAL1 (GAGGTCAA/TTGACCTC) is bound most strongly by EcR/USP, it has never been found in nature in its perfect form. This is probably because natural selection does not necessarily optimize for best binding but rather for best control, in particular, for the option of multiple control and fine tuning. DR1, for instance, is recognized by USP/USP as well as by USP/EcR; the ligand dictates which type of dimer bind predominates (Vögtli et al., 1998). The strength of EcR/USP binding is not strictly in parallel with the strength of transcriptional activation. This is evidenced by a comparison of DR4 with a natural EcRE of the *hsp27* gene (Vögtli et al., 1998). Moreover, negative control of transcription by Ec has been postulated to occur for a long time, i.e., with the so-called "late Ec-controlled genes" (Ashburner et al., 1974). It may be that some of the elements lacking any resemblance to the canonic half site exert a negative transcriptional control on a nearby target gene when occupied by a liganded EcR/USP complex (see Towers and Freedman, 1998).

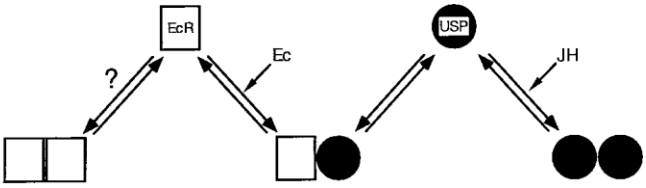

Fig. 1. Scheme illustrating how ecdysone (Ec) and juvenile hormone (JH) could control dimer formation of EcR and USP. EcR and USP monomers and their various dimers are thought to be in an equilibrium that is driven by Ec to the heterodimer (i.e., by binding) while JH promotes the homo-USP-dimeric state. It is still conjectural whether JH does so by binding to USP/USP (see Jones and Sharp, 1997) or indirectly. So far, homo-EcR-dimer formation has only been observed in vitro (Elke et al., 1997; Seibel, unpublished data; Mouillet, unpublished observations).

TRANSACTIVATION

Ligand-Controlled Activation Function AF-2

As shown for a variety of nuclear receptors, the core part of the ligand-controlled transactivation function AF-2 is located at the C-terminal end of helix 12 of the E domain, the major coactivator complex binding region (Masuyama et al., 1997). However, in none of the cloned EcRs and USPs has this function yet been identified, mapped, or characterized experimentally. There is strong circumstantial evidence, though, that it also exists in these members of the nuclear receptor superfamily.

Ligand-Independent Activation Function AF-1

This function usually resides in the A/B domain, at various places exhibiting no consistent sequence characteristics (see, e.g., Rochette-Egly et al., 1997). For the B1 isoform of EcR of *Drosophila melanogaster*, a general AF-1 has recently been mapped, by a one-hybrid approach in yeast to the very N-terminal end of the A/B domain (Mouillet et al., unpublished data). However, other parts of the A/B domain appear to modulate this transactivation function in a way that is not yet understood.

EcR Isoforms Differing in AF-1

In *D. melanogaster*, three EcR isoforms have been identified: B 1, B2, and A (Talbot et al., 1993). Their developmental profiles as well as mutation analyses suggest different stage, tissue, and cell cycle specificities for each of them (reviewed by Henrich et al., 1999). We set out to investigate the molecular basis of these differences starting by a search for AF-1 in their A/B domains. Besides B1, only the B2 isoform appears to exhibit an AF-1, which, however, is weaker. Interestingly, neither *C. tentans* EcR nor any of the USPs tested revealed an AF-1 in their A/B domain when assayed in yeast (Mouillet et al., unpublished data). It is probably not fortuitous that the AF-1 with the broadest host cell compatibility is that of the B1 isoform. Homologs of the *D. melanogaster* B1 isoform of EcR have been found in almost all insect species investigated (Henrich et al., 1999). This suggests that the A/B domain of B1, perhaps due to its very general AF-1, controls a function that is vital not only for insect but also for vertebrate and yeast cells and, thus, has been conserved during evolution.

CYTOPLASMIC ECR AND USP AS PUZZLE-PIECES IN LARGE COMPLEXES

Many nuclear receptors form supramolecular complexes in their cytoplamic state consisting of various heat shock proteins (HSPs), immunophilins, and other proteins (Gehring, 1998). The existence of higher molecular weight complexes was also strongly suggested for EcR by various physical analyses of cytoplasmic extracts (see, e.g., Turberg and Spindler, 1992). In fact, recent studies on prothoracic gland extracts of *Manduca sexta* reveal the presence of an immunophilin (FKBP46), HSP70, and HSP90 in complexes with EcR and USP (Song et al., 1997; L. I. Gilbert, personal communication). It, thus, appears that the Ec receptor follows the general behavior of steroid receptors although it belongs to the heterodimeric type of nuclear receptors, which are thought not to be complexed with HSPs (see, e.g., Dalman et al., 1990).

FITTING THE PUZZLE PIECES WITH ECR AND USP DOMAINS

As mentioned above, not all predicted functions could yet be assigned to specific domains or subdomains of EcR and USP. Conversely, for several domains and subdomains, the specific role in EcR/USP functioning is still mysterious. This holds true particularly for USP. Is the task of USP simply that of a "helper" enabling EcR to bind its ligand (Ec), to recognize its response element (EcRE), and to recruit a specific coactivator complex at its AF-2? Or else, does USP have its "own rights" within the Ec receptor complex in terms of intrinsic functions performed by its own domains? Such questions are usually attacked by investigating the functional capabilities of isolated domains in vitro or by looking in vivo for a loss of function due to mutations in a specific domain. A more subtle way of learning about a domain's role exists in the study of chimeric receptors in which the domain in question is exchanged by that of another species. The rationale thereby is not to use a defective domain but rather one that has proven to be functional, maybe in a slightly divergent manner.

In recent experiments (Henrich et al., unpublished data) chimeras were constructed in which the E and part of the D domain of *D. m elanogaster* USP were exchanged by the homologous sequences of *C. tentans*. When introduced into *D. m elanogaster* embryos lacking endogenous USP, this chimera was able to rescue development up to the prepupal stage, in higher doses even to fertile flies. The resulting animals, though,

exhibited an abdomen that resembled that of *C. tentans* rather than *D. melanogaster* because puparial contraction does not occur normally in chimeric USP-bearing mutants.

Various chimeras made of *D. melanogaster* and *B. mori* EcR domains revealed the specific heterodimerization characteristics and differential ligand responsiveness of *B. mori* EcR to be localized in the D domain as well as in the E2 and E3 subdomains (Suhr et al., 1998).

"Natural" chimeras of USP also exist. In crab (and tick), the heterodimer partner of EcR may be regarded as a chimera consisting of domains more closely related to an (insect) USP and an E domain exhibiting a higher degree of sequence homology to a (vertebrate) RXR (Chung et al., 1998; Guo et al., 1998). Accordingly, crabs respond to retinoids during regeneration. However, JH is also present in these types of animals obviously governing reproduction and the molting type (Laufer et al., 1993, and personal communication).

CONCLUDING REMARKS

The present review focused on just a few aspects of Ec receptor structure and function. Other aspects were not touched upon although they are or might turn out to be of significant importance as well. One of these future points certainly concerns phosphorylation (Rauch et al., 1998; Song and Gilbert, 1998). With regard to this point, we have just realized how important it might be.

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