Arthropod nuclear receptors and their role in molting

By: Yoshiaki Nakagawa and Vincent C. Henrich

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Abstract:

The molting process in arthropods is regulated by steroid hormones acting via nuclear receptor proteins. The most common molting hormone is the ecdysteroid, 20-hydroxyecdysone. The receptors of 20-hydroxyecdysone have also been identified in many arthropod species, and the amino acid sequences determined. The functional molting hormone receptors consist of two members of the nuclear receptor superfamily, namely the ecdysone receptor and the ultraspiracle, although the ecdysone receptor may be functional, in some instances, without the ultraspiracle. Generally, the ecdysone receptor/ultraspiracle heterodimer binds to a number of ecdysone response elements, sequence motifs that reside in the promoter of various ecdysteroid-responsive genes. In the ensuing transcriptional induction, the ecdysone receptor/ultraspiracle complex binds to 20-hydroxyecdysone or to a cognate ligand that, in turn, leads to the release of a corepressor and the recruitment of coactivators. 3D structures of the ligand-binding domains of the ecdysone receptor and the ultraspiracle have been solved for a few insect species. Ecdysone agonists bind to ecdysone receptors specifically, and ligand–ecdysone receptor binding is enhanced in the presence of the ultraspiracle in insects. The basic mode of ecdysteroid receptor action is highly conserved, but substantial functional differences exist among the receptors of individual species. Even though the transcriptional effects are apparently similar for ecdysteroids and nonsteroidal compounds such as diacylhydrazines, the binding shapes are different between them. The compounds having the strongest binding affinity to receptors ordinarily have strong molting hormone activity. The ability of the ecdysone receptor/ ultraspiracle complex to manifest the effects of small lipophilic agonists has led to their use as gene switches for medical and agricultural applications.

Keywords: ecdsyone receptor; ecdysteroids; EcR; insecticides; juvenile hormone; transcription factor; USP

Abbreviations:

20E. 20-hydroxyecdysone; CBP. cAMP response element-binding protein (CREB) binding protein; COUP. chicken ovalbumin upstream promoter; DAH. diacylhydrazine; DBD. DNA-binding domain; DR. direct repeat; DSF. dissatisfaction; EcR. ecdysone receptor; EcRE. ecdysone response element; ER. estrogen receptor; ERR. estrogen-related receptor; FTZ. fusi tarazu; GR. glucocorticoid receptor; GST. glutathione S-transferase; HNF4. hepatocyte nuclear factor 4; HR3. hormone receptor 3; HRE. hormone response element; IR1. inverted repeat 1; JH. juvenile hormone; LBD. ligand-binding domain; MET. methoprene-tolerant; NCoR. nuclear receptor corepressor; NR. nuclear receptor; PAL. palindrome; PE. phytoecdysteroid; PNR. photoreceptor-specific nuclear receptor; PonA. ponasterone A; QSAR. quantitative structure–activity relationships; RAR. retinoic acid receptor; ROR. retinoid-related orphan receptor; RXR. retinoid X receptor; SMRT. silencing mediator for retinoic and thyroid hormone receptor; SMRTER. SMRT EcR-cofactor; SVP. seven up; TLL. tailless; TR. thyroid hormone receptor; USP. ultraspiracle.

Article:

Introduction

Arthropoda is the largest phylum of the animal kingdom, and includes insects, crustaceans, mites, arachnids, scorpions and myriapods [1]. These animals are obliged to remove old shells in order to grow, in a process known as molting. Molting accompanies metamorphosis into the adult stage in some species, and precedes it in others. It has been reported that organisms in other phyla, including Nematoda, also grow through repeated molting in response to the action of a molting hormone [2]. Thus, the animal phylum that grows by repeated molting (or ecdysis) is classified as Ecdysozoa, which are protostomes (versus deuterostomes) and are better known as the molting clade. Ecdysozoa was originally proposed as the result of genetic studies using 18S rRNA genes [3]. Recently, it was reported that broad phylogenomic sampling improves the resolution of the animal tree of life [1]. Ecdysozoa includes species from eight animal phyla: Arthropoda, Onychophora, Tardigrada, Kinorhyncha, Priapulida, Loricifera, Nematoda and Nematomorpha.

The presence of the molting hormone was first recognized in the caterpillar and its chemical structure was proposed later. In 1965, two compounds were purified from tons of dissected pupal brains of the silkworm *Bombyx mori* and their chemical structures were characterized by X-ray crystal structure analysis. Later, it was disclosed that in most cases, the molting hormone is 20-hydroxyecdysone (20E; Fig. 1). Structurally related compounds, such as ponasterone A (PonA) [4], makisterone A (MakA) [5] and ecdysone [6] act as molting hormones in a few organisms. In most insects, ecdysone is the precursor of 20E and is synthesized in the prothoracic gland [7]. Synthesis of ecdysone is stimulated by the action of a prothoracicotropic hormone [8], and ecdysone released from the prothoracic gland is oxidized to 20E in peripheral tissues such as the fat body. However, in the prothoracic gland of Lepidoptera (except for *B. mori*), 3-deoxyecdysone is synthesized and secreted, and then converted to ecdysone by a hemolymph reductase [7]. As insects cannot construct the steroid skeleton *de novo*, they use ingested cholesterol and plant sterols such as stigmasterol, campesterol and β-sitosterol as a precursor, which is then oxidized by several P450 enzymes [9]. The biosynthetic pathway of ecdysone has been examined, and genes encoding the enzymes catalyzing each step have been identified [7]. Ecdysteroids, including ecdysone and 20E, also exist in plants, and nearly 400 phytoecdysteroids have been identified (http://ecdybase.org/).

In 1991, about a quarter of a century after the structural identification of molting hormones, the gene coding the ecdysone receptor (EcR) was first identified in *Drosophila* [10]. The homolog of the retinoid X receptor (RXR), the ultraspiracle (USP), was also characterized in the fruitfly *Drosophila melanogaster* [11,12]. EcR and USP (or RXR) bind to various ecdysone response elements (EcREs) as a heterodimer to transactivate several target genes [13], or in some species such as the scorpion, possibly as a homodimer [14]. The proteins encoded by ecdysteroid-dependent genes subsequently set off a multitiered hierarchy of responses that underlie and accompany cellular changes related to molting and metamorphosis [13,15]. Of course, the recruitment of a coactivator by EcR/USP (or RXR), after the release of corepressor by the binding of ligand molecule to EcR, is necessary for RNA polymerase activity [16].

The ecdysteroid receptor has proven to be a successful target for insecticides. Ecdysone agonists that are not easily metabolized can disrupt the molting process and lead to insect death. Moreover, the synthetic ecdysone agonists show variable levels of potency against EcR/USP from different insect orders [17], so that a specific agonist can be targeted to a subset of pest insect species. Furthermore, EcR/USP (or RXR) complexes have been engineered to respond to nonsteroidal compounds such as diacylhydrazines (DAHs) and act as a gene switch in mammalian and plant systems [18]. The nonsteroidal compounds are particularly useful for this adaptation because these compounds are used as insecticides in agriculture. Furthermore, they are environmentally safe, that is, they evoke little, if any, biological response in mammals and plants except for those responses that are transgenically introduced as responders to the gene switch. In this article, we will briefly review the study of nuclear receptors (NRs) and cofactors, and then summarize the study of arthropod ecdysteroid receptors, including sequences, functions, ligand-binding characteristics and the application of ligand–receptor complexes for agricultural and medical treatments.

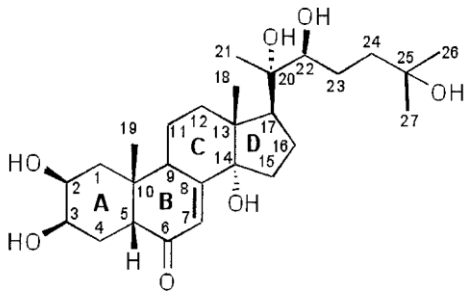

Fig. 1. Structure of 20-hydroxyecdysone. Numbers means the systematic numbering of the basic skeleton according to IUPAC nomenclature.

Nuclear receptors Outline

EcR and USP belong to a family of NRs that form a large family of transcription factors found only in metazoans. Many of these have been shown to play essential roles during the development of *D. melanogaster* and other insects [19]. Among various NRs, the full sequences of the human glucocorticoid receptor (GR) and the estrogen receptor a (ERa) were first determined in the mid-1980s. No NR has been found in the complete genome sequences currently available for plants, fungi, or unicellular eukaryotes, although receptors for some plant hormones exist in nuclei that are not members of the NR superfamily [20,21]. The activity of NRs is often regulated by small molecules (ligands) involved in widely diverse physiological functions such as the control of embryonic development, cell differentiation and homeostasis [22]. NRs also include orphan receptors [23], for which ligands do not exist or have not yet been identified. When transcription factors such as NRs bind to nucleotides within an enhancer sequence that is usually located in the gene promoter region, expression is affected. NRs act as ligand-inducible transcription factors by directly interacting as monomers, dimers, or heterodimers with RXRs via the DNA-response elements of target genes, as well as by 'cross-talking' to other signaling pathways [24]. At present, the gene regulation model for some receptors assumes that the unliganded receptor is bound to the hormone response element (HRE) and silences activity by associating with a corepressor [25]. To activate genes, the ligand molecules and coactivators are necessary through the exchange of corepressor proteins for coactivator proteins [26]. The complete gene network is a patchwork of multiple and independently controlled sites of expression [27].

In the human genome, 48 genes encode NRs [28], and the mouse genome encodes 49 NRs [29], although one more NR gene, plus three NR-related pseudogenes, have also been postulated in the human genome [30]. Because NRs are ligand-activated transcription factors that regulate the transcription of a variety of important target genes, NRs have been exploited as targets for therapy [22,31]. Coupled with tissue-specific promoters, the regulation system using ligands and NRs provides a strategy to address a wide range of human disorders [32].

Classification of arthropod NRs

NRs can be separated into seven groups, based on structural as well as functional data [27,33]. One large family, NR1, includes the thyroid hormone receptors (TRs), retinoic acid receptors (RARs), vitamin D receptors (VDRs) and peroxisome proliferator-activated receptors (PPARs) in mammals. The second family, NR2, contains RXRs and hepatocyte nuclear factor 4 (HNF4). Receptors for mammalian steroid hormones, such as ER and GR, belong to the NR3 family. The insect steroid hormone receptor, EcR, is grouped in NR1, as summarized by Bonneton et al. (Fig. 2) [34]. RXRs can act as the heterodimeric partners of many NR1 family members, including the TRs, VDRs, PPARs and several orphan receptors, as well as EcRs.

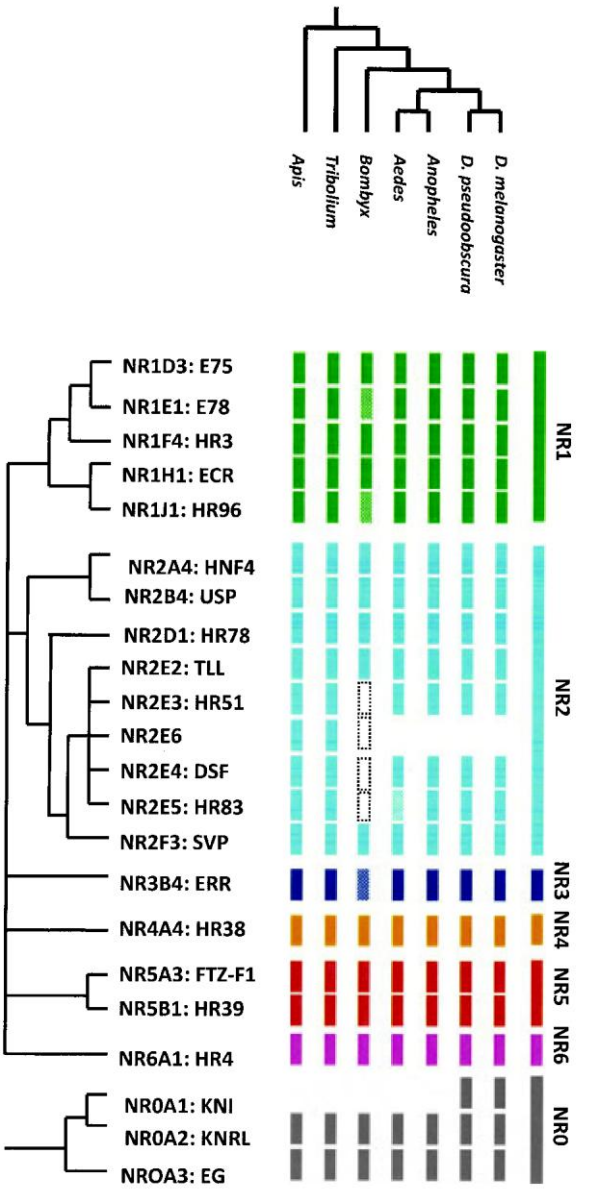

Fig. 2. Classification of nuclear receptors of holometabolous insects. (Modified from the figure from [34] with the permission of Elsevier Ltd.; The original figure is kindly provided by F. Bonneton.)

EcR is officially designated as NR1H1, and other NR1 family members include E75 (NR1D3) and E78 (NR1E1) [35]. In fact, the *EcR* gene was identified through the use of a probe from the E75 gene. To date, the E75 gene has been identified in numerous insects such as *D. melanogaster* [36], the yellow fever mosquito *Aedes aegypti* [37], the greater wax moth *Galleria mellonella* [38], the forest tent caterpillar *Malocosoma disstria* [39], the spruce budworm *Choristoneura fumiferna* [40], the tobacco hornworm *Manduca sexta* [41], *B. mori* [42], the Indian meal worm *Plodia interpunctella* [43], the red flour beetle *Tribolium castaneum* [34] and the honeybee *Apis mellifera* [44] as well as the greasyback shrimp *Metapenaeus ensis* [45]. The E75 gene in *D. melanogaster* encodes three isoforms designated E75A, E75B and E75C [36]. E75 binds to heme and can use this prosthetic group to exchange diatomic gases such as NO and CO [46]. E75 also acts as a repressor of hormone receptor 3 (HR3) which also belongs to NR1 (NR1F4), probably through direct interaction in *B. mori* [47] and *D. melanogaster* [48]. E75 proteins are homologous to the vertebrate orphan nuclear receptors REV-ERBα (NR1D1) [49] and REV-ERBβ (NR1D2) [50, 51]. It was also reported that *Drosophila* HR51 may be either a gas or a heme sensor [52]. Generally, REV-ERB seems to be a gas sensor [53]. In *Drosophila*, inactivation of all E75 functions causes larval lethality, but isoform-specific null mutations reveal different subfunctions for each of the three isoforms [54]. The complex role of E75 is not fully understood, but

expression and hormonal induction data suggest that its involvement in the early ecdysone response may be shared among arthropods. E75 also plays a role during oogenesis and vitellogenesis in *Drosophila* [55], *Aedes* [37] and *Bombyx* [47].

HR3 orthologs have been identified in various insect species, including *D. melanogaster* [56], *A. aegypti* [57], *M. sexta* [58], *G. mellonella* [59], *M. disstria* [39], *C. fumiferana* [60], *P. interpunctella* [61], the mealworm *Tenebrio molitor* [62], the American boll worm *Helicoverpa armigera* [63] and the German cockroach *Blattera germanica* [64], as well as the nematode, *Caenorhabditis elegans* [65]. HR3 is homologous to three retinoid-related orphan receptors (RORs), namely RORα, RORβ and RORγ. These RORs and REV-ERB bind to the same response element, and RORs are thought to be competitors for REV-ERB and are believed to play an important role in circadian rhythms [66]. HR3 plays a key role during metamorphosis by repressing early genes, and directly induces the fusi tarazu (*ftz*) gene that encodes the prepupal regulator FTZ-F1 (NR5A3) [48,67].

Another hormone receptor belonging to the NR1 family is HR96, which binds selectively to the canonical EcRE, the *hsp*27 EcRE. The gene encoding HR96 is expressed throughout the third-instar larval and prepupal development of *Drosophila* [68]. Even though little is known about the function of HR96, it is possible that HR96 requires USP to bind DNA in the same way as EcR [68]. A *Drosophila* HR96 null mutant displays a significant increase in its sensitivity to the sedative effects of phenobarbital as well as defects in the expression of many phenobarbital-regulated genes [69]. Metabolic and stress-response genes are controlled by HR96 in *Drosophila*.

Most of the NR2 proteins are orphan receptors [52]. Insects carry eight genes encoding HNF4 (NR2A4), USP (NR2B4), HR78 (NR2D1), seven up (SVP; NR2F3), tailless (TLL; NR2E2), HR83 (NR2E5), dissatisfaction (DSF; NR2E4) and HR51 (NR2E3). HNF4 is one of the most highly conserved NRs between arthropods and vertebrates, and has been identified in *Drosophila* [70], *A. aegypti* [71] and *B. mori* [72]. HNF4 probably performs similar functions during gut formation, and it has been shown that the mammalian HNF4 binds fatty acids constitutively [73]. High similarity observed for the HNF4 ligand binding domain (LBD) between insects and vertebrates suggests that this type of ligand interaction may occur in insects.

The gene encoding HR78 has been identified in *Drosophila* [68], *B. mori* [74] and *T. molitor* [62], and is distantly related to the vertebrate's orphan receptors TR2 and TR4, and to the nuclear hormone receptor 41 (NHR-41) of *C. elegans* [75] and the NHR-2 of filarial nematode *Brugia malayi* [76]. HR78 is required for ecdysteroid signaling during the onset of metamorphosis of *Drosophila* [68,77]. This receptor is inducible by 20E and binds to more than 100 sites on polytene chromosomes, many of which correspond to ecdysteroid-regulated puff loci. HR78 is associated with a sterile a motif (SAM) domain containing the corepressor, middleman of seventy-eight signalling (Moses), which specifically inhibits HR78 transcriptional activity independently of histone deacetylation. Moses is co-expressed in the same tissues as HR78 [78].

SVP is a member of the chicken ovalbumin upstream promoter (COUP) transcription factor group. The COUP transcription factor exists in a number of different tissues and is essential for expression of the chicken ovalbumin gene. The COUP transcription factor specifically binds to the rat insulin promoter element [79]. The *svp* gene has been identified in *Drosophila* [80], *A. aegypti* [81], *B. mori* [82], *T. molitor* [62] and the grasshopper *Schistocerca gregalia* [83]. Overexpression of *svp* causes lethality in *Drosophila*, but this lethality is offset by the simultaneous overexpression of *usp*. Presumably, SVP competes with USP for heterodimerization with EcR and thereby offsets ecdysone action [84].

The tailless (tll) gene was identified in *Drosophila* [85] and its homologs have been studied in the housefly *Musca domestica* [86] and *T. castaneum* [87]. Nematodes and vertebrates also have a *tll* homolog. TLL is primarily involved in the development of forebrain, and its role in segmentation was probably acquired during the evolution of holometabolous insects. This gene is homologous to the vertebrate photoreceptor- specific nuclear receptor (PNR) [88]. *PNR* gene expression is restricted to the retina and plays a critical role in the development of photoreceptors. Both TLL and PNR play

important roles during vertebrate eye development. According to Laudet and Bonneton, the role of TLL in the formation of the visual system is conserved between insects and vertebrates [89].

The dissatisfaction (*dsf*) gene, which has been identified in *D. melanogaster* [90], the fruitfly, *Drosophila virilis* and *Manduca* [91] encodes DSF (NR2E4), which is necessary for appropriate sexual behavior and sex- specific neural development in both male and female insects [92]. It will be interesting to test whether DSF will prove to be a ligand-dependent activator, assince no sex hormones are known in insects [93].

Recently Sung *et al*. [94] reported the functional analysis of the *unfulfilled*/HR51 gene in *Drosophila*, which is the ortholog of *C. elegans fax*-1 and human PNR. The *fax*-1 gene was first identified in *Caenorhabditis* as a regulator of axon path finding and neurotransmitter expression [95]. Both *fax*-1 and *PNR* mutations disrupt developmental events in a limited number of neurons, leading to behavioral or sensory deficits.

NR3 comprises the receptors for sex and adrenal steroid hormones, such as estrogen, androgen, progesterone, glucocorticoids and mineralocorticoids. In insects, estrogen-related receptor (ERR; NR3B4) is an orphan receptor related to ER [96]. It appears that, with the exclusion of ERR, members of the NR3 family were specifically lost in ecdysozoans.

NR4 is a small group of orphan receptors containing the vertebrate's nerve growth factor-induced clone B (NGFI-B) [97] and nucleus receptor related 1 (NURR1) [98], and insect HR38 (NR4A) [75]. The gene encoding HR38 has been cloned in *Drosophila* [68,99], *A. aegypti* [100] and *Bombyx* [99]. HR38 can bind DNA either as a monomer or through an interaction with USP and outcompetes EcR/USP heterodimerization. HR38 is not directly regulated by 20E, but can participate in the 20E pathway as an alternative partner to USP. Reporter fusion proteins have shown that the HR38-LBD/USP-LBD is responsive to ecdysone and to several 20E metabolites in *Drosophila*, but HR38 and NURR1 lack a conventional ligand-binding pocket and a bona fide AF2 transactivation function [101,102]. In *Drosophila*, HR38 is expressed in the ovaries and during all stages of development. Different mutant alleles have different lethal phases, from larval stages to adults, demonstrating the role of HR38 in metamorphosis and adult epidermis formation [103]. Interestingly, the vertebrate NGFI-B receptors are ligand-independent transcriptional activators and are considered to be true orphans [101].

The NR5 family includes FTZ-F1 (NR5A3) [104, 105] and HR39 (NR5B1) [106] in insects, which are players in the ecdysone-regulated response pathway [107]. FTZ-F1 has two isoforms with different amino- terminal domains (α and β). The FTZ-F1 gene has been cloned across a wide range of insect orders and crustaceans, including Diptera [105], Lepidoptera [108], *A. mellifera* [109], *T. molitor* [62] and the greasyback shrimp, Metapenus ensis [110]. *Drosophila* αFTZF1 is a direct regulator of the pair-rule gene *ftz*, whose product governs the formation of embryonic metameres [111]. The PFTZ-F1 plays a central role during the molting and metamorphosis of *Drosophila* [112]. HR3 temporally regulates FTZ-F1 gene expression, which, in turn, initiates transcriptional activity associated with the onset of metamorphosis. For instance, in the larval salivary gland of *D. melanogaster*, FTZ-F1 is silent during the large 20E peak. Moreover, when the epidermis is cultured with 20E, PFTZ-F1 mRNA is not induced until after the removal of 20E [113]. The general characteristics of FTZ-F1 seem to be well conserved in Lepidoptera such as *Bombyx* [114] and *Manduca* [108,113]. HR39 (NR5B1) has so far been found in *Drosophila* [106] and *Anopheles* [115]. The *Caenorhabditis* genome does not include an HR39 homolog, but a FTZ-F1 gene exists. The HR39 gene of *Drosophila* is induced by 20E and is expressed at every stage of development, with a maximum at the end of the third instar larval and prepupal stages [107]. Recently, *Drosophila* HR39 has been implicated in the regulation of female reproductive tract development, a role that closely resembles the function of the mammalian steroidogenic factor 1 (SF1) homolog [116].

HR4 (NR6A1) belongs to NR6 in insects and is homologous to a vertebrate orphan receptor, germ cell nuclear factor (GCNF). The HR4 gene has been identified in the genome of *Drosophila* [117], *Anopheles*, *Manduca*

[108], *Bombyx* [118], *Trichoplusia ni* [119] and *Tenebrio* [62]. This gene is also identified in nematodes [75]. The HR4 gene is directly inducible by 20E in *Manduca* [113] and *Tenebrio* [119].

Structures of NRs

The basic structure of typical NRs includes several modular domains: A/B, C, D and E regions (or domains). Some receptors, including the EcR of *D. melanogaster*, also have a carboxy-terminal F-region whose function is unknown; however, deletion of the F-domain seems to have no functional consequences in flies [120]. A highly variable amino-terminal A/B region interacts with other transcriptional factors, and this region is responsible for a ligand-independent transcriptional activation function, which function is known as AF1. The modulatory domain can also be the target for phosphorylation mediated by other signaling pathways, and this modification can significantly affect both ligand-dependent and ligandindependent transcriptional activity, as demonstrated in RXRα [121].

The C region is the central DNA-binding domain (DBD) and consists of two highly conserved zinc-finger motifs that are characteristic of the NR superfamily [21]. The core DBD contains two α-helices. The first α-helix binds the major groove of DNA by making contacts with specific bases, and the second α-helix forms at a right angle with the recognition helix [122]. The DBD targets the receptor to specific DNA sequences, called HREs [123], as discussed below. The DBD contains nine cysteines, as well as other structures that are conserved across the NRs and are required for high-affinity DNA binding. The two 'zinc fingers' span about 60–70 amino acids, and a few receptors also contain a carboxy-terminal extension containing T-box and A-box motifs [124]. In each zinc finger, four invariant cysteine residues coordinate tetrahedrically to a zinc ion, and both zinc fingers fold together to form a compact structure [122]. The amino acids required for discrimination of core DNA-recognition motifs are present at the base of the first finger in a region termed the P box, and the D-box of the second finger. D-box is also involved in dimerization of NRs. Although some monomeric receptors can bind to a single hexameric DNA motif, most receptors bind as homodimers or heterodimers to HREs composed of two core hexameric motifs (half-sites). For dimeric HREs, the half-sites can be configured as palindrome (PAL), inverted palindromes, or direct repeats (DRs). In general, the HREs are separated by a gap of one or more nucleotides [125], as will be discussed later (in the section 'Ecdysone response elements'). The AGAACA motif is preferentially recognized by members of the NR3 family, but AGG/TTCA is recognized by other receptors. For example, vertebrate steroid hormone receptors (such as GRs, mineralcorticoid receptors, progesterone receptors and androgen receptors) bind homodimerically to the palindromic elements spaced by three nucleotides (AGAACAnnnTGTTCT) in a symmetrical manner, whereas ERs bind to AGGTCAnnnTGACCT [126].

The D region serves as a hinge between the C and E regions, and harbors nuclear localization signals. Mutations in the D region have been shown to abolish the interaction with NR corepressors [127].

The E region is the LBD and functionally is very unique to NRs. In the case of EcR, the LBD plays roles in (a) receptor dimerization, (b) ligand recognition and (c) cofactor interactions. The 3D structure of the LBD was first analyzed for RAR [128] and RXR [129], followed by other nuclear receptors. The crystal structure of nuclear receptors has indicated that the LBD is formed by 10–12 conserved α-helices numbered from helix-1 (H1) to H12 and there is a conserved β-turn between H5 and H6 [129]. A central core layer of three helices is precisely packed between the other two layers to create the hydrophobic ligand-binding pocket. Several differences are evident when comparing unliganded and ligand-bound receptors [16]. Ligand binding to the receptor (holo-receptor) occurs through contacts with specific amino acid residues in the pocket, promoting a conformational change in which the most carboxy-terminal H12 folds to form a 'lid' over the pocket and also leads to the dissociation of the corepressor. Thus, H12 is able to interact with coactivators and promotes the transcription of target genes in a ligand-dependent (AF2) manner. H12 projects away from the LBD body in unliganded RXR [129], but this helix moves in a 'mouse-trap' that is tightly packed against H3 or H4 in liganded receptors, thus making direct contacts with the ligand [128,130].

Cofactors

As noted above, the function of the ligand–receptor complex is regulated by cofactors (or coregulators), such as coactivators and corepressors [27], which can determine whether a given ligand acts as an agonist or an antagonist. Coactivator and corepressor complexes serve as 'sensors' that integrate signaling inputs to generate precise and complex programs of gene expression [26]. Many coactivators and corepressors are components of the multisubunit cofactor complex that exhibits various enzymatic activities, and these cofactors can be divided into two classes. The first class consists of enzymes that are capable of covalently modifying histone tails through acetylation/deacetylation and methylation/demethylation, protein kinases, protein phosphatases, poly(ADP)ribosylates, ubiquitin and small ubiquitin-related modifier (SUMO) ligases [131]. The second class includes a family of ATP-dependent remodeling complexes [132].

The first coactivator described is a member of the p160 (160 kDa protein) family, which was cloned and identified as a steroid receptor coactivator (SRC-1) [133]. This was followed by the cloning of numerous activators such as SRC-2 and a cAMP response element-binding protein (CREB) binding protein (CBP)/p300. Over the years, cofactors have been identified for a wide range of NRs [134]. The liganded NRs bind members of the p160 family, which recruit a CBP/p300 to a target gene promoter. This recruitment locally modifies the chromatin structure through the CBP/p300 histone acetyltransferase activities. The first corepressors identified were named nuclear receptor corepressor (NCoR) [135] and the silencing mediator for retinoic and thyroid hormone receptor) (SMRT) [136]. Later, other molecules that may be corepressors were identified by several groups [137].

Molting hormone receptors (EcR and USP)

Outline

Puffs appear at specific locations along polytenized chromosomes in response to pulses of 20E. Ashburner and his colleagues proposed a model for puff response that was based on their studies (carried out in 1973) of isolated salivary glands exposed to ecdysone under a variety of conditions. In this model, early genes are induced and late genes are repressed by a hormone– receptor complex. It is now known that these early genes (*E75*, *E74* and *Broad-Complex*) encode transcription factors that are involved in two types of modulations to the primary response mediated by the functional molting hormone receptor, the EcR/USP heterodimer. One secondary response is the repression of early gene transcription by early gene products, while another is the induction of late gene transcription by these same early gene products [138].

The insect steroid hormone receptor identified from *D. melanogaster* was designated as EcR [10]. EcR was verified as an NR based on its amino acid similarity to the first NRs identified, namely GR and ER. The EcR of *D. melanogaster* is described here as DmEcR, designating the species name, and this convention is also used for other species. There are three EcR isoforms in *D. melanogaster* [139], and probably multiple EcR isoforms exist in several, but not all, insect species. In all cases described so far, the isoforms vary in their amino-terminal domain and presumably interact with different transcription factors to mediate gene activity.

The most important heterodimeric partner for EcR is the USP. In the case of *D. melanogaster*, the USP is about 86% identical to RXRα in the DBD and shares 49% identity in the LBD with RXR, but only 24% with RAR. The USP was originally identified from several recessive lethal alleles of *Drosophila* that failed to molt at the transition from the first to the second instar. When maternal USP mRNA is absent, the developmental failure occurs during embryogenesis [140]. Transcript levels of *usp* genes in most species vary modestly through their development, though their profiles vary among them [141]. Expression of the *usp* gene after the lethal phase of *usp* mutants indicates a continuing role for *usp* through metamorphosis. This has been experimentally demonstrated by showing that the expression of normal or modified forms of USP can rescue larval development [142], but that the depletion of wild-type *usp* in the third instar causes premetamorphic lethality. In a similar experiment, an interspecific (chimeric) *Drosophila/Chironomus usp* gene was introduced trans-genically, which substituted the LBD of *Drosophila* USP (DmUSP) with that of the midge *Chironomus tentans*. This gene product rescued larval development in *usp* mutant larvae, but led to the same metamorphic failure as *usp* depletion [143]. In other words, the chimeric USP successfully fulfills a larval USP function in *Drosophila*,

but is unable to replace a function at metamorphosis that involves the DmUSP-LBD; thus, two general points concerning the USP emerge. First, the DmUSP-LBD carries out at least two developmentally distinct functions during the larval stages and metamorphosis. Second, the metamorphic function cannot be carried out by the USP-LBD of a closely related Dipteran species, suggesting that a diversity of regulatory functions are carried out by USPs among species.

The EcR/USP (or RXR) heterodimer regulates a wide variety of physiological functions in development, reproduction, homeostasis and metabolism. In fact, ecdysteroids are known to regulate the transcription of genes encoding several other NRs, which, in turn, carry out individual cellular functions. Even though USP expression varies modestly during larval stages, USPs participate in both the activation and repression of gene expression. The USP forms heterodimers with at least two other orphan receptors in *Drosophila*, namely Drosophila HR38 [99] and SVP [84], which are briefly reviewed above in the section entitled 'Classification of arthropod nuclear receptors'. The USP has a potentially repressive role in eye and neuronal development that is disrupted when the USP-DBD is mutated, although this modified USP maintains its ability to form an active heterodimer with EcR-B1 [144]. However, without its DBD, the USP is unable to form an active dimer with EcR-A and EcR-B2 in cell culture, suggesting that the interaction of USP with EcR is isoform-dependent [145]. The phosphorylation of USP inhibits ecdysteroid biosynthesis in *M. sexta* [146] and is required for normal induction of expression of the 20E gene in the salivary glands of *D. melanogaster* [147].

Primary sequences of EcR and USP

To date, the cDNA sequences for EcR and USP have been cloned not only from insects but also from other arthropods such as crustaceans, mites and a scorpion, and these are summarized in Table 1. In insects such as Diptera, Lepidoptera and Hymnoptera, the imaginal discs differentiate abruptly into adult structures in response to pulses of 20E, whereas the larval tissues die or are remodeled into adult forms responding to the same stimuli. These metamorphic responses of tissues to ecdysteroids show a general correlation with the expression patterns of the EcR isoforms in *Drosophila*. The DmEcR-A isoform is expressed predominantly in the imaginal discs, and the DmEcR-B1 isoform is expressed predominantly in larval tissues [139]. Specific metamorphic responses seem to require particular DmEcR isoforms [148]. Nevertheless, the relationship between isoform expression and function has not been fully verified by genetic analysis [149]. Complex temporal and spatial expression patterns of DmEcR-A and DmEcR-B1 isoforms are correlated with the cell-typespecific response to ecdysteroids [150]. Generally, DmEcR-A predominates when cells are undergoing maturational responses, and DmEcR-B1 predominates during proliferative or regressive responses. Kamimura *et al.* [151] reported that BmEcR-B1 was predominant in most tissues of *Bombyx*, including the wing imaginal disc and larval tissues such as the fat body, epidermis and midgut. In the anterior silk gland, however, BmEcR-A was predominantly expressed. Only small amounts of mRNA species for both isoforms were detected in the middle and the posterior parts of the silk gland. The levels of BmEcR-A mRNA increased when the ecdysteroid titer was basal (20 ng.mL-1) and began decreasing just before the hormone peak [152]. However, the expression of BmEcR-B1 mRNA was low when that of BmEcR-A was high. The expression of mRNA for *T. molitor* (Tm)EcR-A and for TmEcRB1 became evident just before the rise of each ecdysteroid peak, both in prepupae and pupa [153]. A relatively small amount of variation in the expression level of *usp* transcripts was found, whereas the genes for the DmEcR isoforms were expressed in a tissue-restricted pattern in the same stage. The *DmEcR-B1* gene was expressed at higher levels in larval tissues that are destined for histolysis, while *DmEcR-A* predominates in the imaginal discs.

The phylogenetic tree constructed from the EcR sequences is consistent with the taxonomic analysis among insects, as shown in Fig. 3 [14]. EcRs have also been cloned from the Chelicerata phylum that includes mites [154,155] and scorpion [14]. Guo *et al.* [154] isolated cDNAs encoding three presumed EcR isoforms (AamEcR-A1, AamEcR-A2 and AamEcR-A3) from *A. americanum*, but none was equivalent to the B-isoforms in *D. melanogaster*. The AmEcR-A1 amino- terminus shares limited similarity to that of DmEcR-A [139] and to that of the EcR-A of *M. sexta* (MsEcRA) [156], and the amino-terminus of AmEcR-A3 is similar in size to that of DmEcR-B2 [139]. The DBD and LBD of AmEcRs share 86% and 64% identity with the respective domains of insects. The amino-termini are highly divergent and the receptors lack F-domains, whereas

Mecopterida have a very long F-domain [157]. The presence of EcR was also confirmed in the scorpion *Liocheles australasiae* (LaEcR), but LaEcR binds to the ligand molecule with high affinity in the absence of RXR, which is different from the situation in insects [14]. The regulation of glue gene transcription by 20E in the *Drosophila* salivary gland during the mid-third instar requires EcR but does not require USP [158]. In summary, there is growing evidence that EcR can, at least under some conditions, act as a receptor without USP/RXR.

Originally, *usp* genes were identified as *rxr* orthologs in *D. melanogaster*, and the encoded protein was named USP. The rxr genes were also successfully cloned from *A. americanum* [159] and from the soft tick *Ornithodoros moubata* [155], as well as from the scorpion *L. australasiae* [14]. Similarly to EcRs, multiple USP isoforms have been found in *A. aegypti* [160], *M. sexta* [161], *C. tentans* [162] and *A. americanum*, but only a single form has been found in *D. melanogaster* and in several other species. In *A. americanum*, the two isomers are named AamRXR-1 and AamRXR-2 [159]. According to the phylogenetic tree constructed from the sequences of USP (RXR) (Fig. 3), USPs of Lepidoptera and Diptera are distant from RXRs. Interestingly, USPs of mites, scorpions and crustaceans are more similar to the RXR of humans than to the USPs of Diptera and Lepidoptera. Nevertheless, none of the insect USP proteins are functionally activated by known RXR ligands, with the notable exception of the *Locusta* USP whicht is activated by 9-cisRA, suggesting that the arthropod USP is functionally distinct in fundamental ways from vertebrate RXR [163]. It has been reported that methyl farnesoate exhibited high affinity for DmUSP [164].

Table 1. EcRs and USPs (RXRs) successfully cloned to date from Ecdysozoa.

Animals			
Order	Species	EcR or USP (RXR)	Reference[a]
Insects			
Diptera	*Aedes aegypti*	EcR	[262]
	Aedes aegypti	USPa, USPb	[160]
	Aedes albopictus	EcR, USP	[263]
	Bradysia hygida	EcR	AAD21309
	Calliphora vicina	EcR	AAG46050
	Ceratitis capitata	EcR-B1	[264]
	Ceratitis capitata	EcR-A	[265]
	Drosophila melanogaster	USP	[12]
	Drosophila melanogaster	EcR-B1	[10]
	Drosophila melanogaster	EcR-A, EcR-B1, EcR-B2	[139]
	Drosophila melanogaster	USP	[13]
	Drosophila melanogaster	EcR-B1	[266]
	Drosophila pseudoobscura	EcR	[267]
	Lucilia cuprina	EcR	[266]
	Lucilia sericata	EcR	BAD12052
	Sarcophaga crassipalpis	EcR (partial), USP (partial)	[268]
	Sarcophaga similis	EcR	BAD81037
Lepidoptera	*Bicyclus anynana*	Ecdysteroid receptor	CAB63236
	Bombyx mori	USP (CF1)	[269]
	Bombyx mori	EcR-B1	[270]
	Bombyx mori	EcR-B1	[271]
	Bombyx mori	EcR-A	[151]
	Chilo suppressalis	EcR-A, EcR-B1	[272,273]
	Chilo suppressalis	USP	[171]
	Chironomus tentans	EcR1(B1), EcR2, EcR3	[274]
	Chironomus tentans	USP-1, USP-2	[162]
	Choristoneura fumiferana	EcR	[275]
	Choristoneura fumiferana	USP	[276]
	Choristoneura fumiferana	EcR-A, EcR-B	[178]
	Helicoverpa armigera	EcR, USP-1, USP-2	[277]
	Heliothis virescens	EcR-B1	[278]
	Heliothis virescens	EcR-B1, USP	[167]
	Junonia coenia	Ecdysteroid receptor	CAB63485
	Lucilia cuprina	EcR, USP	[277]
	Manduca sexta	EcR-B1, EcR-A	[279]
	Manduca sexta	USP-1, USP-2	[280]
	Orgyia recens	EcR-A, EcR-B1	BAC44996, BAC44997
	Omphisa fuscidentalis	EcR-A, EcR-B1	[281]
	Plodia interpunctella	EcR-B1	[61]
	Spodoptera exigua	EcR	ACA30302
	Spodoptera litura	EcR	ABX79143
	Spodoptera frugiperda	EcR-B1, USP-2	[119]
	Trichoplusia ni	EcR-B1, USP-2	[119]
Hymenoptra	*Apis mellifera*	EcR-A	[282]
	Copidosoma floridanum	Putative EcR	[283]
	Camponotus japonicus	EcR-A, EcR-alpha	[284]
	Leptopilina heterotoma	EcR, USP (partial)	[157]
	Nasonia vitripennis	EcR-A, EcR-B1	NP_001152828 NP_001152829
	Pheidole megacephala	EcR-A, EcR-B	BAE47509, BAE47510
	Polistes dominulus	EcR	[285]
	Scaptotrigona depilis	USP	ABB00308
Coleoptera	*Anthonomus grandis*	EcR (partial)	[286]
	Anthonomus grandis	EcR	ACK57879

Table 1. (Continued).

Animals			
Order	Species	EcR or USP (RXR)	Reference[a]
	Leptinotarsa decemlineata	EcR-A, EcR-B1, USP	[172]
	Tribolium castaneum	EcR-A, EcR-B, USP	[163]
	Tenebrio molitor[a]	EcR-A, EcR-B1	[153]
	Tenebrio molitor	USP	[287]
	Harmonia axyridis	EcR-A, EcR-B1 USP-1, USP-2	(Morishita *et al.*, unpublished)[b]
	Epilachna vigintioctopunctata	EcR-A, EcR-B1 USP-1, USP-2	(Morishita *et al.*, unpublished)[c]
Orthoptera	*Blattella germanica*	RXR-S, RXR-L	[288]
	Blattella germanica	EcR-A	[289]
	Locusta migratoria	EcR	[290]
	Locusta migratoria	RXR	[291]
Hemiptera	*Bemicia tabaci*	EcR, USP (protein)	[285]
	Bemicia tabaci	EcR	[168]
	Bemicia tabaci	EcR, USP	[277]
	Myzus persicae	EcR, USP	[277]
	Acyrthosiphon pisum	EcR-A, EcR-B1	NP_001152831 NP_001152832
Dictyoptera	*Periplaneta americana*	USP (RXR) (partial)	[157]
Collembola	*Folsomia candida*	USP (RXR) (partial)	[157]
Myriapoda	*Lithobius forficatus*	USP (RXR) (partial)	[157]
Urochordata	*Polyandrocarpa misakiensis*	USP (RXR) (partial)	[157]
Trichoptera	*Chimarra marginata*	USP (RXR)	[285]
	Hydropsyche incognita	EcR	[285]
Mecoptera	*Panorpa germanica*	EcR, USP (RXR)	[285]
Siphonaptera	*Ctenocephalides felis*	EcR, USP (RXR) protein	[285]
Strepsiptera	*Xenos vesparum*	EcR, USP	[285]
Crustacean	*Carcinus maenas*	Ecdysteroid receptor	AAR89628
	Daphnia magna	EcR-B, EcR-A1, EcR-A2	[292]
	Gecarcinus lateralis	EcR	[293]
	Gecarcinus lateralis	RXR	[294]
	Marsupenaeus japonicus	EcR, RXR	[295]
	Uca (Celuca) pugilator	EcR, RXR	[296]
	Uca (Celuca) pugilator	EcR-B1, RXR-1	[297]
Mite	*Amblyomma americanum*	EcR-A1, EcR-A2, EcR-A3	[154]
	Amblyomma americanum	RXR-1, RXR-2	[159]
	Ornithodoros moubata	EcR-A	[298]
	Ornithodoros moubata	RXR	[155]
Others			
Scorpion	*Liocheres australasiae*	EcR-B1, RXR	[14]
Nematode	*Dirofilaria immitis*	RXR-1	[2]
Filaria	*Brugia malayi*	EcR-A, EcR-C	ABQ28713, ABQ28714
	Brugia malayi	RXR	ABQ28715
Trematode	*Schistosoma mansoni*	RXR	[299]

[a] Unless published, GenBank accession numbers are listed. [b] Sequences have been submitted to the databank and their GenBank accession numbers are available (HaEcR-B1: AB506665; HaEcR-A: AB506666; HaUSP-1: AB506667; HaUSP-2: AB506668). [c] Sequences have been submitted to the databank and their GenBank accession numbers are available (EvEcR-B1: AB506669; EvEcR-A: AB506670; EvUSP-1: AB506671; EvUSP-2: AB506672).

3D structures of EcR and USPs

The crystal structures of insect NRs were first analyzed in the USPs of the tobacco budworm *Heliothis virescens* [165] and *D. melanogaster* [166]. The overall architecture of the USP-LBD exhibits canonical NR folding with 11 α-helices (H1 and H3–H12) and two short β-strands, which make a three-layered helical sandwich. This crystal structure contains three binding pockets with significantly lower ligand occupancy, which does not correlate with any changes in the conformation of H12 or in the loop between H1 and H3, suggesting that the lipid binding has little effect on the overall structure of the USP-LBD. The region close to H1 has been implicated in cofactor binding [135]. During activation of the EcR/USP receptor complex, H12 of the USP-LBD adopts a conformation that resembles the antagonist conformation in RXRoc. Two helices (H1 and H3)

comprise an outer layer and are less coplanar in USP than in RXRoc. Three helices (H7, H10 and H11) form part of the outer layer, and four helices (H4, H5, H8 and H9) form a central layer. Divergence between USPs and RXRs is observed mainly for the loop in USP that connects H5 to the β-turn and the loop between H1 and H3. The β-turn is longer for USPs than for RXRs and its length varies inside the USP family. By contrast, the length of the loop between H1 and H3 is rather similar for USPs and RXRs, although the amino acid sequence is poorly conserved between them.

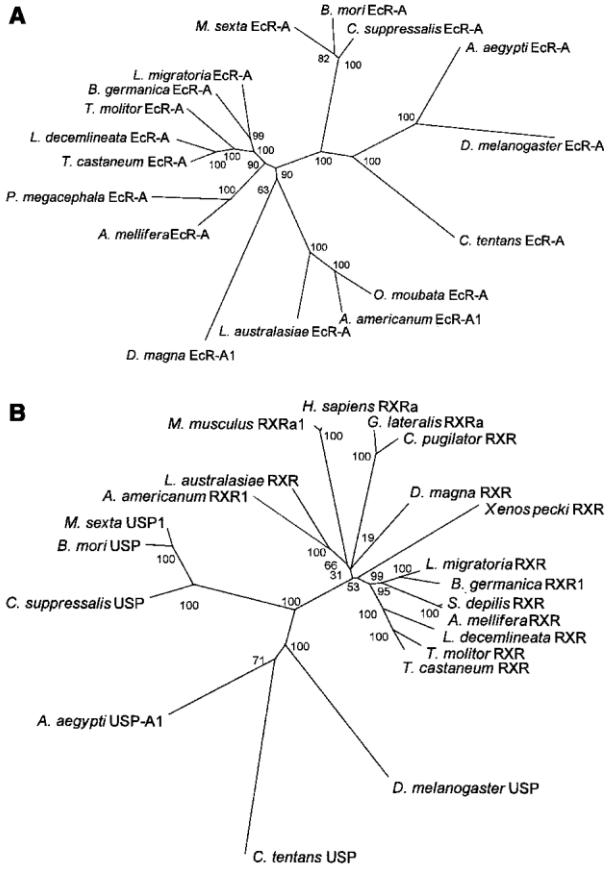

Fig. 3. Phylogenetic trees constructed from primary sequences o the EcR and the USP. (Figure originally published in [14].)

The 3D structure of the PonA-bound EcRLBD/USP-LBD in *H. virescens* was first reported in 2003 [167]. PonA-bound EcR-LBDs from the sweet potato whitefly *Bemicia tabaci* [168] and from *T. castaneum* [163] were also analyzed. The crystal structure of the 20E-bound EcR-LBD has also been solved in *H. virescens*, showing that 20E binds to the same pocket as PonA [169]. The overall structures of the PonA-bound LBDs of HvEcR, BtEcR and TmEcR were similar, as anticipated by their sequence similarity. In the PonA-bound HvEcR-LBD complex, the LBD is composed of 12 helices and three small β-strands, and possesses a long and thin L-shaped cavity extending towards H5 and the β-sheet. The PonA is bound with the steroid A-ring oriented towards H1 and H2, and with the D-ring and the alkyl chain oriented towards the amino-terminus of H3 and H11. The steroid skeleton makes numerous hydrophobic contacts with amino acid residues lining the inside of the pocket. The interaction between the 20-OH group of PonA and the OH group of the tyrosine amino acid residue are observed in three EcRs (Tyr408 of HvEcR, Tyr296 of BtEcR and Tyr427 of TcEcR), illustrating conservation of binding characteristics of the molting hormone across a wide range of ecdysone receptors. Among insect orders, the structure of the USP varies considerably between Mecopterida and non-Mecopterida species [163].

An artificial receptor in which four amino acids located on the hydrophobic surface are mutated (W303Y, A361S, L456S, C483S) was cocrystallized with a nonsteroidal ecdysone agonist, BYI06830 [167]. The structure of this ligand molecule is similar to the commercial insecticide chromafenozide [170]. As stated

above, EcR-LBD in complex with PonA possesses a long and thin L-shaped cavity extending towards H5 and the β-sheet, and is completely buried inside the receptor. By contrast, that of the BYI06830-bound EcR-LBD consists of a bulky V-shaped cavity located close to H7, H11 and H12, with an open cleft between H7 and H10. This opening extends towards the H8– H9 loop of the USP. The amino-terminal part of H7 of the EcR bound to BYI06830 is significantly shifted compared to the PonA complex along with H6. The β-sheet seen in the EcR – PonA complex is drastically affected, resulting in a three-stranded β-sheet in the PonA-bound EcR-LBD, which is replaced by the two stranded β-sheets and a loop with EcR-BYI06830. The dimerization interfaces and the AF2 helix remain identical between PonA and BYI06830-bound EcRs. The tert-butyl group, together with the benzoyl ring (A-ring), corresponds to the hydroxylated side chain of PonA. Along with the differences observed in the receptor scaffold, the EcR-LBD, in complex with the steroidal and nonsteroidal agonists, exhibits different and only partially overlapping ligand-binding pockets. The difference of the binding pocket is easy to understand by looking at the superimposition between PonA and BYI06830 shown in Fig. 4.

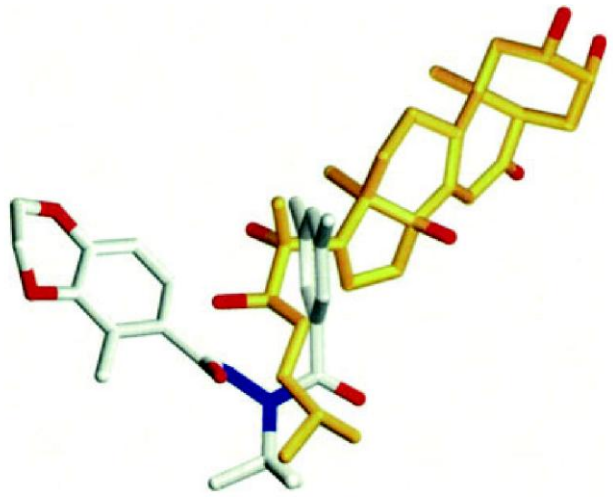

Fig. 4. Superposition between PonA and BYI06830. (Reproduced from [167] with the permission of Nature Publishing Group.)

Ligand-binding affinity

Typically, the specific binding of PonA to EcR is drastically enhanced by the addition of USP [171, 172]. The ligand-binding affinity of EcR has been quantitatively measured against both natural and *in vitro*-translated proteins using radiolabeled PonA [172,173,174]. As shown in Table 2, the binding affinity of PonA to EcR was remarkably enhanced in the presence of USP in the rice stem borer *Chilo suppressalis* [175], the Colorado potato beetle *Leptinotarsa decemlineata* [172] and *D. melanogaster* [176]. The binding affinity of PonA is the same among the EcR isoforms, EcR-A and EcR-B [172,175]. The binding affinity of PonA to scorpion EcR is, however, unaffected by USP [14]. The dissociation constants (K_d) of PonA to the EcR/USP heterodimer have also been determined in *D. melanogaster* [13], *B. mori* [177], *C. fumiferna* [178] and the migratory locust *Locusta migratoria* [179], as shown in Table 2.

Before cDNA clones of the *EcR* and *usp* (*rxr*) genes were obtained, receptor–ligand binding had been performed employing crude receptor extracts prepared by high-speed centrifugation of homogenates of whole tissues, or cytosolic or nuclear fractions [180]. The various physicochemical properties of the ecdysteroid receptor from *D. melanogaster* imaginal discs and Kc cells have been summarized previously [181]. The calculated K_d values were found to fall in the range of 20 – 200 nM for 20E and 0.3–2.0 nM for PonA. Although the binding affinity of ecdysone is generally low compared with the binding affinities of 20E and PonA, ecdysone also showed fairly high binding affinity (K_d = 4–7 nM) and was similar to that of 20E when tested with cell extracts of the crayfish *Orconectes limosus* [182]. Rauch et al. [183] detected three ecdysteroid isotypes (66, 68 and 70 kDa) and several USP bands (55–77 kDa) by western blotting of the homogenate of the epithelial cell line from *C. tentans*. They obtained two classes of high binding affinity (K_{d1} = 0.47 nM and K_{d2} = 7.2 nM) that were competitive either with 20E or muristerone A using a binding assay with [^{3}H]-labelled PonA.

Table 2. Dissociation constants of ponasterone A to the *in vitro*-translated ecdysone receptor proteins.

	Dissociation constants (K_d [nM])		
Insect species	EcR-A/USP (RXR)	EcR-B1/USP (RXR)	EcR
C. suppressalis[a]	1.0	1.2	55
L. decemlineata[b]	2.8	3.7	73
D. melanogaster[c]	0.93	0.85	nd[d]
D. melanogaster[e]	0.9		nd
B. mori[f]	1.1		nd
C. fumiferna[g]	1.87		nd
L. migratoria[h]	1.18		nd
L. australasiae[i]	4.2		3.2

[a] Ref [175]. [b] Ref [172]. [c] Ref [173]. [d] Not determined. [e] Ref [13]. [f] Ref [177]. [g] Ref [178]. [h] Ref [179]. [i] Ref [14].

Full-length EcR and USP clones of C. tentans were prepared as glutathione S-transferase (GST) fusion proteins in Escherichia coli and were then purified by affinity chromatography [184]. According to Grebe and co-workers, the absence of detergents during the purification procedure is essential for retaining the ligand-binding activity. They found two high-affinity binding sites (Kd1 = 0.24 nM and Kd2 = 3.9 nM). The removal of GST had no effect on PonA binding, but altered DNA binding. The presence of USP was necessary for strong ligand–EcR binding, and the presence of cofactors and post-translational modifications also seemed to be important for binding. EcR and USP of L. migratoria were also produced in E. coli as a GST– fusion construct, and bacterial cells were harvested by centrifugation and suspension in the buffer. The binding assay was performed using dextran-coated charcoal [185]. Against this partially purified EcR and USP, the binding affinity of PonA, in terms of Kd, was determined to be 1.2 nM, which is similar to that determined for other insect receptors [179] and in vitro- translated EcR/USP heterodimers, as shown in Table. 2.

The binding assay was once carried out using [^{125}I]- labelled26-iodoponasterone A (I-PonA) instead of [^{3}H]PonA, because the specific radioactivity of the iodo compound is very high [186]. However, the chemical structures are different between PonA and I-PonA, and the K_d value of I-PonA (3.8×10^{-10}M) was 2.6 times higher than that of PonA [186]. This is probably caused by the increased hydrophobicity on the terminal part of the ecdysteroid side chain. This terminal part substituted with the iodine atom corresponds to the *t*-butyl group of dibenzoylhydrazines, as shown in Fig. 4.

Vertebrate RXRs have several ligand molecules, including methoprene acid [187], phytanic acid [188], docosahexaenoic acid [189] and several fatty acids, but no hormone ligand has been conclusively established for USP. The idea that USP could be the receptor of juvenile hormone (JH) or any of its derivatives is attractive because JH might directly modulate the activity of the EcR/USP complex. The docking model of JH to USP-LBD, proposed by Billas and Moras [190], suggested the plausibility of JH binding within the LBD of USP, although there has been no direct evidence that the binding sites derived from the model are functional. Even though JH can bind to the USP and stimulate oligomerization of the USP *in vitro* [161], further experimentation will be required to establish the function of JH as a ligand for USP *in vivo*. In particular, the K_d for the binding of JH to the Drosophila USP is rather high (approximately 500 nM) [161], whereas the typical K_d values for the binding between hormones and NRs are often very low (in the nanomolar range). The ligand affinity of methyl farnesoate, the precursor of JH in *Drosophila* and other insects, is considerably higher (low K_d) [164]. The insect growth regulator and JH mimic, fenoxycarb, is an activator of the USP ligand-binding domain *in vivo*, although this was interpreted as a distinct response from one involving JH, because JH itself was not active inon the same assay [191]. Another interpretation of the role of the USP is based on the finding that many vertebrate NRs possess the low ligand-affinity interactions that serve as ‘sensors’ for cellular titers of ligands. In fact, whereas 9-cis retinoic acid is a high-affinity ligand for RXR, there is evidence that retinoids are not the natural ligand for RXR in cells and that RXR normally plays repressive, as well as inductive, roles [192]. In cell cultures, both JH-HI and several of its precursors, including methyl farnesoate, farnesyl diphosphate and

farnesoic acid, can potentiate the ecdysteroid-induced transcription mediated by EcR⁄USP, although these compounds alone exert no effect on transcriptional activity [145,193,194]. By contrast, Maki *et al*. [195] demonstrated that JH-III and methoprenic acid markedly repressed ecdysone-dependent EcR transactivation through shifting of H12 of the USP without affecting EcR⁄USP heterodimerization or DNA binding.

Ecdysone response elements

As described above, EcR is able to heterodimerize with the USP on the EcRE. Mammalian steroid hormone receptors, however, bind to their response elements as a homodimer, while other nuclear receptors, such as TRs and RARs, make heterodimers with their partner, RXR. The EcRE was first identified in *Drosophila* to be a 23-bp hyphenated dyad lying in the promoter of the heat shock protein gene (*hsp*27), which includes the consensus binding sites for other steroid hormone receptors such as the glucocorticoid response element (GRE), the estrogen response element (ERE) and the progesterone response element (PRE) [196]. Later, it was shown that the USP-DBD acts as a specific anchor that preferentially binds the 5' half site of the pseudo-PAL response element from the *hsp*27 gene promoter and thus locates the heterodimer complex in a defined orientation [197]. USP-DBD is able to bind as a monomer to the inverted repeats of 5-'AGGTCA3' separated by 1 bp βnverted repeat 1 (IR1)] in the absence of EcR-DBD. Moreover, EcR-DBD can bind to IR1 primarily as a homodimer in the absence of USP-DBD [197]. IR1 shows the highest affinity for the DmEcR⁄DmUSP [198,199]. The role of individual amino acids in the putative DNA recognition alpha- helix of DBD and the roles of the base pairs of the response element have been demonstrated [200]. Other EcREs that reside in the promoters of hsp23 (a heat shock protein) [201], *Drosophila Eip*28/29 (an ecdysone-induced polypeptide) [202], *Lsp*-2 (a larval serum protein) [203] and *Drosophila Sgs*-4 (a salivary gland secretion protein) gene [204] are palindromic. It has also been demonstrated that the EcR⁄USP complex is able to recognize the DR element in the promoter of nested gene (*ng*) [205]. The structure of the EcR/USP– DNA complex has been solved by X-ray diffraction, showing that the receptor complex recognizes elements by two 'zinc fingers' that are commonly present in NRs [124].

The order of the relative binding affinity of the EcR⁄USP heterodimer to the various DNA elements was determined to be PAL1 > DR4 > DR5 > PAL0 > DR2 > DR1 > hsp27 > DR3 > DR0 [123]. Interestingly, the mosquito EcR⁄USP complex binds to DR elements separated by 11–13 nucleotide spacers [199], indicating that the spacer length is less important for DRs. Of course, the AaEcR⁄AaUSP heterodimer bound an IR with higher affinity than a DR. Recently, a genomic approach has been utilized to localize regions that harbor binding sites for EcR and/or USP in *D. melanogaster*. In most cases, the two receptors colocalize within over 500 regions, although there are also some sites that are recognized by only one of the two receptors. In turn, many of these regions are proximal to genes that have been shown to be ecdysteroid-inducible [206].

Cofactors (coregulators) for ecdysteroid receptor

As shown above, hormone binding leads receptors to dissociate corepressors and bind coactivators, which in turn mediate gene activation. Many corepressors and coactivators have been identified in vertebrates [26], but their functions are relatively unknown in insects. Based on the experiments with vertebrate NRs, transcriptional cofactors and their roles have begun to be recognized and explored in insect species. Homologs of some vertebrate coactivators, such as p300/CBP [207] and p300/CBP-associated factor (P⁄CAF) [208], have been identified in *Drosophila* [209] and *C. elegans* [210], even though only a few cofactors related to molting are reported. Here only a few cofactors that are able to interact with insect nuclear receptors are discussed.

Taiman (TAI), a homolog of a steroid receptor coactivator of p160 family histone acetyltransferase, was identified in *Drosophila* [211]. TAI colocalizes with EcR and USP in vivo, evokes an elevated ecdysteroid-inducible transcriptional response in cell culture and coprecipitates with EcR [211]. The methyl transferase TRR, the product of the *trithorax-related* (*trr*) gene, has also been reported to be an ecdysone-dependent coactivator in *Drosophila* [212]. Another EcR interacting protein containing the LXXLL motif, Rig (*rigor mortis*), is also required for ecdysteroid signaling during larval development [213]. Rig is required as a coactivator for induction of the E74A isoform, which normally appears as ecdysteroid titers increase, but is not required for E75A, EcR, or USP transcription. Rig is also required for ecdysone responses during larval

development because rig mutants display defects in molting, delayed larval development, larval lethality, duplicated mouth parts and puparium formation, indicative of a failed ecdysteroid response [213].

As stated above, NCoR and SMRT are involved in repression by unliganded THs and RARs, as well as several unrelated transcription factors in mammals [214]. Ebi was first identified as a cofactor that regulates epidermal growth factor receptor signaling pathways during eye development in *Drosophila* [215]. Another corepressor in *Drosophila* is SMRTER; it is structurally divergent from, but functionally similar to, vertebrate SMRT and NCoR [216]. SMRT EcR-cofactor (SMRTER) carries LXXLL amino acid motifs associated with NR interactions, and physical interaction sites with EcR have been mapped [212,216]. Later it was shown that the Ebi–SMRTER complex directly regulates expression of the gene in *Drosophila* eye development [217]. Alien was also reported as a corepressor for selected members of the nuclear hormone receptors [218], which was originally given to a gene in the *Drosophila* genome with unknown function. Alien binds to EcR and SVP, but not to the RAR, RXR/USP, DHR3, DHR38, DHR78, or DHR96 [219]. Another potential cofactor carrying the LXXLL motif is methoprene-tolerant (MET), a member of the basic helix-loop-helix family (bHLH)-period-aryl hydrocarbon receptor/aryl hydrocarbon nuclear translator-single-minded (PAS) of transcriptional regulators that has been shown to interact physically with EcR and USP [220], and has also been shown to be essential for mediating developmental responses to JH in *Tribolium* [221]. The gene was originally identified in *D. melanogaster* as a mutation that confers resistance to the toxic effects of the commercial insecticide and JH analogue, methoprene [222]. Whether a functional interaction can be established between MET and the ecdysteroid receptor *in vivo* remains an important question.

The responsiveness of EcR and USP to a variety of ligands has been tested using a system in which yeast transcription activator protein, GAL4 fusion proteins for both the EcR and the USP LBDs were tested using a transgenic GAL4-responsive upstream activation sequence promoter [191,223]. When fly tissues expressing the GAL4-USP were challenged with JHs and other synthetic analogues such as pyriproxyfen and methoprene, no response was registered by the GAL4-USP, although it was noted that the JH mimic, fenoxycarb, evoked a response. These experiments, however, have not resolved whether USP is a receptor for JH. For instance, the GAL4-USP LBD could be responding indirectly to the effects of a cofactor, such as MET, or a JH response could be inhibited by binding with a corepressor in the test system. Moreover, other studies have shown that the responsive characteristics of USP depend on the promoter element, an issue that cannot be addressed when using the upstream activation sequence promoter [224]. Finally, because the effects of JH on transcription have sometimes been seen only in the presence of an ecdysteroid, there is the possibility that a detectable JH response *in vivo* depends on the presence of both JH and ecdysteroids [145].

Ligand molecules for ecdysone receptors

Ecdysteroids

20E (Fig. 1) is produced at all stages of the insect life cycle, not only in the larval and pupal stages, but also in the egg and adult stages, and it is responsible for regulating processes associated with development, metamorphosis, reproduction and diapause. Ecdysteroids, including 20E, are also found in other animal and plant kingdoms, and ecdysteroids of animals and plants are categorized as phytoecdysteroids (PEs) and zooecdysteroids, respectively. In all naturally occurring PEs, the methyl groups at C-10 (C19) and C-13 (C18) have a β-configuration. The B/C- and C/D-ring junctions are always *trans*, and the A/B ring junction is normally *cis* (5β-H). Most ecdysteroids possess hydroxyl groups at the 2β-, 3β-, 14α-, 20*R*- and 22*R*- positions, which together give rise to the most biologically active ecdysteroid, PonA (25-deoxy-20E). Other modifications are also found in plant steroid hormones known as plant triterpenoids (brassinosteroids, cucurbitacins, withanolides, etc.) [225].

The first isolation of ecdysteroids from insects was coincident with the isolation of ponasterones, PonA from the leaves of *Podocarpus nakaii*, as well as PonB and PonC. At almost the same time, 20E, podecdysone A and inokosterone were isolated from the roots of *Achyranthes fauriei*, the wood of *Podocarpus elatus*, the rhizomes of *Polypodium vulgare* and in the dry pinnae of the bracken fern *Pteridinium aquilinum*. These reports

stimulated natural product chemists to isolate PEs, whose structures are available on a website (http://www.ecdybase.org). In the survey of plant species, it was found that 5–6% of the tested species were positive for ecdysteroids [226]. A number of ecdysteroids with unusual structural features (16-hydroxy, 24-hydroxy or 22,23-epoxide) have been isolated from the mushrooms *Paxillus atrotomentosus* and *Tapinella panuoides* [227], and are designated as mycoecdysteroids. Several unusual ecdysteroids have also been isolated from fungi such as *Polyporus umbellatus* (polyporusterones A–G) [228], *Polyporus versicolor* (polyoxygenated derivatives of ergosterol) [229] and *Lasiosphaera nipponica* (30,14α,17α,20,24,25-hexahydroxy-5α-ergosta-7,22-dien-6-one) [230]. However, it is currently unclear if the ecdysteroids are biosynthesized by the fungi themselves or are taken up from the host plant.

It has long been recognized that PEs possess insect molting hormone activity and could participate in the defense of plants against nonadapted phytophagous invertebrates. This is supported by the fact that the major PE in most ecdysteroid-containing plants is 20E. Monophagous or oligophagous species feeding on PE-negative host plants were either deterred from feeding or showed marked abnormalities in growth and development after incorporation of 20E in their diets. Oligophagous or polyphagous species that feed on host plants from families which are known to contain PE-positive species were found to be able to tolerate low levels of 20E in their diets, but exhibited developmental defects when exposed to high concentrations of 20E [231]. The activity of a PE depends on its affinity for the receptor and the effective concentration at the target site (normally assumed to be the ecdysteroid receptor complex). If it is ingested, its potency also depends on the amount of ingested ecdysteroids, its ability to pass through the gut wall and its rate of inactivation. The binding affinities of representative ecdysteroids to the molting hormone receptors (which are measured using either proteins or whole cells) are listed in Table 3.

As shown in Table 3, structure–activity relationships for ecdysteroids are very similar among insects, whereas those for the binding activity of DAHs are remarkably different among insects, particularly insect orders [17]. As PonA is the most potent ecdysteroid regardless of insect species, the compounds that mimic the binding of PonA to the binding niche of the receptor are presumed to be effective on all insects. To design new compounds, quantitative structure–activity relationships (QSARs) are useful. Dinan and co-workers quantitatively analyzed the activity of ecdysteroids (including phytosteroids) using multidimensional QSARs to predict the pharmacophore [232]. Arai et al. [233] synthesized PonA analogs containing various steroid skeleton moieties and discussed the structure– activity relationship of ecdysteroids. Recently, Harada et al. [234] demonstrated, using models of ligand– receptor complexes, that the binding affinity of ecdysteroids to the receptor proteins is enhanced with an increased number of hydrogen bonds. If a nonsteroidal structure can be applied to the structure of PonA, the newly designed compounds are predicted to be nonselective among insects.

Nonsteroidal compounds

A large number of ecdysteroids have been identified in plants and microorganisms, but none has been marketed for the purpose of insect control. This is because ecdysteroids have a complicated core structure and the poor hydrophobicity is usually unfavorable for use as insecticides. To overcome the problems associated with ecdysteroids, attempts were made to identify nonsteroidal compounds with ecdysone-like activity. In the late 1980s, it was reported that DAHs induce insect molting as well as insecticidal toxicity [235]. After performing intensive structure–activity relationship studies, tebufenozide, methoxyfenozide, halofenozide [236] and chromafenozide were developed for commercial use as insecticides. Other nonsteroidal compounds, such as 3,5-di-*tert*-butyl-4-hydroxy-*N*-isobutylbenzamide [237], tetrahydroquinoline [238], α-acylaminoketone [239], oxazoline [240] and γ-methylene-γ-lactam [241], were identified as nonsteroidal agonists, but none of these have been developed for commercial use. Some of them are structurally similar to DAHs.

Table 3. Binding activity of various ecdysone agonists to *in vitro*-translated various EcR/USP heterodimers and intact cell and tissue, and *in vitro* molting hormone activity[a]. ND, not determined.

	Binding activity [pIC_{50} (M)]									Molting hormone activity [pEC_{50} (M)]			
	In vitro translated			Cell free			Tissue or cells						
Compounds	Cs[b]	Dm[b]	Ld[b]	Ct[c]	Gm[d]	Se[e]	Dm[f]	Sf[g]	Sl[h]	Dm[i]	Dm[j]	Sl[h]	Cs[k]
Ponasterone A	8.08	8.27	8.13	ND	8.40	8.15	8.89	8.05	ND	9.51	7.15	ND	7.53
20-Hydroxyecdysone	6.66	7.03	6.36	6.64	6.92	6.51	7.34	6.78	6.80	8.12	6.74	6.54	6.75
Cyasterone	6.65	6.39	6.29	ND	ND	ND	7.21	6.57	ND	7.49	6.48	ND	6.37
Makisterone A	6.33	5.97	5.74	ND	ND	ND	6.95	6.41	ND	7.89	6.54	ND	5.73
Ecdysone	4.70	4.60	4.98	ND	4.97	ND	5.59	5.63	ND	5.96	< 5.00	ND	5.05
RH-5849	6.50	5.16	4.97	6.56	6.39	5.96	5.24	6.44	ND	5.74	ND	4.35	6.40
Halofenozide	6.92	5.95	5.23	ND	ND	ND	6.17	6.48	ND	6.05	ND	6.33	7.10
Tebufenozide	8.85	6.01	5.18	7.95	ND	7.48	6.39	8.81	7.06	6.28	< 5.00	6.39	8.94
Methoxyfenozide	8.87	6.49	5.94	8.14	ND	ND	6.55	8.46	7.61	ND	ND	7.96	8.95
Chromafenozide	9.13	6.54	5.77	ND	ND	ND	6.83	8.78	ND	ND	ND	ND	8.83

[a] Values means the reciprocal logarithmic value of IC_{50} (M; concentration required to inhibit the binding of [^{3}H]PonA to receptors to 50%), and 50% effective concentration (M, EC_{50}). [b] *In vitro* translated proteins (EcR+USP) of *C. suppressalis* (Cs), *D. melanogaster* (Dm) and *L. decemlineata* (Ld) [174]. [c] GST-bacterial fusion proteins (EcR+USP) of *C. tentans* [248]. [d] Cell-free preparations of *G. mellonella* (Gm) [246]. [e] Cell-free preparations of *S. exigua* (Se) [247]. [f] Intact Kc cells [243]. [g] Intact Sf-9 cells [244]. [h] Imaginal wing disc of *S. littoralis* (Sl) [249]. [i] BII cells from *D. melanogaster* (Dm) [252]. [j] Luciferase reporter assay using SL2 cells [250]. [k] Cultured integument of *C. suppressalis* (Cs) [17,300].

Although the structure–activity relationships for the binding of ecdysteroids to the EcR/USP complex are very similar among insect species, those for nonsteroidal compounds vary [17, 242]. This has revealed the opportunity to develop ecdysone agonists that can be targeted at pest insect species. For instance, DAHs such as tebufenozide, methoxyfenozide and chromafenozide are very potent against *C. suppressalis* (Lepidoptera), being 6–10 times more potent than PonA, as shown in Table 3 (the values are given on a logarithmic scale). However, these compounds are less potent than PonA and 20E against either *D. melanogaster* (Diptera) or *L. decemlineata* (Coleoptera). Even though halofe nozide is registered as an insecticide for the control of both Coleoptera and Lepidoptera, it shows a slightly weaker affinity than methoxyfenozide and chromafenozide against the *in vitro*-translated EcR/USP complex of *L. decemlineata* (Table 3). In cell-based assays, species-specific differences in toxicity are generally confirmed by measurements of transcriptional activity, although the potency of agonists seems to be more predictive of toxicity than maximal fold-induction [193].

As shown in Table 2, the binding affinity of PonA to the EcR/USP complex is enhanced in the presence of USP (or RXR). As we know, EcRs are able to heterodimerize not only with native USP, but also with other insect USPs. The binding activity of various ecdysone agonists against hybrid EcR/USP heterodimers has been quantitatively measured [174]. The activity of all ecdysteroids and nonsteroidal compounds towards DmEcR/DmUSP is slightly decreased by swapping the DmUSP with CsUSP or LdUSP, but the binding activity to LdEcR/LdUSP was slightly enhanced by replacing LdUSP with DmUSP. These observations suggest that the ligand-binding affinity might be enhanced by using different heterodimeric partners, which are supported by a cell-based assay [193]. The transcriptional inducibility mediated by a given species' EcR/USP heterodimer is primarily a function of the EcR. The USP plays a modulatory role that affects the inducibility of a given agonist, as seen with cross-species EcR/USP pairings in cell culture transcriptional assays [193].

Before the establishment of the *in vitro* binding assay using receptor proteins, the ligand-binding affinity of ecdysteroids had been measured in tissues and cells [243,244]. The *in vitro* binding activity of representative ecdysteroids and nonsteroidal compounds evaluated in intact cells are listed in Table 3. Even though concentration required for 50% inhibition (IC_{50}) values are higher in intact cells (Kc cells) than for *in vitro* translated proteins (DmEcR/DmUSP), the structure– activity relationships are similar between them. Minakuchi et al. [245] demonstrated that the structure–activity relationships for the ligand–receptor binding are equivalent between intact cells and the cell-free homogenates. Other groups also measured the binding affinity (K_d) or the

related activity (IC_{50}) of representative compounds against crude receptor preparations of *G. mellonella* [246], *C. suppressalis* [175], *S. exigua* [247], *C. tentans* [248], the cotton leafworm *Spodoptera littoralis* [249], *Drosophila* [250], Kc cells [251], Sf-9 cells and BII cells [252].

QSARs (including classical and 3D QSARs) have been used extensively to investigate various *in vivo* and *in vitro* activities [17]. QSARs of steroidal compounds have also been carried out by Hormann and Dinan, and ligand–receptor docking models have been proposed [252,253]. A large number of nonsteroidal ecdysone agonists (158 active compounds) were analyzed using one of the 3D QSAR methods, comparative molecular field analysis [254]. The comparative molecular field analysis steric and electrostatic views are overlapped on the ligand-binding pocket of the receptor [254], which is modeled from the crystal structure of HvEcR-LBD [167], and confirm that the steric and electrostatic effects are consistent with the milieu of the receptor pocket [255]. The hydrophobic amino acid residues surround the *t*-butyl group of the DAH that corresponds to the terminal moiety of the ecdysteroid side chain. Based on the receptor–ligand docking model, some completely different chemical classes were designed from a knowledge of the shape of the EcR-LBD niche, in which a DAH, BYI06830, is accommodated [256].

Application of the ligand–EcR/USP complex as a gene switch

The appearance of various phenotypes is determined, in part, by genetic switches that do not encode any proteins but regulate when, where and how much a gene is expressed. Noncoding DNA may have no specific function, but many of these regions participate in the very important task of regulating gene expression. Gene expression entails the transcription of the DNA sequence into a mRNA, followed by the translation of that mRNA into a protein sequence. Many genes are expressed only in an organ-, tissue- or cell type-specific manner. Sequence-specific DNA-binding proteins (transcription factors) are components of genetic switches that turn genes 'on' or 'off' at the right time and place in the body. The binding of transcription factors to the enhancers in the nucleus determines whether the gene switches are on or off in that cell. Enhancers usually have hundreds of base pairs and may be located on either side of a gene, or even within a noncoding stretch inside a gene. Small molecules, such as hormones, are also essential to regulate the gene expression or transcription specifically. Thus, gene-regulation systems are potentially applicable in medicine to regulate the expression of therapeutic proteins, and as a tool for functional genomics and drug discovery. These systems are also useful for agriculture [257].

As stated above, EcRs can heterodimerize with USPs (or RXRs) to acquire transcriptional activity in the presence of some ecdysteroids and are sensitive to endogenous RXR when introduced into vertebrate cells. Esengil and co-workers transfected human embryonic kidney (HEK) 293 cells with a cytomegalovirus promoter-driven expression construct. This construct encodes a chimeric transactivator composed of the DBD and the homodimerization domain of GAL4, VP16 (the herpes simplex virus regulatory domain) and the BmEcR-LBD. This construct also carries a GAL4-dependent firefly luciferase reporter and a constitutive *Renilla* luciferase reporter [32]. The zebrafish *Danio rerio* was used to test the expression system, because zebrafish is an excellent animal model for the study of developmental function [258]. The ecdysteroid-inducible gene-expression system derived from the insect-specific EcR using suitable ecdysone agonists may overcome the limitations of gene regulation such as imprecise dosage control, cross-talk with endogeneous signaling pathways, poor inducibility and slow kinetics in the zebrafish.

Chemical-inducible gene-expression systems using EcR-dependent gene switches have also been developed for applications in plants [259]. Transgenes in plants are often controlled by constitutive promoters such as the cauliflower mosaic virus (CaMV) 35S [260], but such promoters are 'always-on'. The disadvantages of the genetic systems using constitutive promoters include (a) the generation of metabolic waste and risk of pleiotropic effects caused by the expression of transgenes at all stages in all tissues, (b) the escape of genes into the environment and (c) the inability to regulate genes whose overexpression is toxic or may block normal plant regulatory processes. To solve these problems, other gene-regulation systems based on plant promoters that increase transgene transcription upon the application of herbicide safeners [261], plant hormones and heat shock treatment have also been developed.

Future studies

EcR and USP (RXR) belong to the NR superfamily and have been identified in various insects and other arthropod species. Even though the ligand molecule of EcR is 20E in most cases, the LBDs vary among insect species. The occurrence of such differences in LBDs may be useful in the design of specific ligands that can be employed as species-specific insecticides. Because EcR and USP (RXR) are ligand-dependent, these receptors can be harnessed as regulators of gene expression in other biological systems using ecdysone agonists as activating agents. Ecdysone agonists are potentially useful for gene therapy in animals and the production of beneficial proteins in plants, because ecdysteroid activity is specific to insects and the agonists are not toxic to mammals. In fact, some ecdysone agonists are used as insecticides precisely because of their low toxicity in mammals and environmental safety. The discovery of new chemicals with binding affinity for NRs such as EcR and USP (RXR) offer potential value in medical and industrial applications as well as in agriculture. NRs, including EcR and USP, themselves may also play roles of biochemical and medicinal mediation. Future studies will focus on the structural basis for receptor differences among insect species and the consequences of these differences on developmental and physiological processes in insects. Finally, the possible interplay of ecdysteroids and insect JH upon the activity of the ecdysteroid receptor remains an important and unresolved question in insect endocrinology.

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