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The *Drosophila* Orphan Nuclear Receptor DHR38 Mediates an Atypical Ecdysteroid Signaling Pathway

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Summary

Ecdysteroid pulses trigger the major developmental transitions during the *Drosophila* life cycle. These hormonal responses are thought to be mediated by the ecdysteroid receptor (EcR) and its heterodimeric partner Ultraspiracle (USP). We provide evidence for a second ecdysteroid signaling pathway mediated by DHR38, the *Drosophila* ortholog of the mammalian NGFI-B subfamily of orphan nuclear receptors. DHR38 also heterodimerizes with USP, and this complex responds to a distinct class of ecdysteroids in a manner that is independent of EcR. This response is unusual in that it does not involve direct binding of ecdysteroids to either DHR38 or USP. X-ray crystallographic analysis of DHR38 reveals the absence of both a classic ligand binding pocket and coactivator binding site, features that seem to be common to all NGFI-B subfamily members. Taken together, these data reveal the existence of a separate structural class of nuclear receptors that is conserved from fly to humans.

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Introduction

Ecdysteroids are arthropod-specific hormones that function as the major inducing signals responsible for postembryonic developmental progression in insects. In *Drosophila*, the ring gland releases two major ecdysteroids, α-ecdysone and 20-deoxymakisterone A, which are thought to be largely inactive (Gilbert et al., 1997; Riddiford, 1996). The conversion of α-ecdysone to 20-hydroxyecdysone (20E) in the peripheral tissues is thought to elicit most of the effects of ecdysteroid pulses, with the remaining metabolites having no known function (Gilbert et al., 2002). The receptor for 20E is a transcription factor comprised of a nuclear receptor heterodimer of the ecdysone receptor (EcR, NR1H1) and Ultraspiracle (USP, NR2B4) (Koelle, 1992; Thomas et al., 1993; Yao et al., 1993). The ecdysone receptor complex acts at the top of a signaling hierarchy that transitions the developing larva through staged molts at the end of the first and second instars, as well as directing its entrance into metamorphosis (Riddiford et al., 2000).

Of the 18 *Drosophila* genes that encode canonical nuclear receptors, only EcR, in conjunction with USP, has been shown to be ligand responsive (Koelle, 1992; Thomas et al., 1993; Yao et al., 1993). Similarly, although a wide spectrum of ecdysteroids with biological activity have been identified in the insect hemolymph (Sommé-Martin et al., 1990, 1988; Grau and Lafont, 1994; Cherbas and Cherbas, 1970), only a small subset of these can activate EcR/USP at physiologic concentrations (Baker et al., 2000; Cherbas et al., 1980). These results have suggested that other signaling pathways, perhaps mediated by one or more orphan nuclear receptors, may participate in ecdysteroid responses (Thummel and Chory, 2002). To that end, we investigated the possibility that DHR38 (NR4A4) may be an ecdysteroid-responsive factor based on the following observations. (1) DHR38, like the ecdysteroid-responsive EcR, is the only other *Drosophila* nuclear receptor known to heterodimerize with USP, the ortholog of the vertebrate retinoid X receptor (RXR) (Sutherland et al., 1995). (2) DHR38 is the *Drosophila* ortholog of the mammalian NGFI-B subfamily of orphan nuclear receptors, which includes NGFI-B (NR4A1), Nurr1 (NR4A2), and NOR1 (NR4A3) (Philips et al., 1997; Wilson et al., 1991; Cheng et al., 1997; Paulsen et al., 1995; Zetterstrom et al., 1996; Perlmann and Jansson, 1995; Forman et al., 1995). Like other orphan receptors that heterodimerize with RXR, the NGFI-B/RXR and Nurr1/RXR heterodimers are ligand responsive, suggesting that the DHR38/USP heterodimer may also be ligand activated (Perlmann and Jansson, 1995). (3) Like *EcR* and *usp*, *DHR38* is broadly expressed during the third instar, prepupal, and pupal stages, suggesting that its temporal specificity may also be conferred by a hor-

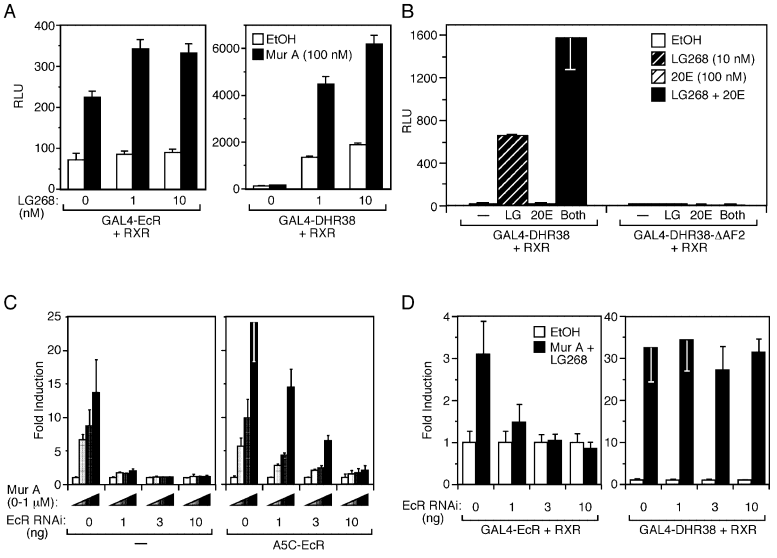


Figure 1. DHR38 and EcR Mediate Distinct Ecdysteroid-Signaling Pathways

(A–D) EcR- and DHR38-dependent transactivation was assayed in SL2 cells cotransfected with insect expression vectors for the various receptor combinations shown (RLU, relative light units). Reporter plasmids used were *adh*-UASx3-Luc (in [A], [B], and [D]) and adh-*hsp27*-EcRE-Luc in (C). (A) The GAL4-EcR/RXR heterodimer (left) and the GAL4-DHR38/RXR heterodimer (right) respond differently to the RXR agonist (LG268) and the ecdysteroid muristerone A (Mur A). (B) GAL4-DHR38/RXR-dependent transcriptional activity requires the DHR38 AF-2 domain. (C) RNAi against EcR abolishes ecdysteroid-dependent transcription of the *hsp27*-EcRE that is mediated by either endogenous (left) or transfected (right) EcR. Increasing amounts of muristerone A (0, 0.1, 0.3, and 1 μM) are indicated by shaded bars. (D) RNAi against EcR selectively eliminates transactivation of GAL4-EcR/RXR (right), but not GAL4-DHR38/RXR (left) induced by LG268 (10 nM) and muristerone A (100 nM).

mone (Fisk and Thummel 1995; Kozlova et al., 1998). (4) Both *DHR38* and *usp* mutant flies have abnormalities in cuticle formation that are not seen in EcR mutants, thereby uncoupling the action of the two receptor heterodimer complexes and suggesting they may govern distinct ecdysteroid signaling pathways (Kozlova et al., 1998; Hall and Thummel, 1998). Taken together, these findings raise the possibility that DHR38 participates in an ecdysteroid response pathway that is different from the one transduced by the EcR/USP heterodimer.

In this paper, we report the characterization of a second ecdysteroid signaling pathway in *Drosophila* mediated by DHR38. This signaling pathway requires heterodimerization with USP and utilizes a mechanism that does not involve direct binding of either ecdysteroids or canonical cofactors to DHR38. To further explore the basis for this atypical mechanism, we also show the X-ray crystal structure of DHR38, which differs from all previously characterized nuclear receptor structures by lacking both the signature ligand binding pocket and coactivator binding site. Extrapolation of the DHR38 structure to other members of the NGFI-B receptor subfamily suggests that this structural feature has been conserved throughout evolution.

Results

DHR38 Functions as an Ecdysteroid Sensor

To investigate the possibility that DHR38 may function in an ecdysteroid-mediated transcriptional pathway, we developed a screening assay in which DHR38 was heterodimerized with ligand-activated RXR. The feasibility of this approach was based on the finding that RXR can substitute for USP as a productive heterodimeric partner for EcR (Thomas et al., 1993; see also Figure 1A). A distinct advantage of substituting RXR for USP is that although ligands for USP are not known, several potent RXR ligands (i.e., rexinoids) have been characterized. We reasoned that assaying DHR38 activity in the presence of rexinoid-activated RXR may be important because previous work has shown that some RXR heterodimers require sensitization with ligand for one receptor before the partner receptor can become ligand responsive (Botling et al., 1997).

To screen for a DHR38 ecdysteroid response, transient cotransfections were performed in the *Drosophila* SL2 cell line using chimeric GAL4-receptor proteins and a GAL4-responsive luciferase reporter gene (Baker et al., 2000). In this assay, GAL4-EcR and GAL4-DHR38 were screened in the presence of RXR, the synthetic rexinoid LG268 (Boehm et al., 1995), and the potent plant ecdysteroid muristerone A (Figure 1A). Similar to previous work (Baker et al., 2000), the GAL4-EcR/RXR heterodimer responded to 100 nM muristerone A as expected but was not activated significantly by LG268 alone (Figure 1A, left). Addition of both ligands resulted in only a modest increase in EcR/RXR activity (Figure 1A, left). In contrast, GAL4-DHR38/RXR, which is known to have a potent basal activity (Baker et al., 2000; Sutherland et al., 1995), was not induced by the addition of ecdysteroid alone but, instead, exhibited a strong, dose-dependent response to LG268 (Figure 1A, right). Rexinoid activation of the DHR38/RXR heterodimer is consistent with the rexinoid response seen with other NGFI-B family members when paired with RXR (Perlmann and Jansson, 1995). Surprisingly, however, there was a significant, 3- to 4-fold response to muristerone A when it was added together with LG268 (Figure 1A, right). A similar response was obtained with the endogenous insect ecdysteroid 20E (Figure 1B). Both rexinoid and ecdysteroid responses were abolished when a GAL4-DHR38 construct was utilized that lacks the ligand-dependent activation function-2 (AF-2) domain. Identical results were obtained (i.e., loss of rexinoid and ecdysteroid response) when the AF-2 domain of RXR was also deleted (data not shown). These data suggest that the DHR38 heterodimeric complex is responsive to ecdysteroid but, like other RXR heterodimers, it requires transactivation of both receptor partners for full agonist activity.

The results above revealed the possible existence of

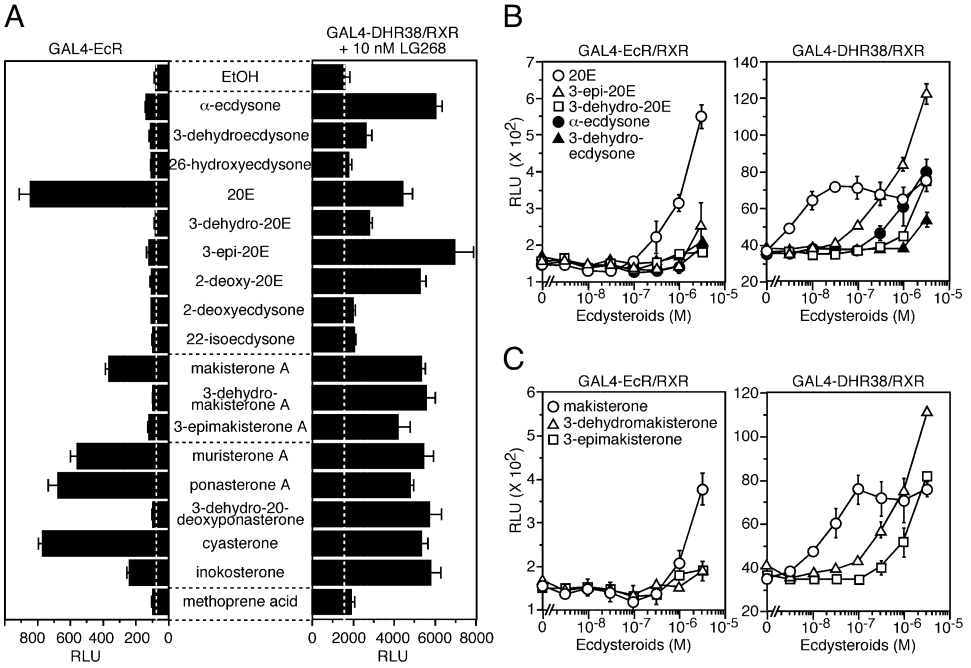


Figure 2. The DHR38/RXR Heterodimer Is a Promiscuous Sensor of Ecdysteroids

(A–C) Activity of GAL4-EcR versus GAL4-DHR38 in transfections performed in SL2 cells. (A) GAL4-EcR and GAL4-DHR38 respond to a distinct set of natural and synthetic ecdysteroids. Note that the ecdysteroid response of GAL4-DHR38 required heterodimerization with RXR and rexinoid (10 nM LG268). Dotted white line represents the basal response in the absence of any ecdysteroid. (B) Dose-response curves for natural ecdysone metabolites activating GAL4-EcR/RXR (left) and GAL4-DHR38/RXR (right) in the presence of 10 nM LG268. (C) Dose-response curves for natural makisterone metabolites activating GAL4-EcR/RXR (left) and GAL4-DHR38/RXR (right) in the presence 10 nM LG268.

two ecdysteroid signaling pathways, one mediated by EcR and the other by DHR38. To begin to delineate the specificity of the DHR38 pathway and show that it functions independently of the EcR pathway, we utilized double-stranded RNA (*ds*RNA) directed against the coding region of the EcR ligand binding domain to reduce the expression of endogenous EcR in the SL2 cell assay. As shown in Figure 1C, treatment of SL2 cells with increasing amounts of EcR *ds*RNA completely eliminated muristerone-A-dependent transcription when tested using endogenous EcR/USP heterodimers on an *hsp27*-EcRE reporter gene. This RNAi-mediated repression of the EcR-dependent response also completely blocked the activity of exogenously transfected EcR (Figure 1C, right) and GAL4-EcR (Figure 1D, left). In contrast, under the same experimental conditions where the EcR response was abolished, the GAL4-DHR38/RXR heterodimer was fully responsive to ecdysteroid and LG268. These results demonstrate that the DHR38 response to ecdysteroids is independent of EcR.

To define the spectrum of potential DHR38 agonists and further delineate the differential ecdysteroidal response of DHR38 and EcR, a panel of naturally occurring *Drosophila* ecdysteroids, phytoecdysteroids, synthetic ecdysteroids, and a synthetic juvenile hormone (methoprene acid) were tested for activity using the reporter gene assay described above. SL2 cells were transfected with either GAL4-EcR or GAL4-DHR38 plus RXR and tested for agonist activity in the presence of 10 nM LG268 (Figure 2A). Consistent with previous results (Baker et al., 2000), GAL4-EcR responded selectively to the endogenous ecdysteroids 20E and makisterone A and the plant ecdysteroids muristerone A, ponasterone A, and cyasterone. In marked contrast, the GAL4-DHR38/RXR response was promiscuous for several different ecdysteroids when LG268 was included as a coagonist (Figure 2A). In addition to the compounds that activated EcR, at least six other ecdysteroids (α-ecdysone, 3-epi-20E, 2-deoxy-20E, 3-dehydromakisterone A, 3-epimakisterone A, and 3-dehydro-20-deoxyponsterone) also exhibited significant DHR38-dependent activity. Dose-response profiles demonstrated that all of these compounds were more potent agonists for DHR38 than for EcR (Figures 2B and 2C). In fact, 20E, which is believed to be the endogenous hormone agonist for EcR, exhibited a 100-fold greater potency for DHR38-dependent transcription. These data suggest that the DHR38/RXR heterodimer is a potent sensor of a distinct class of physiologically relevant ecdysteroids.

Activation of the DHR38/USP Heterodimer by Ecdysteroids Requires Transactivated USP

An unusual characteristic of the DHR38/RXR heterodimer is that it required transactivation of both receptors to elicit an ecdysteroid response. In particular, the DHR38/RXR heterodimer failed to respond to ecdysteroid in the absence of ligand-activated RXR. Interestingly, DHR38 also failed to respond to ecdysteroid when USP, the physiologic partner of DHR38, was used instead of RXR (Figure 3A). These results raise the intriguing possibility that USP, like RXR, must also be transacti-

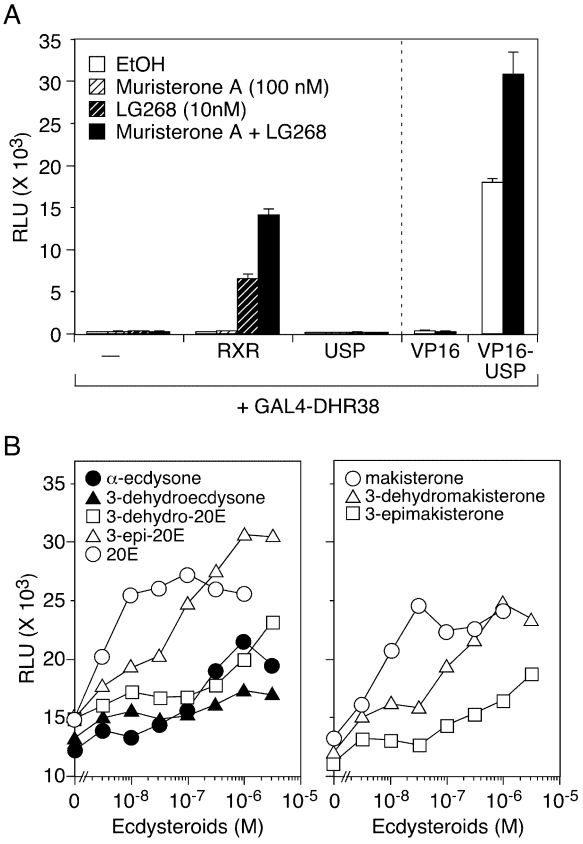


Figure 3. Heterodimerization with Transactivated USP Enables DHR38 Responsiveness to Ecdysteroids

(A) GAL4-DHR38 responds to ecdysteroids when dimerized with VP16-USP, a constitutively active form of USP. Assays were performed in SL2 cells expressing GAL4-DHR38 in combination with RXR, USP, VP16, or VP16-USP. VP16-USP alone showed no activity in this assay.

(B) Dose-response curves for ecdysone metabolites (left) and makisterone metabolites (right) activating the GAL4-DHR38/VP16-USP heterodimer in transfected SL2 cells.

vated (e.g., by ligand) in order to enable the ecdysteroid response. We attempted to address this question by using VP16-USP, a constitutively active form of USP that circumvents the requirement for USP ligand by fusing the strong transcriptional activation domain of the herpes simplex viral protein-16 (VP16) to USP. As expected, in the absence of agonist, the GAL4-DHR38/VP16-USP heterodimer showed a high constitutive level of basal activity that effectively mimicked ligand-activated USP (Figure 3A). Importantly, the addition of muristerone A to the GAL4-DHR38/VP16-USP heterodimer elicited a significant increase in reporter gene activity, analogous to the effect seen with LG268-activated GAL4-DHR38/RXR. Similar to the results obtained above with ligand-activated RXR (Figures 2C and 2D), the GAL4-DHR38/VP16-USP heterodimer responded to a wide variety of ecdysteroids at comparably low concentrations (Figures 3B and 3C). These results support the idea that DHR38 mediates a distinct heterodimer-dependent ecdysteroid signaling pathway.

DHR38 Is Activated by Ecdysteroids in Organ Culture

To test the prediction that DHR38 is activated by ecdysteroids in *Drosophila*, we created transgenic flies that carry a heat-inducible *hs-GAL4-DHR38* transgene in combination with a GAL4-dependent *UAS-nlacZ* reporter gene. Since target genes for DHR38 in the fly are unknown, this model permits us to assay DHR38 transactivation directly in fly tissues. This transgenic fly model has been used to follow the ecdysteroid-dependent activation patterns of the EcR and USP in *Drosophila* and has provided data consistent with the known biochemical and genetic activities of the full-length receptors in vivo (Kozlova and Thummel, 2002). This strategy has also been employed to track ligand-dependent activation of the RAR and RXR ligand binding domains in the mouse central nervous system (Solomin et al., 1998). To determine if GAL4-DHR38 is activated by ecdysteroids, third instar larval organs from this transgenic line were dissected at ~8 hr before puparium formation and cultured in the presence of either α-ecdysone or 3-epi-20E, two ecdysteroids that were shown to activate DHR38, but not EcR in SL2 cells (Figure 2). In the presence of 1 μM α-ecdysone, significant activation above background was seen for GAL4-DHR38 (Figures 4C and 4D). In contrast, these ecdysteroids had no effect on GAL4-EcR (Figures 4A and 4B), although this same transgenic line showed robust activation by 20E (Kozlova and Thummel, 2002). GAL4-DHR38 was also activated by 3-epi-20E in both the epidermis and fat body (Figures 4E–4H), consistent with the ability of this agonist to selectively activate DHR38 in SL2 cells (Figure 2). These organs contain significant amounts of endogenous USP (Henrich et al., 1994), consistent with the interpretation that GAL4-DHR38 ecdysteroid activation is dependent on heterodimerization with a USP partner. Although similar results were seen in several independent experiments, not all *hs-GAL4-DHR38; UAS-nlacZ* animals displayed robust activation, indicating that a specific stage might be competent to respond to the hormone. In agreement with this idea, a complex and dynamic pattern of GAL4-DHR38 activation can be seen in untreated animals (T.K., unpublished data). This observation is consistent with the notion that the endogenous DHR38 response may be spatially and temporally regulated by the presence of a number of factors, including ecdysteroids, DHR38/USP-specific coactivators, and potentially a USP ligand.

Structure of DHR38

The results above suggested that DHR38 may function as an ecdysteroid receptor and, like other nuclear receptors, recruit coactivators upon ligand binding. However, despite several attempts using a variety of techniques, including direct radioligand binding and cofactor recruitment assays (Makishima et al., 1999, 2002), we were unable to demonstrate that DHR38, RXR, or USP (alone or as a heterodimer) directly binds any of the potent ecdysteroid agonists or their metabolites (Baker et al., 2000; data not shown). In addition, we were unable to detect interactions between DHR38 and any of the known nuclear receptor cofactors (Rosenfeld and Glass,

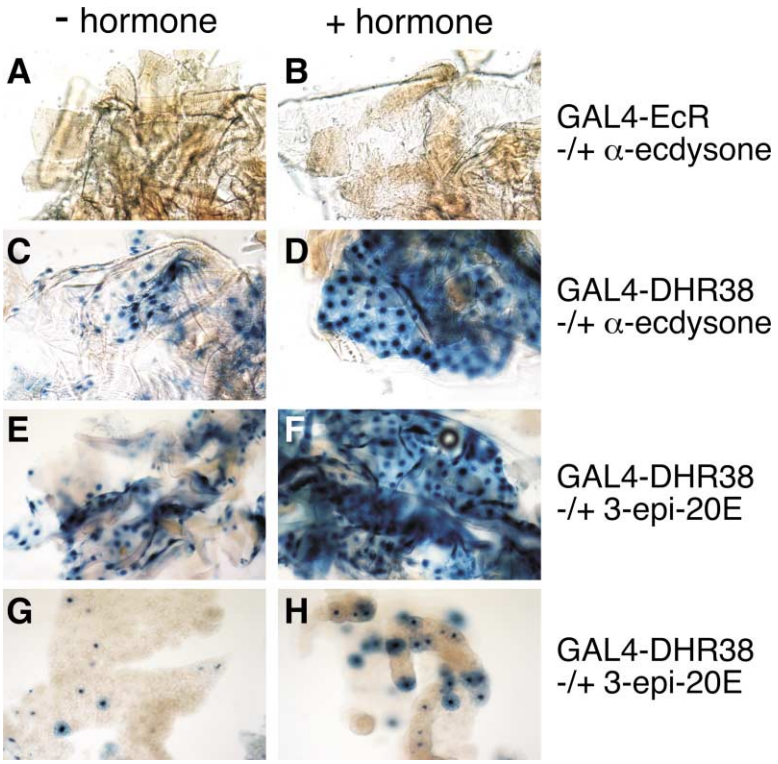


Figure 4. GAL4-DHR38 Is Activated by Ecdysteroids in *Drosophila* Larval Organ Culture

(A–H) Third instar larval organs dissected from either heat-treated *hs-GAL4-EcR*; *UAS-nlacZ* or *hs-GAL4-DHR38*; *UAS-nlacZ* animals were cultured in the absence or presence of ecdysteroids and processed for histochemical staining. GAL4-EcR is not activated in either the absence (A) or presence (B) of 1 μM α-ecdysone. In contrast, GAL4-DHR38 is significantly activated by 1 μM α-ecdysone in epidermis (D) above its basal level (C). GAL4-DHR38 is also activated by 1 μM 3-epi-20-hydroxyecdysone (F and H) above its basal level (E and G) in epidermis (E and F) and fat body (G and H). The *UAS-nlacZ* reporter alone is not ecdysteroid responsive either in vivo or in organ culture (data not shown).

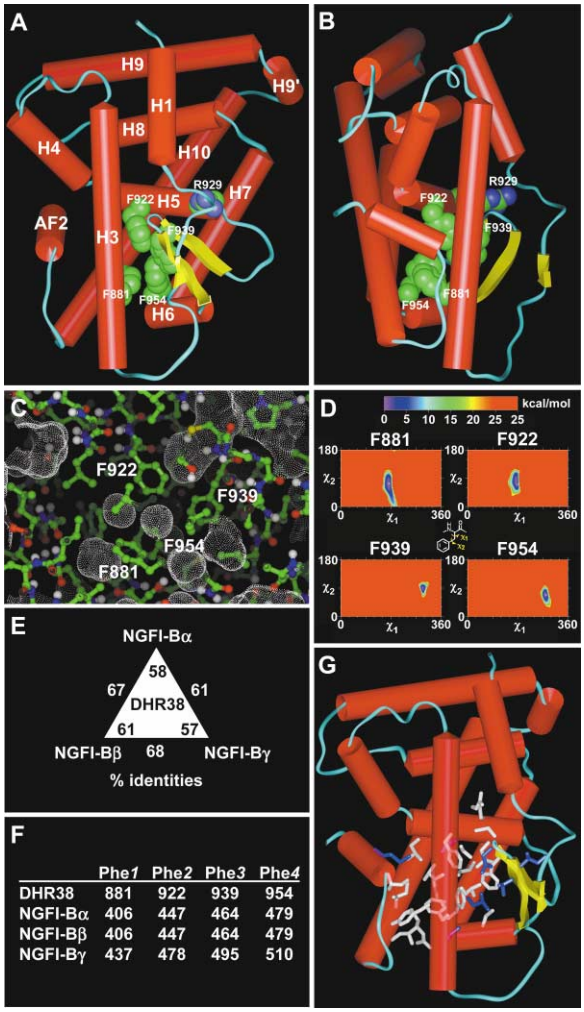
2001) including SRC-1, GRIP1, ACTR, or NCoR (data not shown). Likewise, mammalian NGFI-B family members have also been shown to lack interactions with known cofactors (Castro et al., 1999; Wansa et al., 2002; Maira et al., 2003), which has been suggested to be due to the lack of a coactivator interaction surface on the receptor (Wansa et al., 2002). Taken together, these findings suggest the existence of an atypical signal transduction mechanism that governs DHR38 transactivation and that apparently does not require direct binding of ligand or known coactivators. To begin to explore this mechanism in more detail, the X-ray crystal structure of the *Drosophilia* DHR38 ligand binding domain was solved (residues 841–1073 in Kozlova et al., 1998) to an R factor of 20% at 2.3 Å resolution using MAD phasing (Table 1). Crystals of DHR38 have the space group $P2_12_12_1$, with cell dimensions a = 78.38 Å, b = 82.23 Å, c = 84.15 Å, α = β = γ = 90° and two molecules in the asymmetric unit. Continuous electron density was observed throughout the protein except for the nine amino acids of the 6×His tag at the N terminus and the last four amino acids at the C terminus. No significant differences were observed between the two molecules in the asymmetric unit.

The overall architecture of the DHR38 ligand binding domain (Figures 5A and 5B) is similar to other members of the nuclear receptor family (Bourguet et al., 1995). The structure consists of 11 α helices with a small three-stranded β sheet arranged in a three-layer helical sandwich. Helices H4, H5, H8, and H9 are packed between helices H1 and H3 on one side, with H7 and H10 on the other side. DHR38 does not contain helix H2 but has an

Table 1. Crystallographic Data and Refinement Statistics

Protein	Native	Selenomethionine		
Wavelength (Å)	1.54	0.9792	0.9794	0.9840
Resolution (Å)	50.0–2.3	50.0–2.3	50.0–2.3	50.0–2.3
Observations	189296	202035	193530	189325
Unique reflections	23421	25602	25410	25166
Completeness[b] (%)	94.6	99.3	98	98
R_{sym}[a,b] (%)	7.5	7.7	9.3	6.6
R_{factor}[c] (%)	20			
R_{free}[d] (%)	24			
Rms bonds	0.007 Å			
Rms angles	1.11°			

[a] $R_{sym} = \Sigma_{hkl}|I-\langle I\rangle|I\Sigma I$, where I is the observed intensity and $\langle I\rangle$ is the average intensity from observations of symmetry-related reflections.
[b] Value in parentheses is for the highest resolution shell.
[c] $R_{factor} = \Sigma_{hkl}|Fobs|-|F_{calc}||/\Sigma|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively, for the *hkl* reflections.
[d] R_{free} is calculated for a set of reflections that were not included in atomic refinement.



	Phe1	Phe2	Phe3	Phe4
DHR38	881	922	939	954
NGFI-Bα	406	447	464	479
NGFI-Bβ	406	447	464	479
NGFI-Bγ	437	478	495	510

Figure 5. Structure of the DHR38 Ligand Binding Domain

(A and B) The DHR38 ligand binding domain is composed of 11 α helices (red tubes) and a small three-stranded β sheet (yellow arrows) arranged in a three-layer helical sandwich. Four Phe residues that fill the ligand binding site and Arg929 are shown in space filling representation colored by atom type (carbon, green; nitrogen, blue). The major α helices are numbered according to convention (Bourguet et al., 1995).

(C) Closeup view of the ligand pocket. The four Phe residues that fill the pocket are labeled. The white dot surface represents the calculated volume of the residual cavity within the ligand binding domain.

(D) Ramachandran plot of the quantum mechanical energy surface for the four Phe residues. Conformations of χ_1 and χ_2 angles with the lowest energy are colored blue, with higher energies colored according to the scale, where energies are given in kcal/mole.

(E) Sequence identities of the ligand binding domains within the DHR38/NGFI-B family. Pairwise identities between DHR38 and the human NGFI-B subtypes are shown at the vertices of the triangle. Pairwise identities between the NGFI-B subtypes are shown along the faces of the triangle.

(F) Conservation of the four Phe residues that fill the binding pocket. The amino acid numbering for each of the phenylalanine residues in DHR38 and the human NGFI-B subtypes is given.

(G) The DHR38 ligand binding domain showing the residues that compose the core of the ligand binding pocket in wire-frame representation. Residues that are conserved between DHR38 and the NGFI-B family members are shown in white, while nonconserved residues are shown in dark blue.

additional short helical segment between helices H9 and H10. The AF-2 helix of DHR38 (residues 1060–1069) was found in the "active" conformation, characteristic of agonist bound nuclear receptors.

In many nuclear receptor ligand binding domain crystal structures, the protein exists as a homo- or heterodimer with an extensive dimer interface along helix H10 (Bourguet et al., 1995; Brzozowski et al., 1997; Nolte et al., 1998; Bourguet et al., 2000; Gampe et al., 2000; Wisely et al., 2002). In the DHR38 structure, the protein appears as a monomer despite the fact that two molecules are found in the asymmetric unit. DHR38 is known to bind to specific response elements as a monomer as well as a heterodimer with USP. No significant crystal contacts were observed in the structure that would suggest a biologically relevant homodimer interface. This finding is consistent with the results of size exclusion chromatography using the crystallography construct. Although the structural basis of DHR38's ability to heterodimerize with USP must await the structure of the DHR38/USP complex, DHR38 does contain the consensus heterodimerization motif of ϕ ψKψψ ψKψψ Σ ψRψψ in the first half of helix H10, where ϕ = the hydrophobic aromatic residues Phe, Trp, or Tyr; ψ = a hydrophobic aliphatic residue with preference for Met, Leu, Val, Pro, Ala, or Ile; and Σ = the acidic residue Asp or Glu (Gampe et al., 2000). This motif corresponds to F SRLL GKLP E LRSL in DHR38 (residues 1027–1040).

DHR38 Lacks a Conventional Ligand Binding Pocket

Of significant interest was the finding that the apo-DHR38 structure does not contain a well-defined ligand binding site. The side chains of four phenylalanines (F881, F922, F939, and F954) point into the interior of the pocket (Figures 5A and 5B) and essentially fill the entire space. Calculation of the volume of the cavity within the putative binding pocket revealed that the largest contiguous pocket is only ~30 Å^3, which is too small to allow binding of any organic small molecule (Figure 5C). Three of the four phenylalanines adopt the only available low energy conformations, while the fourth can only move within a single plane before encountering a significant steric barrier (Figure 5D). Thus, it does not appear that any simple structural changes are available that might open up the binding pocket for access to a potential ligand.

Most nuclear receptors contain a conserved arginine within helix H5 that serves to anchor and correctly position ligands within the predominantly hydrophobic ligand binding pocket. While this arginine is conserved in DHR38 (R929), its side chain points out toward solvent rather than toward the interior of the protein. The β sheet packs tightly against the end of helix H5, excluding R929 from the potential binding pocket (Figures 5A and 5B). The absence of a ligand binding pocket is consistent with our inability to detect ecdysteroid binding to DHR38. We conclude from these data that ecdysteroid activation of the DHR38/USP heterodimer must occur through an alternative mechanism that does not involve direct binding of the agonist to the receptor complex. The details of this mechanism are currently under study.

Conservation between DHR38 and Mammalian NGFI-B Family Members

DHR38 has high sequence identity with the ligand binding domain of the three human NGFI-B subfamily members. A pairwise analysis of these sequences showed that DHR38 has the same level of conservation with each of the human NGFI-B family members as the human receptor subtypes have amongst themselves (Figure 5E), suggesting that each of these four receptors evolved from a common ancestor. Further analysis of the DHR38 and mammalian sequences revealed that the four phenylalanines that fill the ligand pocket are conserved (Figure 5F), as are almost all the amino acids that make up the core of the ligand binding domain (Figure 5G). The remarkable conservation between these receptors suggests that the vertebrate NGFI-B subfamily members also do not have a conventional ligand binding pocket.

DHR38 Lacks a Conventional Coactivator Binding Site and Has an Atypical AF-2 Helix

The coactivator binding site in other nuclear receptors consists of a hydrophobic groove formed by helices H3, H4, H5, and the AF-2 helix. In these receptors, the LXXLL motif of the coactivator is positioned within the groove by a charge-clamp interaction involving a highly conserved glutamic acid on AF-2 and a lysine on helix H3 (Darimont et al., 1998; Nolte et al., 1998; Shiau et al., 1998; Gampe et al., 2000; Xu et al., 2001). Sequence alignments of the DHR38/NGFI-B family with other nuclear receptors indicates that the charged clamp residues are not conserved. The conserved glutamic acid on the AF-2 is an asparagine in DHR38 (N1065) and a lysine in the mammalian NGFI-B receptors. The conserved lysine on helix H3 is a glutamic acid in both DHR38 (E897) and its mammalian orthologs. Interestingly, in the DHR38 structure, the AF-2 helix is shifted by one turn relative to its position in other nuclear receptor ligand binding structures (Figure 6A). The AF-2 helix is held in this position by a series of hydrophobic contacts with the main body of the ligand binding domain. The side chains of I1063 and M1066 are buried completely at the interface, with the M1066 side chain packing in a hydrophobic depression created by the side chains of L885, L918, L1045, and I1048. Due to the shift of the AF-2 helix in DHR38, A1061, rather than N1065, sits at the same position as the conserved glutamic acid of the charge clamp. These features result in the loss of the charge clamp in DHR38. In addition, the hydrophobic cleft that makes up the LXXLL motif binding site is blocked by a number of hydrophilic residues in DHR38. The side chains of L893 and N1065 point into the groove, partially blocking one of the leucine binding pockets and closing off one end of the groove (Figures 6B and 6C). Thus, it is unlikely that the DHR38 AF-2 helix can stabilize the binding of coactivator proteins through the LXXLL coactivator motif. These observations may explain why none of the well-characterized coactivator proteins for all other nuclear receptors have been shown to interact with any members of the DHR38/NGFI-B family. Taken together with the finding that these orphan nuclear receptors lack a conventional ligand binding pocket, the structural analysis supports the notion that these receptors must use an alternate mechanism for effecting transactivation of gene expression.

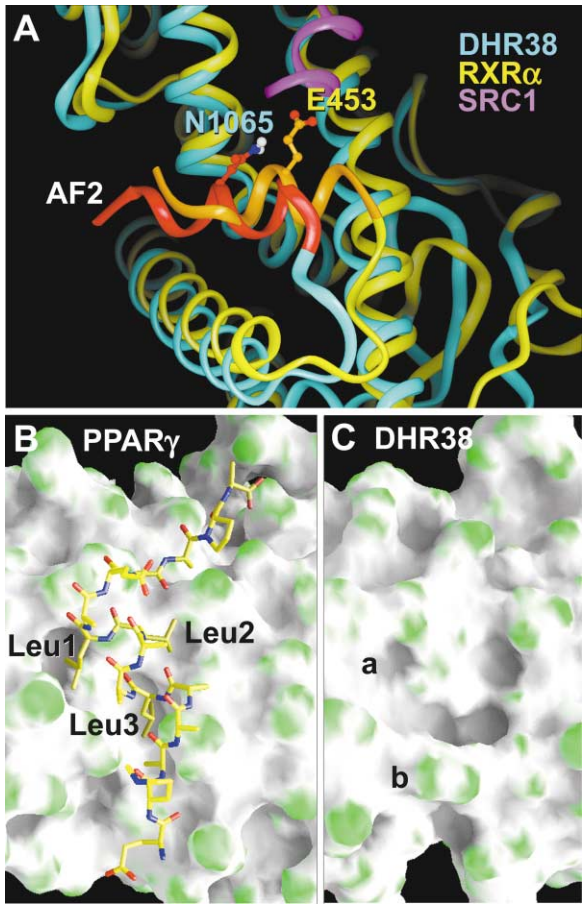


Figure 6. Structural Features that Preclude Binding of Coactivator Proteins to DHR38

(A) Overlay of DHR38 with the RXRα/SRC1 complex (Gampe et al., 2000) showing the shift in the C-terminal AF-2 helix. The DHR38 protein backbone is shown as a blue ribbon, with the AF-2 helix in red. The RXRα protein backbone is shown as a yellow ribbon, with the AF-2 helix in gold. The SRC1 coactivator fragment is shown in magenta. N1065 from DHR38 and E453 from RXRα are highlighted. (B and C) DHR38 lacks a binding groove for LXXLL motifs. (B) Space-filling view of the surface of PPARγ with the SRC1 fragment shown as a stick model. Note that the Leu residues of the LXXLL motif are buried into the groove along the surface of PPARγ that is formed by hydrophobic pockets. (C) The corresponding surface of DHR38 is shown. One of the Leu binding pockets (a) is partially blocked, and the bottom of the coactivator groove (b) is not accessible.

Discussion

In the present work, we provide evidence for the existence of an ecdysteroid signaling pathway mediated by the orphan nuclear receptor DHR38. The existence of this pathway is supported by four independent experimental findings. First, transactivation assays in insect cells demonstrated that a distinct group of endogenous ecdysteroids, several with no previously known function, can potentiate DHR38-dependent transcription when heterodimerized with a preactivated partner (i.e., rexinoid bound RXR or VP16-USP). Second, organ explants from transgenic flies bearing a DHR38-specific reporter gene were shown to be similarly responsive to ecdysteroids, indicating that this pathway can function

in vivo. Importantly, the specificity of the DHR38 ecdysteroid activators and the use of RNAi methodology have excluded the involvement of EcR in mediating this response. Third, neither the ecdysteroid agonists nor any of the known nuclear receptor coactivators were capable of binding directly to DHR38. Fourth, X-ray crystallographic structure analysis of the DHR38 ligand binding domain showed that DHR38 lacks the classic binding sites for either a ligand or a conventional coactivator, features that are hallmarks of all other known inducible nuclear receptors. As discussed below, these findings provide compelling evidence for an atypical nuclear receptor transcriptional signaling pathway that mediates ecdysteroid responses in insects.

Integration of the DHR38 Signal Transduction Pathway with Known Ecdysteroid Responses

The insect hemolymph carries a wide range of endogenous ecdysteroids, some of which are only present at specific stages during development. These may be supplemented by phytoecdysteroids that can enter the animal through its diet (Riddiford, 1996; Dinan, 2001; Gilbert et al., 2002). Until recently, it was thought that the vast majority of these compounds were unable to elicit a biological response. Mounting evidence, however, indicates that alternate transcriptional pathways exist that are driven by ecdysteroids other than 20E. Coordinate changes in ecdysteroid-regulated gene expression occur at several stages in the *Drosophila* life cycle at times when the 20E titer is known to be low (Andres and Cherbas, 1992; Andres et al., 1993; Benyajati et al., 1983; Mougneau et al., 1993; A. Sullivan and C.S.T., unpublished data). In addition, the *let-7* and *miR-125* small temporal RNAs are induced at puparium formation in precise synchrony with the *E74A* 20E-inducible gene, but in a manner that is independent of either 20E or EcR (Bashirullah et al., 2003). Of particular relevance to *DHR38* functions, Champlin and Truman (1998) have shown that α-ecdysone drives neuroblast proliferation during early pupal development in the hornworm *Manduca sexta*, providing in vivo evidence that this hormone is responsible for a specific response in insects. Similarly, 3-dehydro-20E was shown to have a potency indistinguishable from 20E in *Manduca* (Hiruma et al., 1997) and was also observed to have high activity in *Drosophila* larval fat body (Sommé-Martin et al., 1990), while Rachinsky et al. (1990) noted that makisterone A and not 20E is the major ecdysteroid present during the last larval instar of the honeybee. Given the reported activity of these ecdysteroids, it seems reasonable to expect that at least one of the pathways governing these responses is mediated by the DHR38 pathway described here. Further support for the hypothesis that a DHR38/USP heterodimer may play an essential role in ecdysteroid signaling comes from the observation that DHR38 and USP are each required for ecdysteroid-induced cuticle formation during *Drosophila* development (Perrimon et al., 1985; Kozlova et al., 1998; Hall and Thummel, 1998). A key to the future characterization of this developmental pathway will be the use of the DHR38/USP heterodimer and ecdysteroid agonists as tools to identify downstream target genes, which at present remain unknown.

An interesting feature of the DHR38 response is the broad specificity and increased sensitivity that a number of ecdysteroids have for DHR38 compared to the previously described signaling pathway mediated by EcR. Indeed, even the response to 20E appears to be an order of magnitude more potent for DHR38 than for EcR. Thus, discovery of the DHR38 response pathway may not only provide a mechanism of action for other ecdysteroids in insects, but may also provide a means of augmenting the ecdysteroid-mediated functions of EcR at specific stages in the life cycle.

Another striking feature of the DHR38 response is that it requires coactivation of its heterodimer partner to become competent for transcriptional activation via ecdysteroids. The finding that VP16-USP was able to substitute for ligand-activated RXR in our transfection assays is intriguing and suggests that in vivo, wild-type USP is capable of activation by ligand or some other coactivation mechanism. The existence of a ligand for USP is supported by X-ray crystal data on the USP ligand binding domain showing the presence of a large hydrophobic pocket that can be occupied by lipophilic ligands (Billas et al., 2001; Clayton et al., 2001). The observation that the DHR38 ecdysteroid response can occur in larval organs that contain wild-type USP supports this hypothesis (Figure 4). Identification of the USP ligand and/or coactivator represents a critical next step toward defining the mechanism of DHR38 action.

Biochemical and Structural Studies Reveal a Nonclassical Mechanism of Nuclear Receptor Activation

DHR38's distinct ecdysteroid-regulated activity points to a role that is substantially different from that of EcR, both in terms of ligand specificity and mechanism of action (Koelle, 1992; Thomas et al., 1993; Yao et al., 1993). Although both receptors require heterodimerization with USP to be ecdysteroid responsive, only the EcR response appears to require conventional binding of the ecdysteroid agonist. Furthermore, the role of USP in the EcR heterodimer is that of a silent partner (i.e., the transcriptional activity of USP is dispensable for the ecdysteroid response). In contrast, we have shown that the DHR38 pathway requires transcriptional activation of both itself and its heterodimeric partner. Surprisingly, however, this response occurs in the absence of ecdysteroid binding directly to receptor, implying the existence of a nonclassical mechanism of action.

The structure of the DHR38 ligand binding domain offers an intriguing framework from which several clues about the mechanism of DHR38 action can begin to be elucidated. Although we cannot formally rule out the possibility that a ligand could bind to DHR38 by an induced-fit mechanism or to an allosteric site, we consider both possibilities unlikely. The tight spatial constraints forced upon the protein by the four phenylalanines within the conventional ligand binding pocket (Figures 5C and 5D) almost completely exclude the induced-fit possibility. Likewise, our inability to demonstrate any type of specific ligand binding to the protein under a variety of conditions (e.g., in the presence or absence of activated heterodimer partner) using a number of assays argues against the existence of a second

binding site on the protein. An equally important finding is the loss of the charge clamp (Figure 6), which fundamentally excludes the DHR38 ligand binding domain from interacting with the p160 family of coactivators in a conventional fashion (Rosenfeld and Glass, 2001). This finding is consistent with our inability to observe any interactions with these coactivators in either cell-based or biochemical assays (data not shown). Taken together, our results provide strong evidence that the ecdysteroid response by the DHR38/USP heterodimer occurs through a mechanism that is different from the well-documented, direct binding paradigm that has been exhibited for numerous other RXR heterodimers (Chawla et al., 2001). Therefore, the signaling pathway between ecdysteroid and DHR38-mediated transcription must be transduced in an atypical fashion. This mechanism, however, still appears to require the AF-2 domain of DHR38 (Figure 1). Although it is not clear how the AF-2 contributes to receptor transactivation, our data support a model in which ecdysteroids may indirectly activate DHR38, perhaps by recruiting a specific cofactor to the DHR38/USP heterodimer. In this model, it is tempting to speculate that ecdysteroids may activate the cofactor through a direct interaction or through a second message pathway. Regardless, the requirement for a DHR38 cofactor is implicit in our findings and its future characterization will be important to fully understand the mechanism of this new signal transduction pathway.

Perspectives

The principles of DHR38 action may be of help in characterizing its mammalian orthologs, the NGFI-B family of receptors. Like DHR38, these orphan receptors can function as monomers or RXR heterodimers and be activated by RXR ligands (Giguere, 1999). However, little is known about the agonist or cofactor specificity of these proteins or the mechanistic details of how they promote transactivation. Our analysis shows that the overall conservation between DHR38 and the three mammalian NGFI-B family members is well conserved in the putative ligand binding pocket. Indeed, as shown for DHR38, modeling of the 3D structure of the NGFI-B receptors predicts the absence of both a ligand binding pocket and a coactivator binding site, suggesting that a common mechanism of action may exist for governing these receptors in mammals. Given these similarities between DHR38 and its vertebrate counterparts, it should not be surprising that, like many other insect signaling pathways, there is a lot to learn from the fly.

Experimental Procedures

Ecdysteroids

Ecdysteroids used were a generous gift from J.T. Warren and L.I. Gilbert (University of North Carolina). Stock solutions of compounds were dissolved in a 1:1 mixture of ethanol to DMSO or in 100% ethanol.

Cell Culture and Plasmids

SL2 Schneider cells were grown in Schneider's *Drosophila* medium (Gibco) supplemented with 6.5% super-stripped fetal bovine serum (Gemini) and 1% antibiotic-antimycotic (Gibco) in atmospheric conditions at 24°C. Insect cell expression vectors pA5C-EcR, pA5C-GAL4-DHR38, pADH-hspEcRE-LUC, and pADH-UAS-LUC were as described (Baker et al., 2000). For pA5C-GAL4-EcR, the ligand binding domain, including hinge region, of EcR (amino acids 330–878) was amplified by PCR and inserted in pA5C vector using the EcoRV restriction site. The following oligonucleotides PCR primers were used to construct A5C-GAL4-DHR38ΔAF-2: 5′-ATAGATATCGT CAAGGAAGTGGTGCGCAC-3′ and 5′-TATGCTAGCTATGGTGCGG GTACCAGGTCCTC-3′, which utilize EcoRV and NheI restriction sites, respectively. For A5C-VP16-USP, a CMX3′ primer and the following oligonucleotide was used: 5′-AAAGGTACCAGGATGGA CAACTGCGACCAG-3′, which utilizes an ASP718 restriction site. The PCR products from each of these reactions was then cloned into A5C with standard techniques.

Transfection Assays

SL2 cells were plated at a density of 8×10^5 cells/ml (100 μl/well) in 96-well opaque plates with clear bottom (Costar) and allowed to grow overnight. After ~17 hr of growth, 20 μl of transfection mix was added per well. Transfection mix was prepared using the standard calcium phosphate method with 1×HEPES (pH 7.4) and contained 15 ng of each receptor, 50 ng of LUC reporter, 20 ng of internal control βGAL plasmid (which drives high-level expression of the *E. coli* β-galactosidase protein), and carrier DNA (pGEM) up to 150 ng total DNA for each well that was transfected. Cells were dosed with 20 μl of the indicated compounds in media 6 hr posttransfection and harvested 17 hr after dosing with hormone. The media in each well was replaced with 50 μl of luciferase lysis buffer (3 mM tricine [pH 7.8], 0.8 mM magnesium acetate, 0.02 mM EDTA, 0.15 mM ATP, 100 mM 2-mercaptoethanol, 1% Triton X-100, 0.5 mM Coenzyme A (Sigma), and 0.5 mM D-luciferin, sodium salt (Molecular Probes), and the plates were incubated at room temperature under aluminum foil for 60 s. Light units were then read with a Dynatech MR5000 luminometer. After reading light units, 125 μl of ONPG buffer (60 mM Na_2HPO_4 and 40 mM NaH_2PO_4) containing 2 mg/ml ONPG was added/well. After color development at 37°C, the plate was read on a Dyntech MR5000 plate reader (test filter 410 nm, reference filter 630 nm). Relative light units (RLU) reported were calculated as ([light units/OD 420] × reaction time in minutes).

RNAi

For RNAi, the following oligonucleotides were generated against the EcR LBD, which incorporate a 5′ T7 RNA polymerase binding site: 5′-TTAATACGACTCACTATAGGGAGAGGAGGTCACGTCCTC CTC-3′ and 5′TTAATACGACTCACTATAGGGAGACTTCTTCGCATC GCAGCT-3′. Primers were used to PCR a fragment off of the A5C-EcR plasmid. The product was purified and quantified by OD 260 and used to generate dsRNA with the T7 MEGAscript kit (Ambion). RNA was resuspended in water and directly added to the normal tranfection mix described above.

Generation of the *hs*-GAL4-DHR38 Transgenic Flies and Larval Organ Culture

Hs-GAL4-EcR is described in Kozlova and Thummel (2002). To generate the hs-GAL4-DHR38 construct, DNA encoding the *Drosophila* DHR38 LBD, including the hinge region (amino acids 287–527), was amplified by PCR from the cTK11 cDNA clone (Sutherland et al., 1995), and this fragment was inserted between the EcoRI and BamHI sites of pCaSpeR-hs-GAL4act (Kozlova and Thummel, 2002). The junction between *GAL4* DBD and *DHR38*, as well as *DHR38* LBD sequences in the resulting construct, were verified by DNA sequence analysis. This P element construct was introduced into the germline of w^{1118} flies by standard transformation procedures. A homozygous viable *hs-GAL4-DHR38* insertion on the third chromosome was used for all studies reported here. *Hs-GAL4-DHR38; UAS-nlacZ* or *hs-GAL4-EcR; UAS-nlacZ* third instar larvae were maintained on food containing 0.5% bromophenol blue and partial blue gut animals (Andres and Thummel, 1994) were heat treated for 30 min at 37°C in a water bath in plastic vials with food. Animals were selected at ~8 hr before puparium formation, and larval organs were cultured in the presence of 10^{-6}M α-ecdysone or 10^{-6}M 3-epi-20E as described (Kozlova and Thummel, 2002). Tissues were then stained with X-gal for β-galactosidase expression as described (Kozlova and Thummel, 2002).

Subcloning and Protein Purification

The ligand binding domain (residues 841–1073) of DHR38 (Kozlova et al., 1998) was PCR amplified with oligonucleotides containing the subcloning restriction enzyme sites (NdeI–BamHI). The PCR fragment was subcloned into a T7 *E. coli* expression vector, pRSETa (Invitrogen), at the corresponding sites. The N-terminal PCR primer also included a 6×His-tag sequence MKKGHHHHHHG that will generate a His-tagged fusion protein with DHR38. The DHR38/pRSETA plasmid was transfected into the BL21(DE3) strain of *E. coli* and grown in 1 liter cultures in shake flask at 22°C for 16 hr. The cell pellet was resuspended, lysed by sonication, and clarified by centrifugation. The cleared supernatant was loaded on to ProBond nickel-chelating resin (Invitrogen) and washed with the resuspension buffer (50 mM imidazole [pH 7.5] and 150 mM NaCl) to the baseline. The DHR38 protein was eluted from the column with an imidazole gradient (50–400 mM) at pH 7.5. Fractions containing the DHR38 protein were pooled and loaded on to a 50 ml SP-Sepharose FastFlow (Pharmacia) column. The DHR38 protein was eluted from the column at 200 mM NaCl in an increasing salt gradient. Again, the fractions containing the DHR38 protein were pooled, concentrated using Centri-prep 30 units (Amicon), and subjected to size exclusion using Sepharose S-75 resin (Pharmacia). The appropriate column fractions containing the DHR38 protein were pooled and stored on ice. Purity of the DHR38 protein was estimated at >95% as assessed by SDS-PAGE with Coomassie Blue staining.

Crystallization and Data Collection

Both native and selenomethione protein were concentrated to 2–4 mgs/ml in 10 mM Tris HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 5 mM DTT, and 1 mM nervonic acid. Crystals of native DHR38 were grown using the hanging drop vapor diffusion method (Gilliland and Davies, 1984) in VDX trays. Protein was mixed with an equal volume of well solution containing 0.1 M HEPES (pH 7.5) and 1.5 M lithium sulfate (Crystal Screen I, Hampton Research; Jancarik and Kim, 1991). Crystals appeared within a few days at 10°C. Selenomethionine-labeled DHR38 crystals were obtained using the Cyberlab C240 crystallization robot with NeuroProbe hanging drop trays. The reservoirs contained 80 μl of the precipitating agent (0.1 M Bis Tris Propane [pH 7.0] and 1.5 M ammonium sulfate [Salt Rx Screen, Hampton Research]). Drops were composed of 1 μl protein (2–4 mg/mL in 10 mM Tris HCl [pH 8.0], 500 mM NaCl, 1 mM EDTA, and 5 mM DTT) and 1 μl precipitating agent. Trays were incubated at 10°C. Prior to data collection, crystals were transferred to paraffin oil and flash frozen in liquid nitrogen. Crystals belong to the space group $P2_12_12_1$ with unit cell dimensions a = 78.38 Å, b = 82.23 Å, c = 84.15 Å, $\alpha = \beta = \gamma = 90°$ and two molecules in the asymmetric unit. Data were collected on the undulator beamline 17-ID at the APS using an ADSC detector. Data were collected on a single crystal at three wavelengths corresponding to the peak and inflection point for selenium as well as a remote wavelength. All data was processed and scaled with HKL2000.

Structure Determination and Refinement

Eight of the possible ten selenium sites were found with Shake and Bake (Weeks and Miller, 1999) and refined with SHARP (De La Fortelle et al., 1996). Models were built using Quanta and refined with CNX (Brunger et al., 1998).

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References

Andres, A.J., and Cherbas, P. (1992). Tissue-specific ecdysone reponses: regulation of the *Drosophila* genes *Eip28/29* and *Eip40* during larval development. Development *116*, 865–876.

Andres, A.J., and Thummel, C.S. (1994). Methods for quantitative analysis of transcription in larvae and prepupae. Methods Cell Biol. *44*, 565–573.

Andres, A.J., Fletcher, J.C., Karim, F.D., and Thummel, C.S. (1993). Molecular analysis of the initiation of insect metamorphosis: a comparative study of *Drosophila* ecdysteroid-regulated transcription. Dev. Biol. *160*, 388–404.

Baker, K.D., Warren, J.T., Thummel, C.S., Gilbert, L.I., and Mangelsdorf, D.J. (2000). Transcriptional activation of the Drosophila ecdysone receptor by insect and plant ecdysteroids. Insect Biochem. Mol. Biol. *30*, 1037–1043.

Bashirullah, A., Pasquinelli, A.E., Kiger, A.A., Perrimon, N., Ruvkun, G., and Thummel, C.S. (2003). Coordinate regulation of small temporal RNAs at the onset of *Drosophila* metamorphosis. Dev. Biol., in press. Published online March 25, 2003. 10.1016/S0012-1606(3) 00063-0

Benyajati, C., Spoerel, N., Haymerle, H., and Ashburner, M. (1983). The messenger RNA for alcohol dehydrogenase in *Drosophila melanogaster* differs in its 5′ end in different developmental stages. Cell *33*, 125–133.

Billas, I.M.L., Moulinier, L., Rochel, N., and Moras, D. (2001). Crystal structure of the ligand-binding domain of the ultraspiracle protein USP, the ortholog of retinoid X receptors in insects. J. Biol. Chem. *276*, 7465–7474.

Boehm, M.F., Zhang, L., Zhi, L., McClurg, M.R., Berger, E., Wagoner, M., Mais, D.E., Suto, C.M., Davies, J.A., Heyman, R.A., et al. (1995). Design and synthesis of potent retinoid X receptor selective ligands that induce apoptosis in leukemia cells. J. Med. Chem. *38*, 3146–3155.

Botling, J., Castro, D.S., Oberg, F., Nilsson, K., and Perlmann, T. (1997). Retinoic acid receptor/retinoid X receptor heterodimers can be activated through both subunits providing a basis for synergistic transactivation and cellular differentiation. J. Biol. Chem. *272*, 9443–9449.

Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995). Crystal structure of the ligand binding domain of the human nuclear receptor RXR-α. Nature *375*, 377–382.

Bourguet, W., Vivat, V., Wurtz, J.M., Chambon, P., Gronemeyer, H., and Moras, D. (2000). Crystal structure of a heterodimeric complex of RAR and RXR ligand-binding domains. Mol. Cell *5*, 289–298.

Brunger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.-S., Kuszewski, J., Nigles, M., Pannu, N.S., et al. (1998). Crystallography and NMR System: a new software suite for macromolecular structure determination. Acta Crystallogr. D Biol. Crystallogr. *54*, 905–921.

Brzozowski, A.M., Pike, A.C., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., Ohman, L., Greene, G.L., Gustafsson, J.A., and Carlquist, M. (1997). Molecular basis of agonism and antagonism in the estrogen receptor. Nature *389*, 753–758.

Champlin, D.T., and Truman, J.W. (1998). Ecdysteroid control of cell proliferation during optic lobe neurogenesis in the moth *Manduca sexta*. Development *125*, 269–277.

Castro, D.S., Arvidsson, M., Bolin, M.B., and Perlmann, T. (1999). Activity of the Nurrl carboxyl-terminal domain depends on cell type and integrity of the activation function 2. J. Biol. Chem. *274*, 37483–37490.

Chawla, A., Repa, J.J., Evans, R.M., and Mangelsdorf, D.J. (2001).

Nuclear receptors and lipid physiology: opening the X-files. Science *294*, 1866–1870.

Cheng, L.E., Chan, F.K., Cado, D., and Winoto, A. (1997). Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T-cell apoptosis. EMBO J. *16*, 1865–1875.

Cherbas, L., and Cherbas, P. (1970). Distribution and metabolism of alpha-ecdysone pupae of the silkworm *Antherea polyphemus*. Biol. Bull. *138*, 115–128.

Cherbas, L., Yonge, C., Cherbas, P., and Williams, C. (1980). The morphological response of Kc-H cells to ecdysteroids: hormonal specificity. Roux's Archives Dev. Biol. *189*, 1–15.

Clayton, G.M., Peak-Chew, S.Y., Evans, R.M., and Schwabe, J.W.R. (2001). The structure of the ultraspiracle ligand-binding domain reveals a nuclear receptor locked in an inactive conformation. Proc. Natl. Acad. Sci. USA *98*, 1549–1554.

Darimont, B.D., Wagner, R.L., Apriletti, J.W., Stallcup, M.R., Kushner, P.J., Baxter, J.D., Fletterick, R.J., and Yamamoto, K.R. (1998). Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev. *12*, 3343–3356.

De La Fortelle, E., Fourme, R., and Bricogne, G. (1996). A maximum-likelihood parameter refinement program for MIR and MAD phasing. Trans. Am. Crystallogr. Assoc. *30*, 85–97.

Dinan, L. (2001). Phytoecdysteroids: biological aspects. Phytochemistry *57*, 325–339.

Fisk, G.J., and Thummel, C.S. (1995). Isolation, regulation, and DNA-binding properties of three *Drosophila* nuclear hormone receptor superfamily members. Proc. Natl. Acad. Sci. USA *92*, 10604–10608.

Forman, B.M., Umesono, K., Chen, J., and Evans, R.M. (1995). Unique response pathways are established by allosteric interactions among nuclear hormone receptors. Cell *81*, 541–550.

Gampe, R.T., Jr., Montana, V.G., Lambert, M.H., Miller, A.B., Bledsoe, R.K., Milburn, M.V., Kliewer, S.A., Willson, T.M., and Xu, H.E. (2000). Asymmetry in the PPARγ/RXRα crystal structure reveals the molecular basis of heterodimerization among nuclear receptors. Mol. Cell *5*, 545–555.

Giguere, V. (1999). Orphan nuclear receptors: from gene to function. Endocr. Rev. *20*, 689–725.

Gilbert, L.I., Song, Q., and Rybczynski, R. (1997). Control of ecdysteroidogenesis: activation and inhibition of prothoracic gland activity. Invert. Neurosci. *3*, 205–216.

Gilbert, L.I., Rybczynski, R., and Warren, J.T. (2002). Control and biochemical nature of the ecdysteroidogenic pathway. Annu. Rev. Entomol. *47*, 883–916.

Gilliland, G.L., and Davies, D.R. (1984). Protein crystallization: the growth of large-scale single crystals. Methods Enzymol. *104*, 370–381.

Grau, V., and Lafont, R. (1994). Metabolism of ecdysone and 20-hydroxyecdysone in adult Drosophila melanogaster. Insect Biochem. Mol. Biol. *24*, 49–58.

Hall, B.L., and Thummel, C.S. (1998). The RXR homolog ultraspiracle is an essential component of the Drosophila ecdysone receptor. Development *125*, 4709–4717.

Henrich, V.C., Szekely, A.A., Kim, S.J., Brown, N.E., Antoniewski, C., Hayden, M.A., Lepesant, J.A., and Gilbert, L.I. (1994). Expression and function of the ultraspiracle (usp) gene during development of *Drosophila melanogaster*. Dev. Biol. *165*, 38–52.

Hiruma, K., Böcking, D., Lafont, R., and Riddiford, L.M. (1997). Action of different ecdysteroids on the regulation of mRNAs for the ecdysone receptor, MHR3, dopa decarboxylase, and a larval cuticle protein in the larval epidermis of the tobacco hornworm, *Manduca sexta*. Gen. Comp. Endocrinol. *107*, 84–97.

Jancarik, J., and Kim, S.-H. (1991). Sparse matrix sampling: a screening method for crystallization of proteins. J. Appl. Crystallogr. *24*, 409–411.

Koelle, M.R. (1992). Molecular analysis of the *Drosophila* ecdysone receptor complex. PhD thesis, Stanford University, Stanford, California.

Kozlova, T., and Thummel, C.S. (2002). Spatial patterns of ecdysteroid receptor activation during the onset of Drosophila metamorphosis. Development *129*, 1739–1750.

Kozlova, T., Pokholkova, G.V., Tzertzinis, G., Sutherland, J.D., Zhimulev, I.F., and Kafatos, F.C. (1998). Drosophila hormone receptor 38 functions in metamorphosis: a role in adult cuticle formation. Genetics *149*, 1465–1475.

Maira, M., Martens, C., Batsche, E., Gauthier, Y., and Drouin, J. (2003). Dimer-specific potentiation of NGFI-B (Nur77) Transcriptional activity by the protein kinase A pathway and AF-1-dependent coactivator recruitment. Mol. Cell. Biol. *23*, 763–776.

Makishima, M., Okamoto, A.Y., Repa, J.J., Tu, H., Learned, R.M., Luk, A., Hull, M.V., Lustig, K.D., Mangelsdorf, D.J., and Shan, B. (1999). Identification of a nuclear receptor for bile acids. Science *284*, 1362–1365.

Makishima, M., Lu, T.T., Xie, W., Whitfield, G.K., Domoto, H., Evans, R.M., Haussler, M.R., and Mangelsdorf, D.J. (2002). Vitamin D receptor as an intestinal bile acid sensor. Science *296*, 1313–1316.

Mougneau, E., von Seggern, D., Fowler, T., Rosenblatt, J., Jongens, T., Rogers, B., Gietzen, D., and Beckendorf, S.K. (1993). A transcriptional switch between the *Pig-1* and *Sgs-4* genes of *Drosophila melanogaster*. Mol. Cell. Biol. *13*, 184–195.

Nolte, R.T., Wisely, G.B., Westin, S., Cobbs, J.E., Lambert, M.H., Kurokawa, R., Rosenfeld, M.G., Willson, T.M., Glass, C.K., and Milburn, M.V. (1998). Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-γ. Nature *395*, 137–143.

Paulsen, R.F., Granas, K., Johnsen, H., Rolseth, V., and Sterri, S. (1995). Three related brain nuclear receptors, NGFI-B, Nurr1, and NOR-1, as transcriptional activators. J. Mol. Neurosci. *6*, 249–255.

Perlmann, T., and Jansson, L. (1995). A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. Genes Dev. *9*, 769–782.

Perrimon, N., Engstrom, L., and Mahowald, A.P. (1985). Developmental genetics of the 2C-D region of the *Drosophila* X chromosome. Genetics *111*, 23–41.

Philips, A., Lesage, S., Gingras, R., Maira, M.H., Gauthier, Y., Hugo, P., and Drouin, J. (1997). Novel dimeric Nur77 signaling mechanism in endocrine and lymphoid cells. Mol. Cell. Biol. *17*, 5946–5951.

Rachinsky, A., Strambi, C., Strambi, A., and Hartfelder, K. (1990). Caste and metamorphosis: hemolymph titers of juvenile hormone and ecdysteroids in last instar honeybee larvae. Gen. Comp. Endocrinol. *79*, 31–38.

Riddiford, L.M. (1996). Hormones and *Drosophila* development. In The Development of *Drosophila melanogaster*, M. Bate and A. Martinez-Arias, eds., (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 899–939.

Riddiford, L.M., Cherbas, P., and Truman, J.W. (2000). Ecdysone receptors and their biological actions. Vitam. Horm. *60*, 1–73.

Rosenfeld, M.G., and Glass, C.K. (2001). Coregulator codes of transcriptional regulation by nuclear receptors. J. Biol. Chem. *276*, 36865–36868.

Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A., and Greene, G.L. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell *95*, 927–937.

Solomin, L., Johansson, C.B., Zetterstrom, R.H., Bissonnette, R.P., Heyman, R.A., Olson, L., Lendahl, U., Frisen, J., and Perlmann, T. (1998). Retinoid-X receptor signalling in the developing spinal cord. Nature *395*, 398–402.

Sommé-Martin, G., Colardeau, J., and Lafont, R. (1988). Conversion of ecdysone and 20-hydroxyecdysone into 3-dehydorecdysteroids is a major pathway in third instar *Drosophila melanogaster* larvae. Insect Biochem. *18*, 729–734.

Sommé-Martin, G., Colardeau, J., Beydon, P., Blais, C., Lepesant, J.A., and Lafont, R. (1990). P1 gene expression in Drosophila larval fat body: induction by various ecdysteroids. Arch. Insect Biochem. Physiol. *15*, 43–56.

Sutherland, J.D., Kozlova, T., Tzertzinis, G., and Kafatos, F.C. (1995). Drosophila hormone receptor 38: a second partner for Drosophila USP suggests an unexpected role for nuclear receptors of the nerve

growth factor-induced protein B type. Proc. Natl. Acad. Sci. USA *92*, 7966–7970.

Thomas, H.E., Stunnenberg, H.G., and Stewart, A.F. (1993). Heterodimerization of the Drosophila ecdysone receptor with retinoid X receptor and ultraspiracle. Nature *362*, 471–475.

Thummel, C.S., and Chory, J. (2002). Steroid signaling in plants and insects—common themes, different pathways. Genes Dev. *16*, 3113–3129.

Wilson, T.E., Fahrner, T.J., Johnston, M., and Milbrandt, J. (1991). Identification of the DNA binding site for NGFI-B by genetic selection in yeast. Science *252*, 1296–1300.

Wansa, K.D.S.A., Harris, J.M., and Muscat, G.E.O. (2002). The activation function-1 domain of Nur77/NR4A1 mediates trans-activation, cell specificity, and coactivator recruitment. J. Biol. Chem. *277*, 33001–33011.

Weeks, C.M., and Miller, R. (1999). The design and implementation of SnB v2.0. J. Appl. Crystallogr. *32*, 120–124.

Wisely, G.B., Miller, A.B., Davis, R.G., Thornquest, A.D., Jr., Johnson, R., Spitzer, T., Sefler, A., Shearer, B., Moore, J.T., Miller, A.B., et al. (2002). Hepatocyte nuclear factor 4 is a transcription factor that constitutively binds fatty acids. Structure *10*, 1225–1234.

Xu, H.E., Lambert, M.H., Montana, V.G., Pluket, K.D., Moore, L.B., Collins, J.L., Oplinger, J.A., Kliewer, S.A., Gampe, R.T., Jr., McKee, D.D., et al. (2001). Structural determinants of ligand binding selectivity between the peroxisome proliferator-activated receptors. Proc. Natl. Acad. Sci. USA *98*, 13919–13924.

Yao, T.P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.D., McKeown, M., Cherbas, P., and Evans, R.M. (1993). Functional ecdysone receptor is the product of EcR and Ultraspiracle genes. Nature *366*, 476–479.

Zetterstrom, R.H., Solomin, L., Mitsiadis, T., Olson, L., and Perlmann, T. (1996). Retinoid X receptor heterodimerization and developmental expression distinguish the orphan nuclear receptors NGFI-B, Nurr1, and Nor1. Mol. Endocrinol. *10*, 1656–1666.

Accession Numbers

The PDB ID for the DHR38 structure is 1PDU.