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Molecular Chaperones Activate the *Drosophila* Ecdysone Receptor, an RXR Heterodimer

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Summary

The steroid hormone 20-hydroxyecdysone coordinates the stages of *Drosophila* development by activating a nuclear receptor heterodimer consisting of the ecdysone receptor, EcR, and the *Drosophila* RXR receptor, USP. We show that EcR/USP DNA binding activity requires activation by a chaperone heterocomplex like that required for activation of the vertebrate steroid receptors, but not previously shown to be required for activation of RXR heterodimers. Six proteins normally present in the chaperone complex were individually purified and shown to be sufficient for this activation. We also show that two of the six (Hsp90 and Hsc70) are required in vivo for ecdysone receptor activity, and that EcR is the primary target of the chaperone complex.

Introduction

The nuclear receptor superfamily contains over 150 related transcription factors, many of which bind small lipophilic signaling molecules, including steroids, thyroid hormone, and retinoids. These receptors mediate the biological responses to their ligands by modulating the expression of target genes (reviewed by Mangelsdorf et al., 1995). Nuclear receptors are characterized by two domains: a highly conserved DNA binding domain (DBD) containing two zinc fingers, by which the receptor binds to specific DNA sequences called hormone response elements, or HREs; and a less well conserved ligand binding domain (LBD) located carboxy-terminal to the DBD. For several ligand-regulated nuclear receptors, ligand binding causes the release of associated corepressor proteins and allows receptor association with coactivator proteins that function to either modify chromatin structure or link the nuclear receptors to the transcription machinery (reviewed by Xu et al., 1999). This receptor family can be divided into the following three classes: (1) the vertebrate homodimeric steroid class, (2) the RXR heterodimeric class, and (3) the less well defined orphan receptors. The vertebrate steroid receptors bind HREs as homodimers; the RXR class bind HREs as heterodimers in which an RXR receptor is coupled with a ligand-specific nuclear receptor; and the orphan receptors bind DNA either as monomers or dimers and have no known ligand.

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In this article we focus on the receptor for 20-hydroxyecdysone (hereafter called ecdysone), the insect steroid hormone that triggers and coordinates the successive stages of the insect's life cycle (reviewed by Riddiford, 1993). In *Drosophila*, the receptor for ecdysone is a heterodimer of the proteins encoded by the *EcR* and *usp* genes (Koelle et al., 1991; Koelle, 1992; Yao et al., 1992; Thomas et al., 1993). *EcR* encodes three ecdysone receptor isoforms, EcR-A, EcR-B1, and EcR-B2 (Talbot et al., 1993), whereas *usp* encodes a single protein that is the *Drosophila* homolog of the vertebrate RXR receptors. The active EcR/USP heterodimer is therefore structurally akin to the vertebrate RXR heterodimeric class that includes the nonsteroidal thyroid (TR), retinoic acid (RAR), and vitamin D receptors (VDR) (reviewed by Mangelsdorf and Evans, 1995), rather than to the vertebrate homodimeric steroid receptors, such as those for glucocorticoid (GR), mineralcorticoid (MR), progesterone (PR), and androgens (AR) (reviewed by Beato et al., 1995). Although placed in the RXR heterodimeric class, the three EcR isoforms differ from most vertebrate members of this class in requiring heterodimerization not only for DNA binding but also for ecdysone binding. (See Koelle, 1992 and Yao et al., 1993 for EcR-B1; and Arbeitman, 1998 for all three isoforms.) Since Kliewer et al. (1998) have recently shown that a vertebrate member of the RXR heterodimer class, the pregnanes receptor, can also be activated by a steroid hormone, this distinction is not invertebrate specific.

Activation of the vertebrate steroid receptors requires interaction with a molecular chaperone-containing heterocomplex (MCH) (reviewed by Pratt and Toft, 1997). The molecular chaperones (Hsp90 and Hsc70), molecular chaperone interacting proteins (Hop, Hip, and p23), and peptidyl-prolyl isomerases (FKBP51, FKBP52, Cyp-40) are components of the MCH that have been shown to interact with the vertebrate steroid receptors. The MCH is thought to facilitate the folding of proteins and multiprotein complexes as well as to refold denatured proteins. In the case of the progesterone receptor (PR) and the glucocorticoid receptor (GR), the MCH is required for maturation to the aporeceptor state. For example, PR alone has no ligand or DNA binding activity. PR is activated by the MCH in an ATP- and Mg^{2+}-dependent manner to the aporeceptor state, which can bind ligand but not DNA. Upon binding ligand, the MCH is released and the receptor acquires DNA binding activity. These biochemical steps correlate with the subcellular location of the receptors. Prior to ligand binding, the receptors are in the cytoplasm in a complex with the MCH. Upon ligand binding and MCH release, the receptor is translocated into the nucleus (reviewed by DeFranco et al., 1998). It has been postulated that RXR heterodimeric receptors do not require the MCH for activity because TR and RAR do not copurify with Hsp90 under conditions where GR does (Dalman et al., 1990, 1991). However, genetic evidence suggests that RAR and RXR expressed in yeast require Hsp90 for transcriptional activity (Holley and Yamamoto, 1995). Furthermore, the demonstration that in crude extracts ATP

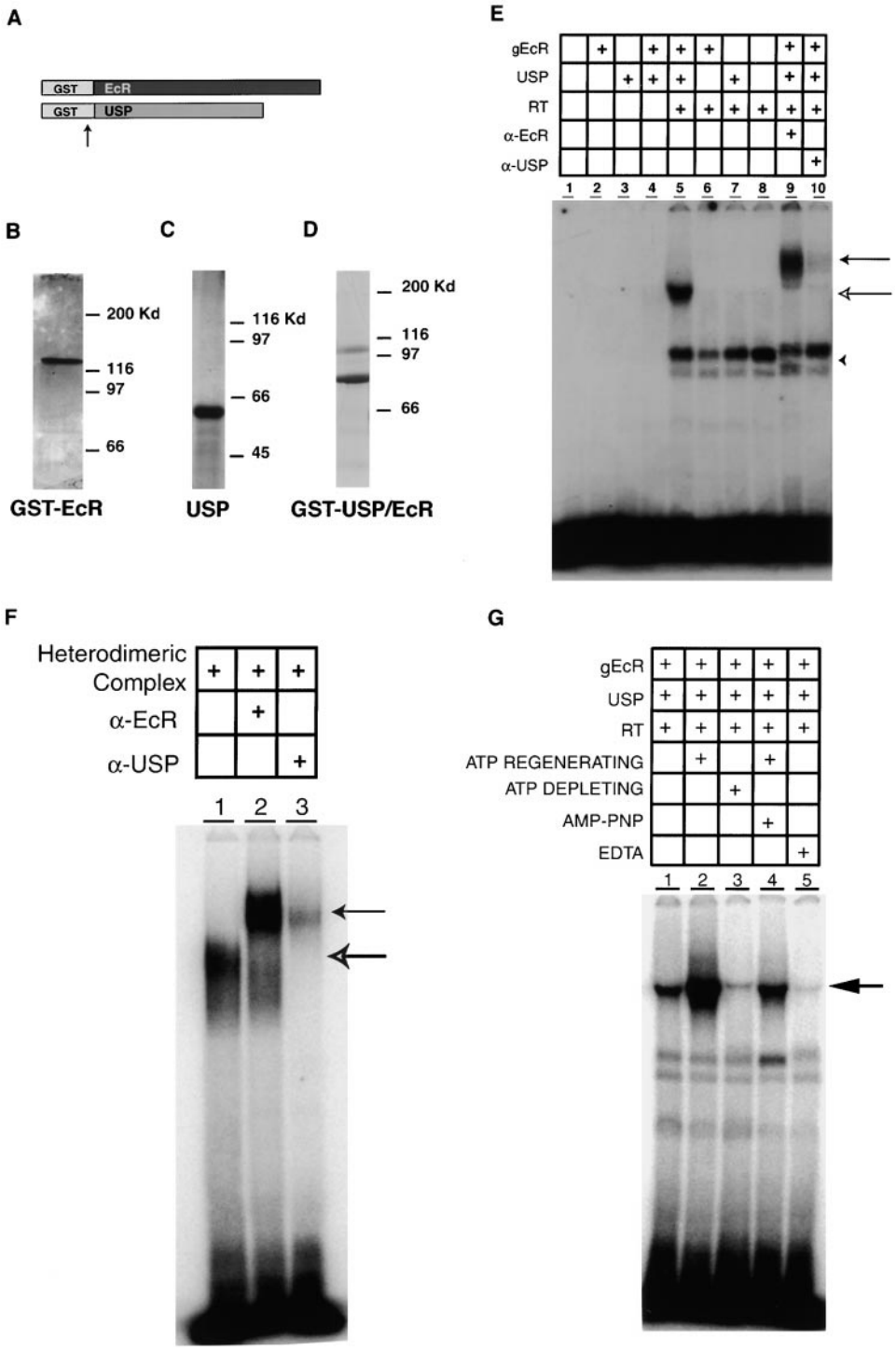

Figure 1. Purification and Activities of gEcR, USP, and an EcR/gUSP Heterocomplex

(A) Schematic diagram of recombinant EcR and USP proteins. Thrombin cleavage site resides amino-terminal to the USP and EcR open reading frames (indicated by arrow). Purified GST-EcR (gEcR) protein (B) and purified USP protein (C) electrophoresed on a 7% SDS-PAGE gel and silver stained. Purified GST-USP/EcR heterocomplex (D) electrophoresed on a 7% SDS-PAGE gel and Coomassie stained. The upper band in (D) is EcR (without the GST tag) and the lower band is GST-USP. The mobility of marker proteins and their molecular weights are indicated. (E) EcR and USP require auxiliary factors for EcRE DNA binding, which can be provided by a reticulocyte lysate (RT, 5 μl lysate). The purified ecdysone receptor components (B and C) were tested for EcRE DNA binding activity using a gel mobility shift assay. The open arrow marks the DNA/protein complex that requires both EcR and USP. This complex shows a slower mobility when an anti-EcR (10 μl AG10.2) or anti-USP (10 μl AB11) antibody is present (lanes 9 and 10; marked by a closed arrow). The arrowhead marks DNA/protein complexes that are present with RT alone. (F) A purified heterodimeric complex (EcR/gUSP) is sufficient for DNA binding. The purified receptor heterocomplex (D) was tested for EcRE DNA binding activity using a gel mobility shift assay. The open arrow marks the DNA/protein complex

modulates ecdysone receptor DNA binding activity and that Hsc70 is associated with EcR suggested that the ecdysone receptor, like the vertebrate steroid receptors, may be activated by an MCH (Koelle, 1992).

In this paper we show that a mixture of purified EcR-B1 and USP has hormone binding activity but lacks ecdysone response element (EcRE) DNA binding activity. DNA binding activity can be generated by the addition of purified components of the MCH—namely, Hsp90 (in *Drosophila* called Hsp83), Hsc70, Hip, Hop, FKBP52, and p23. Once having achieved the DNA binding state, the MCH components can be dispensed with as the activated EcR/USP heterodimer is sufficient for EcRE DNA binding. We also show that Hsp90 and Hsc70 are required for ecdysone receptor activity in vivo. Finally, we show that EcR, prior to associating with USP, can associate with Hsp90 and Hsc70, and that this association is an early step in achieving an active DNA binding state. Our results demonstrate a requirement for MCH in the activation of the ecdysone receptor that is distinct from that required for the homodimeric steroid receptors, thereby revealing a new activation pathway for a nuclear receptor.

Results

Auxiliary Factors Are Required to Transform the Ecdysone Receptor to the DNA Binding State

The functional ecdysone receptor is a heterodimer between the nuclear receptors EcR and USP, and this heterodimer is required for binding both ecdysone response element DNA (EcRE) and ecdysteroids (Koelle et al., 1991; Koelle, 1992; Yao et al., 1992, 1993; Thomas et al., 1993; Arbeitman, 1998). Since these conclusions were derived from results obtained using crude extracts containing EcR or USP, other proteins present in the extracts could be required for ecdysone receptor activity. To address whether EcR-B1 and USP are sufficient for ecdysone receptor activity, each protein was overexpressed as a recombinant glutathione S-transferase (GST) fusion protein using a baculovirus expression system. These fusion proteins contain a thrombin cleavage site carboxy-terminal to the GST sequence (Figure 1A). Both GST-EcR-B1 and GST-USP were purified by glutathione affinity chromatography; EcR-B1 was eluted as the GST-fusion protein, and USP was eluted by thrombin cleavage (see Experimental Procedures). A single major band was observed for each protein, indicating a high degree of purity (Figures 1B and 1C).

The DNA binding activities of purified GST-EcR-B1 (gEcR) and purified USP were tested on the *hsp27* EcRE DNA using a gel mobility shift assay. Consistent with previous observations, neither purified gEcR nor purified USP had EcRE DNA binding activity (Figure 1E, lanes 2 and 3). Furthermore, no EcRE DNA binding activity is evident when purified gEcR is mixed with purified USP (Figure 1E, lane 4). We then tested whether additional proteins added to the mixture of purified gEcR and USP could reconstitute EcRE DNA binding activity. For this purpose we used rabbit reticulocyte lysate (RT) because of its ability to transform purified vertebrate steroid receptors, such as PR, to the aporeceptor state (Johnson and Toft, 1994). *Drosophila* extracts were unsuitable due to their endogenous ecdysone receptor activity.

As shown in Figure 1E (lane 5, marked by open arrow), RT restores EcRE DNA binding activity to a mixture of purified gEcR and USP. Both proteins participate in DNA binding, since each must be present (Figure 1E, lane 6 and 7), and since addition of either an anti-EcR antibody or an anti-USP antibody (Figure 1E, lanes 9 and 10) supershifts the complex (closed arrow). The lower bands (indicated by an arrowhead) are present with RT alone (Figure 1E, lane 8). Similar results obtained with thrombin-cleaved GST-EcR showed that this requirement for additional factors is not due to the N-terminal GST moiety (data not shown). These results indicate that additional factors are required for ecdysone receptor DNA binding activity.

To distinguish between factors that form a complex with EcR/USP and participate in DNA binding, and factors that transform EcR/USP to a DNA binding state but do not participate directly in DNA binding, the ecdysone receptor was purified as a heterodimeric complex and was tested for DNA binding activity. The complex was assembled on a solid support as follows. First, GST-USP was bound to a glutathione sepharose column. Next, a crude extract from EcR-expressing baculovirus-infected Sf9 cells was allowed to bind to the column. The column was then washed, and the bound proteins eluted by the addition of excess free glutathione. Only two major proteins are observed with Coomassie stain, and these exhibited apparent molecular weights corresponding to EcR and GST-USP (gUSP) (Figure 1D). Western analyses using anti-EcR and anti-USP antibodies detected both EcR and gUSP at the appropriate molecular weights (data not shown).

Results from a gel mobility shift assay show that this purified complex has EcRE DNA binding activity (Figure 1F, lane 1). The shifted complex contains both EcR and USP, since antibodies directed against either protein will supershift the complex (Figure 1F, lanes 2 and 3). Since only EcR and USP are present in the eluted fraction, we conclude that the auxiliary factors are required transiently to transform the EcR/USP heterodimer to the DNA binding state, and thus these factors are not part of the complex that binds DNA. These data therefore demonstrate that EcR and USP are sufficient for DNA binding activity once transformation to the DNA binding state has been effected.

containing the purified heterocomplex. This complex displays a slower mobility when an anti-EcR or anti-USP antibody is present (closed arrow; lanes 2 and 3). In (E) and (F) the decrease in DNA binding activity observed after addition of the anti-USP antibody is inferred to be caused by an interference by the anti-USP antibody with the DNA/protein complex. (G) ATP hydrolysis and Mg^{2+} are required for EcRE DNA binding activity. Gel mobility shift assay of purified gEcR and USP and RT (5 μl) in the presence of an ATP regenerating system (lane 2), an ATP depleting system (lane 3), an ATP regenerating system, and the nonhydrolyzable ATP analog AMP-PNP (lane 4) and EDTA (lane 5; final concentration 10 mM).

Transformation of the Ecdysone Receptor to the DNA Binding State Requires ATP and Mg^{2+}

A good candidate for the ecdysone receptor activating factor is the MCH implicated in the conversion of PR to the aporeceptor state (reviewed by Pratt and Toft, 1997). Given that the MCH requires ATP and Mg^{2+} for this activity, we asked whether ATP and Mg^{2+} are required for activation of the ecdysone receptor to the DNA binding state. Figure 1G shows that a >10-fold increase in EcRE DNA binding is observed when an ATP regenerating system is added to the RT (compare lanes 1 and 2). Conversely, if an ATP depleting system is added to RT, a decrease in EcRE DNA binding activity is observed (Figure 1G, compare lanes 1 and 3). Furthermore, if the nonhydrolyzable ATP analog, AMP-PNP, is added to the RT in the presence of an ATP regenerating system, the amount of EcRE DNA binding is much less than with just an ATP regenerating system (Figure 1G, compare lanes 2 and 4). Evidently, ATP hydrolysis is required for the enhancement that is observed with the ATP regenerating system. If EDTA is added to RT, a >10-fold decrease in reconstitution activity is observed (Figure 1G, compare lanes 1 and 5), indicating that Mg^{2+}—the only divalent cation in the buffer system—is required for the reconstitution. Taken together, these results demonstrate that both ATP and Mg^{2+} are involved in the reconstitution of the ecdysone receptor to the EcRE DNA binding state, and suggest that the MCH may be required for transformation of the ecdysone receptor to the DNA binding state. Since transformation of the ecdysone receptor to the DNA binding state does not appear to require ecdysone, it apparently occurs by a different mechanism from that which transforms PR to the DNA binding state (Johnson and Toft, 1994).

Ecdysteroid Binding Does Not Require Auxiliary Factors

To address the question of whether auxiliary factors are also required for ligand binding, the hormone binding activity of purified gEcR and purified USP was tested using a ^{3}H-labeled high-affinity ecdysone analog, ponasterone A (PonA). Consistent with previous observations, neither purified gEcR alone, nor purified USP alone contained detectable ecdysteroid binding activity (Figure 2A). However, in contrast to EcRE DNA binding activity, a mixture of purified gEcR and purified USP has significant ecdysteroid binding activity, resulting in signals >200-fold above the signals observed with either purified gEcR or purified USP alone (Figure 2A)—demonstrating that additional factors are not required for hormone binding activity. Not surprisingly, when the receptor is purified as the EcR/gUSP heterodimeric complex that has DNA binding activity, hormone binding activity is also observed (Figure 2B) with signals >70-fold above background. Taken together, these results demonstrate that EcR and USP are sufficient for ecdysone binding activity, independent of their ability to bind DNA. Interestingly, an approximate 2.3-fold enhancement of hormone binding activity is observed if RT, supplemented with an ATP regenerating system (RT-ATP), is added to purified gEcR and USP mixture; neither RT-ATP, nor RT-ATP mixed with either gEcR or USP has significant ecdysteroid binding activity (Figure 2A). One explanation for this result is that the addition of RT-ATP causes a change in the conformation of the hormone binding cavity, resulting in a receptor with higher affinity for PonA. Alternatively, some fraction of the purified receptor may be inactive for hormone binding and the addition of RT-ATP could then activate this fraction.

To distinguish between these possibilities, the dissociation constant (K_d) for PonA when purified gEcR is mixed with purified USP was determined, in both the presence and absence of RT-ATP. To determine the K_d, each mixture was assayed over a range of PonA concentrations and the data were fit to a standard binding equation. Mixing purified gEcR with USP produced PonA binding activities with a K_d of 2.6 $\pm$ 0.6 nM, which were not changed significantly by the addition of RT (K_d of 2.5 $\pm$ 0.5 nM, Figure 2C). These values are similar to the 3–6 nM K_d previously measured for PonA binding activities using crude *Drosophila* extracts (Maroy et al., 1978; Yund et al., 1978) and mixtures of EcR and USP overexpressing yeast extracts (Arbeitman, 1998). Given that RT-ATP had no effect on PonA affinity, it would appear that the increase in PonA binding resulting from the addition of RT-ATP is due to activation of inactive receptor by the RT-ATP. Since these experiments were performed under conditions similar to those that activate the receptor to the DNA binding state, activation to the DNA binding state does not appear to alter the affinity of the receptor for ecdysteroids.

Purified Components of the MCH Transform the Ecdysone Receptor to the DNA Binding State

The obvious next question is whether purified components of the MCH can substitute for RT in transforming a mixture of purified gEcR and USP to the EcRE DNA binding state. The components of the MCH that were tested are the *Drosophila* Hsp83 (corresponding to Hsp90) and Hsc70 proteins and the human Hop, Hip, FKBP52, and p23 proteins for which the corresponding *Drosophila* genes had not been cloned. The proteins were purified (see Experimental Procedures), fractionated by SDS-PAGE and visualized by Coomassie stain. Only one major band is observed for each protein, indicating a high level of purity (Figures 3A and 3B).

Figure 3C shows that a mixture of purified Hsp90, Hsc70, Hop, Hip, FKBP52, and p23 is sufficient to reconstitute purified gEcR and purified USP to the DNA binding state (Figure 3C, lane 5). This reconstitution is analogous to that seen with RT in that it requires both an ATP regenerating system (Figure 3C, lane 6) and Mg^{2+} (Figure 3C, lane 7). No DNA binding is observed in the absence of gEcR and USP, demonstrating that the MCH proteins have no intrinsic EcRE DNA binding activity (Figure 3C, lane 8). Furthermore, both gEcR and USP are present in the shifted complex because antibodies directed against EcR (Figure 3C, lane 9) and USP (Figure 3C, lane 10) supershift the complex. When the ATP regenerating system is the only component added to the mixture of purified gEcR and USP, a very small amount of reconstitution of DNA binding is observed (Figure 3C, lane 3), perhaps due to contaminants either in the EcR or USP preparations, or in the ATP regenerating system. We cannot therefore rule out a minor contribution of proteins other than Hsp90, Hsc70, Hop, Hip, FKBP52, and p23 to this reconstitution.

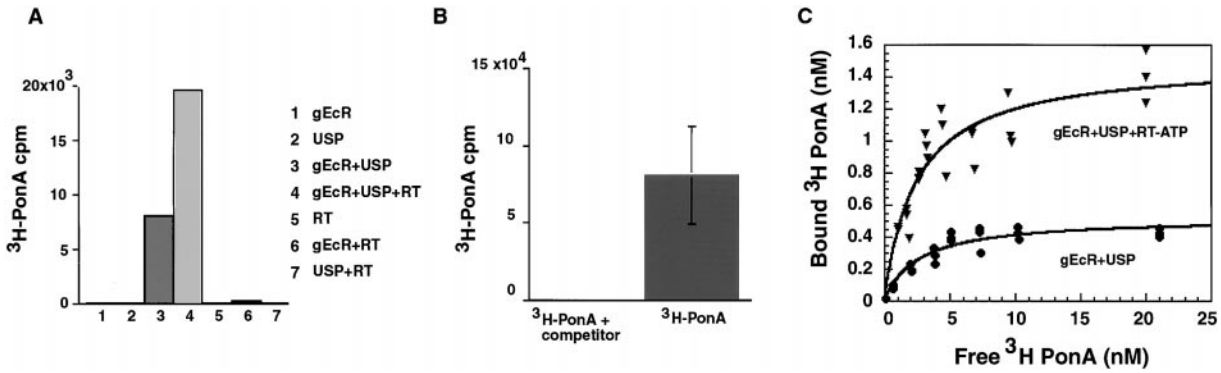

Figure 2. Ecdysteroid Binding Activity of gEcR, USP, and an EcR/gUSP Heterocomplex

(A) gEcR (column 1, SD = 36), USP (column 2, SD = 10), a mixture of gEcR and USP (column 3, SD = 177), a mixture of gEcR, USP and RT-ATP (column 4, SD = 342), RT-ATP (column 5, SD = 14), a mixture of gEcR and RT-ATP (column 6, SD = 7) and a mixture of USP and RT-ATP (column 7, SD = 32) were assayed for ecdysteroid binding. SD is standard deviation. (B) The GST-USP/EcR heterodimeric complex (50 μl of 0.12mg/ml purified heterocomplex) was assayed for ecdysteroid binding activity in the presence or absence of cold competitor. In both (A) and (B) the average of three duplicate assays are shown and ^{3}H PonA is at a final concentration of 6 nM. (C) Ecdysteroid binding activity of purified receptor components with and without RT-ATP. Ecdysteroid binding activity of a mixture of purified EcR and USP without added RT-ATP (K_d = 2.65+.6 nM) and with RT-ATP (K_d = 2.52+.5 nM). K_ds were derived using the Kaleidagraph software and the binding equation [LR]/[L][R] = K_d, where [LR] = concentration of ligand receptor complex, [L] = concentration of free ligand, and [R] = concentration of free receptor. The error of the K_ds is the error of the fit of the curve.

Figure 3D shows results of experiments that address the question of whether each of six MCH proteins is required for reconstitution of the ecdysone receptor. Removal of any one of these components decreases but does not eliminate the enhancement of DNA binding activity, with Hip, Hop, and FKBP52 having the smallest effect (Figure 3D). It therefore appears that these factors have partially redundant functions, which is consistent with the observation that some have been shown to have chaperone activity for protein refolding in the absence of any additional factors (Bose et al., 1996; Freeman et al., 1996). Each of the six MCH factors has also been tested individually and found to have little or no effect on the binding activity (data not shown). Other combinations of the six factors have not been examined.

Geldanamycin, an Hsp90-Specific Inhibitor, Prevents Activation of EcR/USP to the DNA Binding State

Geldanamycin is a benzoquinoid antibiotic that acts as a highly specific and potent inhibitor of Hsp90 activity by binding to a highly conserved ATP-binding pocket in Hsp90 (Prodromou et al., 1997; Stebbins et al., 1997). To test whether Hsp90 is critical to the DNA binding activity of the EcR/USP heterodimer in a more natural condition, EcR and USP were separately overexpressed in Sf9 cells and extracts thereof were treated with geldanamycin for 1 hr at 25°C, or as a control, with DMSO in which the geldanamycin was dissolved. Similarly treated extracts of EcR and USP were then mixed and tested for EcRE DNA binding activity, with results shown in Figure 4A (lanes 5 and 6) along with untreated controls (lanes 2–4). The almost complete inhibition of DNA binding activity by geldanamycin (58-fold by phosophorimager analysis) indicated that Hsp90 is required for DNA binding activity in these extracts.

EcR, Not USP, Is the Target of the MCH

Figure 4B shows the results obtained when the extract from the EcR-overexpressing Sf9 cells used in the preceding section, or that from USP-overexpressing cells, was preincubated with geldanamycin prior to mixing with untreated USP- or EcR-containing extracts, respectively. Strong inhibition of EcRE DNA binding is observed when EcR is pretreated with geldanamycin (lane 5), but not when USP is pretreated (lane 6). This result indicates that Hsp90 acts via association with EcR and not USP. Furthermore, another component of the MCH, Hsc70, appears to be preassociated with EcR rather than USP as Hsc70 does not copurify with USP under conditions in which it copurifies with EcR (Arbeitman, 1998).

To test further the proposition that EcR is the primary target of the MCH, we examined whether preincubation of purified gEcR with RT-ATP and subsequent addition of purified USP, results in an earlier appearance of EcRE DNA binding activity than the reciprocal experiment in which the USP is preincubated with the RT-ATP and the gEcR added subsequently.

Figure 4C shows that when gEcR was preincubated with RT-ATP for 30 min at 25°C and the USP then added, EcRE DNA binding activity was detected within 5 min (lane 3) and full binding was observed by 30 min (compare lanes 6 and 2). By contrast, when USP and RT-ATP were preincubated for 30 min and EcR then added, little DNA binding was observed until 30 min, when the amount of DNA binding approximated that bound 15 min after USP was added to the preincubated gEcR-RT-ATP mixture (as estimated by phosphorimager analysis). These results confirm that an interaction of the MCH with EcR is an early step toward activation to the DNA binding state, and also indicate that this interaction can occur before EcR and USP associate, as we have emphasized in the model given in the discussion.

Ecdysone Receptor Activity In Vivo Required Both Hsp90 and Hsc70

To test whether Hsp90 is required for ecdysone receptor activity in vivo, the effect of geldanamycin on ecdysone-induced gene activity in *Drosophila* cell culture was examined. A cell line that contains endogenous ecdysone

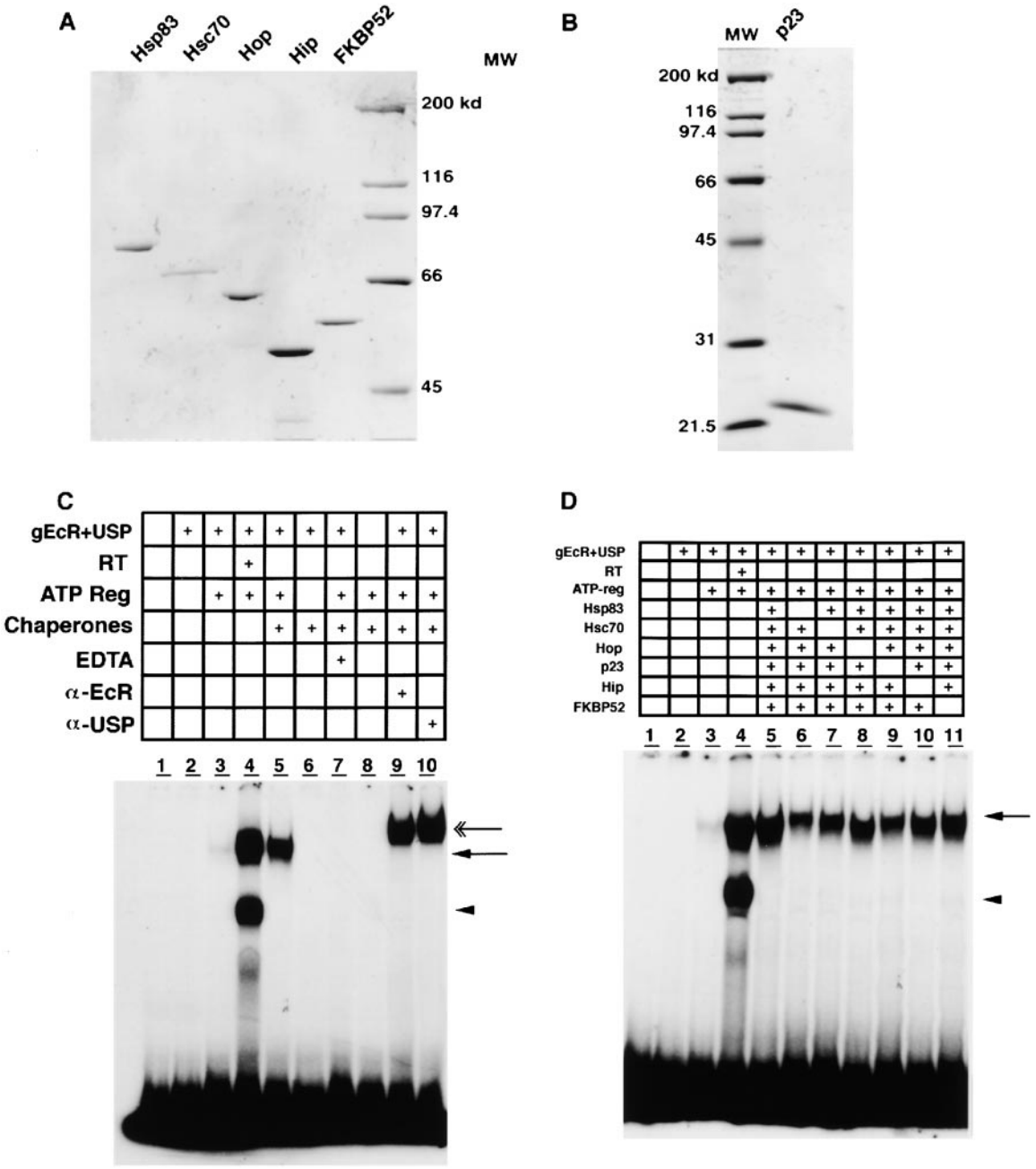

Figure 3. Purification of MCH Components and Reconstitution of DNA Binding Activity using Purified MCH Components

Purified Hsp83, Hsc70, Hop, Hip, FKBP52 (A), and p23 (B) were analyzed on a 7% and 10% SDS-PAGE gel, respectively, and the proteins were visualized with Coomassie stain. Marker proteins and their molecular weights in kilodaltons are indicated (MW). (C) A mixture of EcR and USP (indicated as "gEcR+USP" in the matrix; 0.1 μM EcR, 0.2 μM USP) were tested for EcRE DNA binding using a gel mobility shift assay. Reactions that included reticulocyte lysate (RT), an ATP regenerating system (ATP-reg; supplemented to a final concentration of 2.5 mM ATP), components of the MCH (0.4 μM, Hsp90; 0.01 μM, Hsc70; 0.1 μM, Hop; 0.4 μM, Hip; 0.1 μM, FKBP52; and 0.2 μM, p23; indicated as chaperones in the matrix), EDTA (final concentration 10 mM), an anti-EcR antibody (α-EcR; AG10.2; 6 μl), or an anti-USP antibody (α-USP; AB11, 12 μl) are indicated. (D) Maximal DNA binding activity is observed when Hsp90, Hsc70, Hop, Hip, FKBP52, and p23 are all mixed with purified gEcR and USP. Reactions that include a reticulocyte lysate (RT), an ATP regenerating system (ATP-reg), and MCH components are indicated in the matrix. In (C) and (D) the arrow marks the DNA/protein complexes that contain gEcR and USP, the arrowhead marks the DNA/protein complexes present with RT alone, and the double arrow marks the DNA/protein complexes with slower mobility that are present after the addition of the EcR and USP antibodies.

receptor activity and a reporter construct in which transcription of the *E. coli lacZ* reporter gene is under control of multiple EcREs (Koelle et al., 1991) were treated with 0.1 μg/ml or 1.0 μg/ml of geldanamycin for 5 hr. Ecdysone was then added to these two experimental cell cultures as well as to two control cultures, one of which contained the same amount of the DMSO solvent as the two geldanamycin experimental cultures, the other

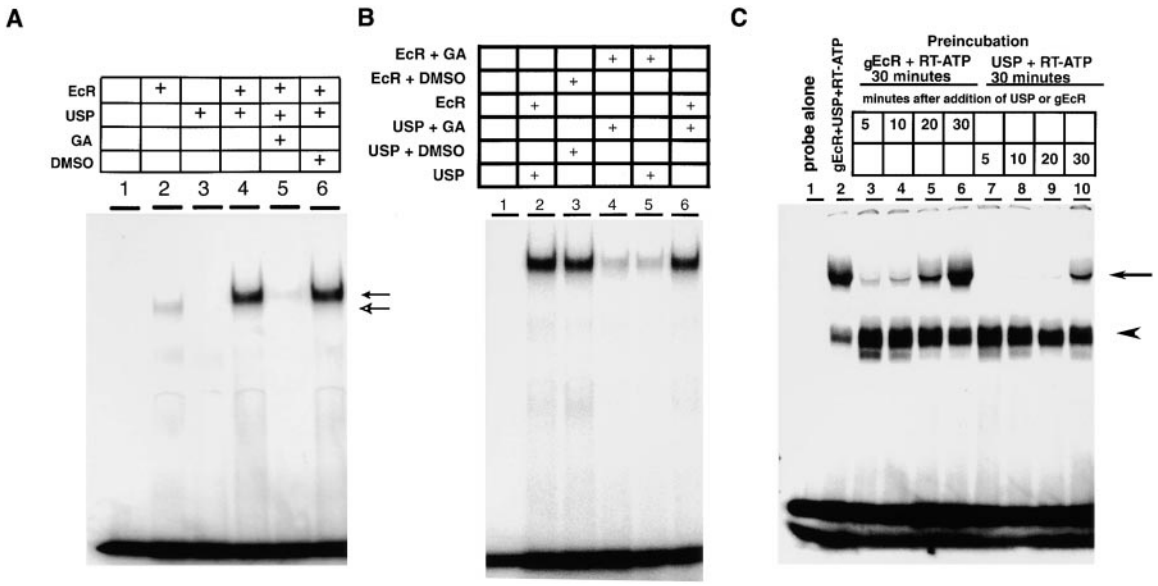

Figure 4. Components of the MCH Are Preassociated with EcR and Not USP

Inhibition of EcRE DNA binding activity by geldanamycin is through EcR- and not USP-containing extracts. (A) and (B) EcR and/or USP containing extracts were pretreated with geldanamycin (GA), or DMSO for 1 hr, mixed, and then tested for EcRE DNA binding activity. The open arrow marks the DNA/protein complex that contains *Drosophila* EcR and USP from Sf9 cells. The closed arrow marks the DNA/protein complexes that contain *Drosophila* EcR and *Drosophila* USP. (C) Preincubation of gEcR with RT-ATP results in an earlier appearance of EcRE DNA binding activity than when USP is pretreated. Purified gEcR (lanes 3–6; 0.05 μM) or USP (lanes 7–10; 0.1 μM) were pretreated with RT-ATP for 30 min. Then the purified USP was added to the gEcR/RT-ATP and purified gEcR was added to the USP/RT-ATP and the reactions proceeded for the indicated amounts of time. Lane 1 shows free probe. Lane 2 shows a mixture of EcR, USP, and RT-ATP that was mixed immediately and then incubated for 60 min. The arrow marks the DNA/protein complexes that contain gEcR and USP and the arrowhead marks the DNA/protein complexes present with the RT-ATP.

experiencing no other additions. These cultures were then incubated for an additional 12 hr before being assayed for β-*galactosidase* activity, with results shown in Figure 5.

The two controls exhibited essentially the same activity, while the cultures treated with 0.1 and 1.0 μg geldanamycin/ml exhibited 71% and 21% of that activity, respectively, each experiment having been repeated three

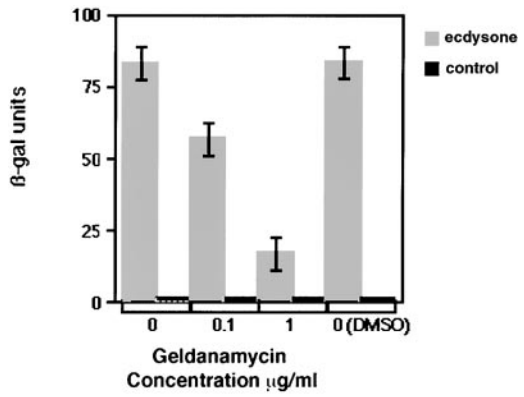

Figure 5. Inhibition of Ecdysone-Induced Gene Expression by Geldanamycin

The CMK7 cell line, which contains an ecdysone inducible *lacZ* reporter gene, was pretreated with either geldanamycin or DMSO for 5 hr and then ecdysone or ethanol (the solvent for ecdysone, labeled as control) was added to the cells. The cells were incubated for an additional 12 hr and then harvested. Extracts made from these cells were assayed for β-galactosidase activity.

times. This inhibition is not due to an overall toxicity of geldanamycin, since the expression of a constitutive reporter gene was not similarly effected by the addition of geldanamycin (data not shown). These results indicate that Hsp90 functions in the conformational maturation of the ecdysone receptor in vivo.

Our evidence that Hsc70 is involved in the activation of the ecdysone receptor in vivo derives from genetic interactions we have observed between mutations in the *Drosophila EcR* gene and those in the *hsc4* gene, which encodes a *Drosophila* cytoplasmic Hsc70 protein that is widely expressed throughout development (Palter et al., 1986). We first note that animals that are transheterozygous for *EcR* and *usp* mutations exhibit adult deformities characterized by blistered wings and malformed legs (M. Bender and D. S. H., unpublished experiments)—phenotypes similar to those exhibited by animals with mutations in other known components of the ecdysone regulatory heirarchy (Kiss et al., 1988; Tsai et al., 1999). By contrast, animals singly heterozygous for either *EcR* or *usp* are wild type (Perrimon et al., 1985; Henrich et al., 1994; Bender et al., 1997).

Since the *EcR/usp* transheterozygous phenotypes are presumably due to a reduction in receptor activity, we reasoned that if Hsc70 is involved in activating the ecdysone receptor in vivo, then animals that are transheterozygous for *hsc4* and *EcR* might display phenotypes similar to those observed in *EcR/usp* transheterozygotes. Genetic interactions were observed in animals transheterozygous for $hsc4^{195}$, a dominant-negative *hsc4* allele (Hing et al., 1999), and each of three different *EcR* alleles

Table 1. Genetic Interaction between Components of the Ecdysone Receptor and *hsc4*

Genotype	Blistered Wings		Malformed Legs	
	n	%	n	%
EcR^{C284Y}/+;$hsc4^{195}$/+	79	48	61	3
EcR^{C300Y}/+;$hsc4^{195}$/+	186	40	94	9
EcR^{D491N}/+;$hsc4^{195}$/+	68	38	101	21
EcR^{C300Y}/+;P[hsc4];$hsc4^{195}$/+	27	0	27	0
EcR^{C284Y}/+;P[hsc4];$hsc4^{195}$/+	45	0	45	0
EcR^{D491N}/+;P[hsc4];$hsc4^{195}$/+	25	0	25	0
cn bw/+;$hsc4^{195}$/+	145	1	145	0

n is the number of transheterozygous animals scored for the phenotype. The percent of animals in the transheterozygous class with the phenotype is given adjacent to n. *cn bw* is the parental chromosome for the *EcR* mutant alleles. *P[hsc4]* is a P element transgene that contains a segment of genomic DNA that includes the wild-type *hsc4* gene and its regulatory elements (Hing et al., 1999).

(Table 1), all of which are null for *EcR* activity (Bender et al., 1997). Figure 6A shows the blistered wing phenotype exhibited by 43% ± 5% of each of three EcR/+; $hsc4^{195}$/+ transheterozygotes (Table 1), which also exhibited a higher frequency of the notched wing phenotype seen in the $hsc4^{195}$ single heterozygote. Figure 6C shows the malformed leg phenotype (compare to the wild-type leg in Figure 6B) that was also seen in all three transheterozygotes with frequencies varying from 3% to 21% depending on the *EcR* mutation (Table 1). Importantly, a P element transgene containing a segment of genomic DNA that includes the wild-type *hsc4* gene and its regulatory elements suppressed the phenotypes observed in the *EcR*/$hsc4^{195}$ transheterozygotes (Table 1). By contrast, animals transheterozygous for *hsc4* and *usp* mutations do not display the blistered wing and malformed leg phenotypes (Arbeitman, 1998), an observation consistent with our observation that MCH components interact with EcR rather than with USP proteins.

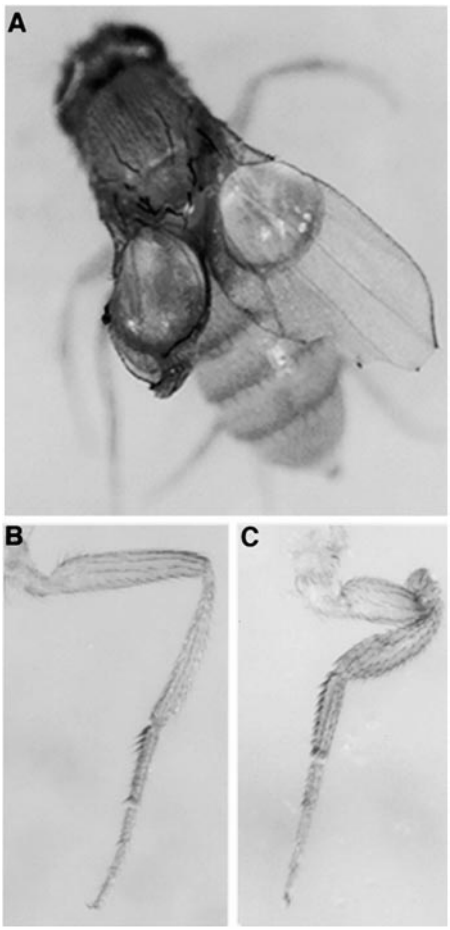

Figure 6. *EcR* and *hsc4* Transheterozygotes Display Blistered Wings and Malformed Legs

(A) Animal with blistered wings (*EcR^{C284Y}/+; $hsc4^{195}$/+*).
(B) Wild-type leg (Canton-S).
(C) Malformed leg (*EcR^{D491N}/+; $hsc4^{195}$/+*).

Discussion

Prior reports about the nature and function of the heterodimeric ecdysone receptor in *Drosophila* were based on experiments carried out in vivo or with crude extracts containing the EcR and USP components of this receptor (Koelle, 1992; Yao et al., 1992, 1993). These experiments led to the postulate that the active receptor is an EcR/USP heterodimer formed directly from the two nuclear receptors without prior activation or stabilization by chaperones. Lack of prior activation was assumed not only because there was no evidential need to think otherwise, but also because EcR/USP belongs to the class of ligand-activated RXR heterodimers, which in vertebrates did not appear to require additional proteins for activation.

By contrast the homodimeric vertebrate steroid receptors were found in association with a complex set of chaperones, which included Hsp90 and Hsp70, and which appear to maintain these receptors in a state activatable for DNA binding by their ligands (reviewed favorably by Pratt and Toft, 1997; and critically by Ylikomi et al., 1998). It was the apparent lack of association of the retinoid, thyroid and vitamin D receptors with such a set of chaperones, particularly with Hsp90, that provided the major argument that chaperones were not required for the maintenance and/or activation of the RXR heterodimers (Dalman et al., 1990, 1991). Our work contradicts the generality of such an argument, as do the recent findings that Hsp90 is required for efficient activation of the vertebrate retinoic acid receptor in yeast (Holley and Yamamoto, 1995), and that the vitamin D receptor contains specific binding sites for Hsp70 (Craig et al., 1999). In addition, by the time the work reported here began, observations by Koelle (1992) in this laboratory indicated that the *Drosophila* Hsc70 chaperone was bound to EcR during its purification by immunoaffinity chromatography.

Multiple States of the EcR/USP Heterodimer

Our results show that the generation of a fully functional ecdysone receptor is a multistep process. The ability to bind ligand is not inherent to EcR or USP alone but arises by an interaction between these two proteins that does not require accessory factors (Figure 2). Such a

naive heterodimer formed from purified receptors is different from the purified steroid receptors for progesterone (PR) or glucocorticoid (GR), which can not bind ligand or DNA prior to chaperone activation (reviewed by Pratt and Toft, 1997). This naive heterodimer was transformed to the fully functional EcR/USP heterodimer (defined here by its ability to bind both ligand and EcRE DNA) by exposure to six purified chaperones and an ATP regenerating system (Figure 3). This is an artificial system in that four of the six purified chaperones (Hip, Hop, FKBP52, and p23) are from humans rather than *Drosophila*. It is also redundant in that removal of any one of the six decreases the yield but does not eliminate activation. The important point at this stage is not what combination of these chaperones is most efficient for the in vitro transformation of the naive heterodimer to the fully active state; rather, it is that such a transformation requires only chaperones and an ATP regenerating system. Our data do indicate the two *Drosophila* chaperones Hsp90 and Hsc70 are required for activation of the EcR/USP heterodimer in vivo (Figures 5 and 6, and Table 1).

Two additional characteristics of the EcR/USP activation reaction deserve emphasis. One is that activation to the fully functional state can occur in the absence of EcRE DNA since we have produced and purified such a fully functional heterodimer under this condition (Figures 1D and 1F). The second is the finding that EcR is the target of the MCH (Figures 4B and 4C)—a finding that is considered in the following last section of this Discussion.

A Minimum Model for the Activation of EcR/USP Heterodimer to the DNA Binding State

Given that almost all the data presented in this paper concerns events that occur after the synthesis of all the polypeptides involved in the formation of the fully functional ecdysone receptor, the following model is limited to events after that completion. We assume that the USP polypeptide folds appropriately into a relatively stable configuration that is not further stabilized by chaperones. By contrast, the EcR polypeptide folds into an unstable configuration easily subject to irreversible unfolding or protease degradation. This postulated difference in the behavior of the two polypeptides derives in large part from our observation that overexpression of each in Sf9 or *E. coli* cells yields much more USP than EcR. Similar observations have been reported by Li et al. (1997), for RAR and RXR in *E. coli*, where RAR is the unstable entity.

Our assumption is that the unstable EcR interacts with appropriate *Drosophila* chaperones including Hsp90 and Hsc70, which stabilize EcR in a configuration appropriate for formation of EcR/USP heterodimers capable of binding EcRE DNA sequences. We postulate that formation of the active heterodimer is accompanied by dissociation of the chaperones from EcR, accounting for our isolation of an EcRE DNA-binding form of the EcR/USP heterodimer. USP binding is postulated to stabilize EcR in a DNA binding configuration, which is enhanced by chromosomal EcRE binding, and can be further enhanced by binding of EcR to other chromatin binding proteins.

Experimental Procedures

Expression Constructs

Coding sequences for *EcR-B1* (Koelle et al., 1991), *usp* (Oro et al., 1990), *hsc4* (Palter et al., 1986), and *hsp83* (Ding et al., 1993) were each inserted into the baculovirus expression vector pAcGHLT-A and recombinant baculoviruses were generated following the manufacturer's protocol (PharMingen). Recombinant baculoviruses were made encoding GST-EcR (bv60), GST-USP (bv59), and GST-83 (bv80). Bacterial expression plasmids Hip/pET28a, FKBP52/pET, and Hop/pET28a were kindly provided by Dr. David Smith. The bacterial expression plasmid pNT2 (p23) was kindly provided by Dr. David Toft.

Cell Culture and Maintenance

Spodoptera frugiperda (Sf9) cells were grown at 25°C as monolayer cultures in supplemented TMN-FH media (Sigma; supplemented with penicillin 50 units/ml, streptomycin 0.05 mg/ml; and 10% fetal bovine serum, GIBCO-BRL).

Purification of Recombinant Proteins

Sf9 cells were infected with high titer recombinant baculovirus stock, and were incubated at 27°C for 3 days. Five 150 cm^2 plates, seeded with 2×10^7 cells, were harvested by low-speed centrifugation. The cell pellet was then resuspended in 5 ml ice-cold Buffer A (100 mM KCl, 50 mM HEPES-KOH, pH 7.0, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 2 μg/ml leupeptin, and 2 μg/ml pepstatin). All subsequent steps were performed at 4°C. The cells were sonicated and the lysate cleared by high-speed centrifugation. The supernatant was applied to a column containing 0.25 ml glutathione-sepharose 4B (Pharmacia, equilibrated in Buffer A) for 1 hr with gentle rocking. The column was eluted and washed with 100 ml Buffer B (500 mM KCl, 50 mM HEPES-KOH, pH 7.0, 1 mM $MgCl_2$). GST-EcR was eluted from the column by the addition of 0.4 ml glutathione elution buffer (10 mM glutathione, 50 mM HEPES-KOH, pH 8.0, 100 mM KCl, 50% glycerol) and the eluate was dialyzed for 4 hr in Buffer C (100 mM KCl, 50 mM HEPES-KOH, pH 7.0, 10% glycerol, 5 mM $MgCl_2$). USP, Hsc70, and Hsp83 were eluted from the glutathione-Sepharose 4B column by the addition of 0.5 ml thrombin cleavage buffer (100 mM KCl, 50 mM HEPES-KOH, pH 7.0, 10 NIH units thrombin) incubated for 2 hr at 4°C. Then the eluate was applied to a 0.4 ml benzamidine sepharose 6B (Pharmacia) column equilabrated in Buffer C and dialyzed for 4 hr in Buffer C. A final concentration of 1 mM dithiothreitol (DTT) was added to each dialyzed eluate and the samples were snap frozen and stored at −80°C.

The GST-USP/EcR heterodimer was purified as follows. Sonicates of cells infected with baculovirus encoding GST-USP or EcR were prepared as above. The supernatant containing GST-USP was applied to a column containing 0.5 ml glutathione-sepharose 4B equilibrated in Buffer A. The EcR-expressor supernatant was then applied to the column. The column was washed with three column volumes of buffer (200 mM KCl, 25 mM HEPES-KOH, pH 7.0, 1 mM $MgCl_2$), and then one column volume of Buffer A (supplemented to 1 mM $MgCl_2$). The heterodimer was eluted by the addition of glutathione elution buffer (no glycerol) and dialyzed into Buffer C (supplemented with 1 mM $MgCl_2$).

Hop-6H, 6H-Hip, 6H-FKBP52, and p23 expression plasmids were transformed into the *E. coli* strain BL21. Recombinant proteins were induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG; 0.4 mM) for 2 hr at 37°C. The following procedures were performed at 4°C. The cells from a 200 ml saturated culture were harvested by centrifugation. For Hop-6H, 6H-Hip, and 6H-FKBP52, the cells were resuspended in 8 ml ice-cold binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The cells were sonicated and then cleared by high-speed centrifugation. The supernatant was applied to a column containing 2.5 ml charged Ni-NTA resin (Novagen) that was equilibrated in binding buffer. The column was washed with 10 column volumes of binding buffer and then 6 column volumes of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The protein was eluted from the column by the addition of 6 column volumes elute buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The purified sample was dialyzed into Buffer C for 4 hr, aliquoted, and snap frozen.

For p23, the cells were resuspended in 15 ml ice-cold buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM thioglycerol, 0.5 mM PMSF, 2 μg/ml leupeptin, and 2 μg/ml pepstatin), sonicated, and then the lysate was cleared by centrifugation. The supernatant was applied to a 10 ml DEAE-sepharose column equilibrated in Buffer D (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The column was washed with 10 column volumes of Buffer D. A 100 ml gradient (0 M KCl–0.5 M KCl supplemented Buffer D) was applied to the column, and 110 drop fractions were collected. p23 eluted in fraction 21, which was further fractionated in a centricon-50 column (Amicon). The filtrate was dialyzed into Buffer C.

Gel Mobility Shift Assays

Each gel shift reaction contained 2 μg polydeoxyinosinic-deoxycytidylic acid (poly dI-dC), ~0.03 fmol of α-^{32}P-labeled oligonucleotide probe DNA, and the indicated amount of extract. After a 1 hr incubation at 25°C, reactions were electrophoresed as described by Koelle (1991). The oligonucleotide probe was prepared by annealing the following oligonucleotides: 5′GTGTGAGACAAGGGTTCAATGCAC TTGTCCAATG3′ and 5′GTGTCATTGGACAAGTGCATTGAACCCT TGTCTC3′, containing the *hsp27* EcRE, followed by extension with Klenow in the presence of [α-^{32}P]dATP. For antibody supershift experiments, the reactions contained tissue culture supernatant of the monoclonal antibodies anti-EcR AG10.2 (Talbot et al., 1993) or anti-USP AB11 (Christianson et al., 1992). The untreated reticulocyte lysate (Promega) was dialyzed into Buffer C for 4 hr at 4°C, aliquoted, and stored at −80°C. Reactions that included an ATP regenerating system were supplemented with 5 mM phosphocreatine, 1.2 mM dithiothreitol, and 0.12 mg/ml creatine phosphokinase. Reactions that contain an ATP depleting system were supplemented with 1 μl of 500 units/ml glycerol kinase. Reactions that included AMP-PNP were supplemented to a final concentration of 3 mM AMP-PNP. Reactions were brought to a final volume of 30 μl unless otherwise indicated by the addition of Buffer C. Quantitation was performed with a phosphorimager (Molecular Dynamics).

Tissue Culture Experiments

The CMK7 cell line was cultured as described in Koelle (1991). Five milliliters of CMK7 cells was plated in 60 mM dishes at a density of 0.8×10^6 cells/ml, and incubated at 25°C overnight. Five microliters of 0.1 mg/ml, or 1 mg/ml geldanamycin, or 5 μl DMSO was added to the cells and incubated for 5 hr at 25°C. Then 1 μl of 10 mg/ml ecdysone (dissolved in ethanol, Sigma) or ethanol was added to the cells. The cells were harvested 12 hr later. Extracts were prepared and β-gal assays were as described in Koelle (1991). β-gal activities are expressed as [OD units/(μg of protein) (minutes of reaction)] multiplied by 10,000.

Ecdysteroid Binding Assays

Ecdysteroid binding activity was determined using the high-affinity ecdysone agonist ^{3}H Ponasterone A (PonA) (170 Ci/mMol, Dupont NEN). The cold competitor used was the high-affinity ecdysone agonist muristerone (Sigma). In Figures 2A and 2C, gEcR and USP are at a final concentration of 0.2 μM and each reaction contains 30 μl of RT-ATP (20 μl reticulocyte lysate, supplemented with 10 μl ATP regenerating system) or 30 μl Buffer C. Mixtures of purified EcR and purified USP or mixtures of purified EcR, purified USP and RT-ATP (80 μl total volume) were incubated for 1 hr at 4°C before the addition of hormone. Then the protein mixtures were added to hormone and incubated for 2 hr at 25°C. In Figure 2C, each reaction contains 10 μl of 10× ^{3}H PonA and contains either 10 μl 5×10^{-4} M muristerone (cold competitor) or 10 μl Buffer C. After the incubation, reactions were spotted on a dry GF/C Whatman filter, and after 30 s the filters were washed by using a vacuum to draw 50 ml of buffer (50 mM HEPES-KOH, pH 7.0, 100 mM KCl) through the filter. The filter was dried, and placed in 2 ml scintillation fluid (Econosafe), and quantified. In Figures 2A and 2C the hormone binding activities shown are specific binding, calculated as the total binding activity measured in assays with no cold competitor, minus the binding activities measured in the assays with unlabeled cold competitor. The dissociation constant was calculated by fitting the specific binding to a standard equilibrium binding equation using Kaleidagraph software (Abelbeck Software).

Geldanamycin Pretreatments

Extracts were made from Sf9 cells infected with bv54 (EcR) or bv55 (USP) (White et al., 1997) and were diluted to 1 mg/ml in buffer (150 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, 50 mM HEPES-KOH, pH 7.4, 1 mM dithiothreitol, .5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). One microliter of 17 mM geldanamycin (dissolved in DMSO, GIBCO) or 1 μl of DMSO was added to 50 μl of extract and incubated at 25°C for 1 hr. Gel shift reactions were performed as described above. Each reaction in Figure 4 contains 5 μl of EcR and/or 5 μl USP containing extracts. Reactions were brought to a final volume of 50 μl with Buffer C, and were maintained at a final concentration of 0.34 mM geldanamycin (2% DMSO), or 2% DMSO.

***Drosophila* Stocks**

All animals were maintained at 25°C on standard medium. The *EcR* mutant stocks *EcR*^{C300Y}, *EcR*^{C284Y} and *EcR*^{D491N} are described in Bender (1997). The *st Ki p hsc4*^{195}*/TM3* and *hsc4* transgenic line *P [hsc4]* (5–22 line) are described in Hing et al. (1999).

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