Evidence That the Co-chaperone p23 Regulates Ligand Responsiveness of the Dioxin (Aryl Hydrocarbon) Receptor*

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The dioxin (aryl hydrocarbon) receptor is a ligand-dependent transcription factor that induces expression of a number of genes encoding drug metabolizing enzymes. In the absence of ligand the dioxin receptor is present in the cytoplasmic compartment of the cell associated with the molecular chaperone hsp90, which has been implicated in regulating the correct folding of the ligand binding domain of the receptor. In this study we have examined a potential role of the hsp90-associated p23 protein in the activation process of the dioxin receptor to a DNA binding form. In an in vitro model we show that addition of ligand alone to the dioxin receptor fails to induce release of hsp90 from the dioxin receptor. In the presence of ligand, this release was, however, induced upon addition of purified preparations of Arnt. Interestingly, p23 was also found to be associated with the non-activated form of the dioxin receptor. Following fractionation on sucrose gradients p23 was dissociated from the receptor-hsp90 complex generating a receptor form, which showed ligand-independent release of hsp90 by Arnt and, consequently, ligand-independent activation of the DNA binding activity of the dioxin receptor. Ligand dependence was reconstituted in the presence of molybdate, a transition metal ion known to stabilize the interaction between the molecular chaperone hsp90 and p23. Taken together these experiments suggest a role of p23 in modulating ligand responsiveness in the activation process of the dioxin receptor.

In the absence of ligand the dioxin receptor is present in a latent conformation in the cytoplasmic compartment of the cell (11) associated with the molecular chaperone hsp90 (12). Hsp90 is required both for maintaining the dioxin receptor in a latent non-DNA binding state and a ligand binding conformation (13). Expression of the dioxin receptor in mutant yeast cells containing reduced levels of hsp90 abolishes ligand responsiveness demonstrating the critical importance of hsp90 for dioxin receptor function (14, 15). The nuclear form of the dioxin receptor interacts with Arnt (16–18) and does no longer posses the ability to bind ligand and does not interact with the molecular chaperone hsp90 (12, 13). This form of the receptor specifically binds to enhancer elements known as XREs (xenobiotic response elements) of a number of genes encoding drug metabolizing enzymes (17, 18). Release of hsp90 from the latent form of the dioxin receptor is therefore a critical step in the activation process of the dioxin receptor.

Recently the hepatitis virus X protein-associated protein (XAP-2) also known as ARA 9 or AIP of 38 kDa has been shown to interact with the latent form of the dioxin receptor (19–21). This protein has been reported to increase the transcriptional activity of the dioxin receptor, although the mechanism of action has not been elucidated (22). The co-chaperone p23 (23) has been shown to be associated with the N-terminal ATP binding domain of hsp90 (24). p23 has also been found to be associated with non-activated form of selected members of the steroid receptor superfamily such as the glucocorticoid and progesterone receptors (25). Moreover, p23 has been reported recently to be associated with the dioxin receptor (26). The role of p23 in modulating target protein function is, however, not clear. Given the background that interaction with p23 correlates with high affinity ligand binding activity of certain steroid receptors (27), it seems plausible that p23 is involved regulation and stabilization of the ligand binding conformation of these receptors. In addition to hsp90 and p23, other protein factors are involved in the formation of the high affinity ligand binding steroid receptor form. In fact, formation of such a complex appears to involve p60 (28), also known as HOP (Histone organizing Protein), several immunophilins, and possibly factors that are found in association with proteins such as hsp70 (25).

We have observed previously that ligand-dependent release of hsp90 in vitro requires the interaction of the dioxin receptor with additional cellular factors, including Arnt (29). Interestingly, in the present study, fractionation of cellular extracts through sucrose density gradients yielded an hsp90-associated form of the dioxin receptor, which did not require ligand to generate the DNA binding complex with Arnt. This loss of ligand dependence correlated with dissociation of p23 from the dioxin receptor-hsp90 complex. It was possible to reconstitute ligand dependence in receptor activation by addition of molybdate, an agent that has been shown to stabilize the interaction between hsp90 and p23. Thus, these results indicate a role of...
Mechanism of Activation of the Dioxin Receptor

**Materials and Methods**

Recombinant Plasmids and Protein Expression—The construction of pGemArnt, pSF72 mDR, and pGEX 4T3 hArnt has been described previously (17, 30). In vitro translation of the dioxin receptor and Arnt was performed according to the manufacturer’s recommendations using coupled transcription/translation reactions in rabbit reticulocyte lysate (Promega Biotech). Bacterial expression of glutathione S-transferase-tagged Arnt has been described in detail elsewhere (30).

Cells and Preparation of Cellular Extracts—Hepa 1C1C7 mouse hepatoma cells and the mutant derivative C4 were grown in minimum essential medium as described previously (12). Cells were grown to near confluence at an atmosphere of 5% CO2 until harvested. Cytosolic extracts were prepared by scraping untreated cells and washing them twice in phosphate-buffered saline and once in TEG buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% (w/v) glycerol, and 1 mM dithiothreitol). After washing the pellet, the cells were homogenized in 1 volume of TEG buffer and centrifuged at 120,000 × g for 45 min. The resulting supernatant was taken as the cytosolic fraction and used either immediately or frozen in small aliquots at −70°C.

Fractionation of Cytosol by Sucrose Density Gradient Centrifugation—Cytosolic extracts (400 µl; about 2–10 mg of protein/ml of cytosol) or in vitro translated dioxin receptor was layered on 10–40% (w/v) linear sucrose gradients prepared in TEG buffer containing 25 mM NaCl and 10 mM sodium molybdate as indicated. The gradients were centrifuged at 300,000 × g for a cumulative centrifugal effect of 1.7 × 10^22 radian^2/s in a Beckman L8-80 ultracentrifuge. Fractions were collected by gravity flow starting from the bottom of the gradients. IgG (6.6 S) and bovine serum albumin (4.4 S) were used as external sedimentation marker proteins.

In Vitro DNA Binding Assay—The dioxin receptor was noncovalently labeled by incubation of crude or fractionated cytosol for 2.5 h at 30°C with 10 nM [3H]TCDD (Chemysyn, Lenexa, KS) or 10 nM radioactive dioxin. Dioxin receptor-dependent DNA binding activities were analyzed by a gel mobility shift assay (EMSA) performed essentially as described (31). Briefly, DNA binding reactions were assembled with the indicated protein fractions in 10 mM Hepes, pH 7.9, 5% (v/v) glycerol, 0.5 mM dithiothreitol, 2.5 mM MgCl2, 1 mM EDTA, 0.08 (w/v) Ficoll, and NaCl to a final concentration of 60 mM. The total volume of the DNA binding reactions ranged between 20 and 50 µl. A 32P-labeled, double-stranded 36-base pair oligonucleotide XRE (32) spanning a dioxin-responsive XRE element of the rat cytochrome P450IA1 upstream/promoter region was added to the reactions as specific probe in the presence of 1 µg of nonspecific poly(dI-dC) competitor DNA. The reactions were incubated for 15 min at 25°C, and protein-DNA complexes were resolved on 4% (acrylamide/bisacrylamide ratio of 29:1) low ionic strength native polyacrylamide gels at 30 mA and 0–4 °C using a Tris-glycine EDTA buffer (31). In indicated DNA binding experiments, polyclonal antibodies against the dioxin receptor (17, 33) or Arnt (34), or preimmune serum, were added to the binding reaction mixtures together with protein fractions and the radiolabeled XRE probe to assess the specificity of protein-DNA complexes.

Co-immunoprecipitation Experiments—In vitro [35S]methionine-labeled dioxin receptor or Arnt were mixed with 3G3 anti-hsp90 antibodies (Santa Cruz Technologies), anti-p23 (J33) antibodies (23), or anti-dioxin receptor polyclonal antibodies. Subsequently 40 µl of 50% protein A-Sepharose in phosphate-buffered saline in J33 or anti-dioxin receptor antibody experiments or Sepharose-coupled IgM antibodies in 3G3 antibody experiments was added and incubated under gentle rotation for 1 h at 0–4°C. Bound material was washed five times in phosphate-buffered saline, and proteins were eluted by addition of SDS-polyacrylamide gel electrophoresis sample buffer.

**Results**

Ligand-independent Activation of the Dioxin Receptor to a DNA Binding Form—Fractionation of the dioxin receptor on sucrose density gradients provides a convenient tool to separate different functional forms of the receptor. Typically the dioxin receptor can be recovered both in the 9 S region of the gradient corresponding to an approximate molecular mass of 300 kDa or in the 5–6 S region with an approximate mass of 200 kDa (12, 13, 35). These different complexes represent distinct functional forms of the dioxin receptor; the 9 S form represents the ligand binding, hsp90-containing receptor complex, whereas the 5–6 S form represents the DNA binding heterodimeric complex between the receptor and Arnt (12, 17). In the present experiments we have used cytosolic extracts from Hepa-1 C4 (36) cells expressing a mutant form of Arnt (37) that does not enable the dioxin receptor to bind DNA (8). Addition of wild-type Arnt to this extract restores the DNA binding activity of the receptor in vitro (16, 17). The Hepa-1 C4 cytosolic extract was treated with 10 nM TCDD for 2 h at 25°C and subsequently fractionated on a 10–40% linear sucrose density gradient. The individual fractions were incubated with Arnt for 30 min, and XRE binding activity was assayed by EMSA. The only region of the gradient that displayed XRE binding activity were fractions in the 9 S region of the gradient (Fig. 1A), corresponding to the sedimentation position of the receptor-hsp90 complex (12). Thus, consistent with the model that ligand alone is not sufficient to induce release of hsp90 but requires functional Arnt (29), ligand treatment did not induce any change in the sedimentation profile of the dioxin receptor from 9 S to the 5–6 S region of the gradient, corresponding to the sedimentation position of hsp90-free receptor forms (13).

To assess the role of ligand in activating the receptor to an XRE binding form, we next fractionated untreated Hepa-1 C4 cytosolic extracts on sucrose gradients and pooled the receptor-
containing fractions in the 9 S region of the gradient. This material was incubated with 10 nM dioxin or vehicle alone for 2 h at 25 °C in the presence of bacterially expressed glutathione S-transferase-Arnt, and XRE binding activity was monitored by EMSA. Interestingly, an XRE binding complex was generated both in the presence or absence of ligand (Fig. 1B, compare lanes 3 and 5). This complex harbored both the dioxin receptor and Arnt, as assessed by specific antibodies (data not shown). In contrast, the dioxin receptor in non-fractionated extracts requires ligand to interact with in vitro translated (38) or bacterially expressed (data not shown) Arnt to generate the XRE binding complex. Thus, these data suggest that the ligand-dependent mechanism of receptor activation had been lost upon fractionation of the receptor.

Given the loss of ligand dependence to induce DNA binding activity by the dioxin receptor, we examined the effect of Arnt on ligand binding activity by sucrose gradient-fractionated receptor preparations. As schematically outlined in Fig. 2A, we fractionated in vitro translated dioxin receptor on a sucrose gradient and pooled the dioxin receptor-containing fractions in the 9 S region of the gradient. The fractions were then dialyzed to remove sucrose and incubated in the presence or absence of Arnt for 1 h at 30 °C. This material was subsequently incubated with 10 nM [3H]TCDD for 2 h at 25 °C and loaded on a second sucrose density gradient. Following centrifugation the individual fractions were assayed for radioactivity. As shown in Fig. 2A the fractionated dioxin receptor was able to bind [3H]TCDD. However, upon addition of Arnt, ligand binding activity was significantly decreased. In view of these results we also examined the effect of Arnt to induce release of hsp90 from in vitro translated [35S]labeled dioxin receptor upon fractionation on sucrose gradients. Consistent with the results using nonfractionated receptor material (29), we observed no release of hsp90 from the 9 S dioxin receptor upon incubation of the 9 S receptor with ligand alone in the absence of Arnt (Fig. 2B, compare lanes 2 and 3). Interestingly, however, addition of Arnt to the isolated 9 S dioxin receptor form induced dissociation of hsp90 from the receptor both in the presence or absence of dioxin (Fig. 2C, compare lanes 2–5), consistent with the observed reduction in ligand binding activity (Fig. 2A).

The Dioxin Receptor Interacts with the Co-chaperone p23—The experiments presented above suggest that the latent form of the dioxin receptor becomes destabilized upon fractionation on sucrose gradients, resulting in ligand-independent release of hsp90 and ensuing receptor activation by Arnt. It has been extensively documented that hsp90 binds additional co-chaperone proteins, which are important for functional activities of certain hsp90 associated proteins, such as a number of steroid receptors (25). We therefore investigated the potential involvement of hsp90-associated factors in stabilization of the dioxin receptor-hsp90 complex. To this end we in vitro translated the dioxin receptor in the presence of [35S]methionine and performed co-immunoprecipitation experiments with p23 antibodies. The dioxin receptor was efficiently co-precipitated by p23 antibodies, whereas in control experiments Arnt, which does not interact with hsp90, failed to interact with p23 (Fig. 3A, compare lanes 3 and 6). We next studied the role of ligand and Arnt on release of p23 from the dioxin receptor complex. In vitro translated [35S]methionine-labeled receptor was incubated in the absence or presence of 10 nM dioxin, Arnt, or both treatments. Interestingly, addition of Arnt or ligand alone did not induce release of p23 from the dioxin receptor complex (Fig. 3B, compare lanes 3–5). Only in the presence of a combination of Arnt and ligand we observed partial dissociation of p23 from the dioxin receptor (Fig. 3B, compare lanes 3 and 6).

Loss of Ligand-inducible XRE Binding Activity Correlates with Dissociation of p23 during Gradient Centrifugation—In view of the background that p23 has been shown to stabilize binding between hsp90 and other target proteins (27), we investigated whether sucrose gradient centrifugation affected the interaction of p23 with the 9 S dioxin receptor form. In vitro translated [35S]methionine-labeled dioxin receptor was fractionated on a sucrose density gradient, and receptor-containing fractions in the 9 S region were pooled and used in p23 immu-

![Mechanism of Activation of the Dioxin Receptor](image)
noprecipitation experiments. As expected, experiments performed with nonfractionated *in vitro* translated dioxin receptor showed highly efficient co-immunoprecipitation of the labeled receptor using p23 antibodies (Fig. 4A, compare lanes 1–3). However, following gradient centrifugation a significant decrease in recovery of dioxin receptor by precipitation with p23 antibodies was detected (Fig. 4A, compare lanes 3 and 6). In control reactions, dioxin receptor antibodies precipitated similar amounts of dioxin receptor from either the unfractonated material or the 9 S gradient fraction (Fig. 4B, compare lanes 3 and 6). In conclusion, these experiments indicate that the association of the dioxin receptor-hsp90 complex with p23 becomes destabilized upon fractionation, correlating with the loss of requirement of ligand to induce XRE binding activity.

**Reconstitution of Ligand-dependent Activation of the 9 S Dioxin Receptor Form—** Molybdate ions and other transition metal oxides have been shown to stabilize the interaction between hsp90 and a number of hsp90 interacting proteins, most notably different members of the steroid hormone receptor family (27). We therefore supplemented cytosolic extracts from Hepa-1C4 cells with 10 mM MoO\(_3\) prior to fractionation through sucrose density gradients containing 10 mM MoO\(_3\). As expected, the dioxin receptor was recovered as a 9 S sedimenting complex. This material was subsequently incubated with Arnt both in the presence or absence of 10 nM TCDD and induction of the XRE binding activity of the dioxin receptor-Arnt complex was monitored by EMSA. Upon exposure to Arnt the dioxin receptor-Arnt complex did not require ligand to bind the XRE target sequence following fractionation of the receptor in a sucrose density gradient lacking MoO\(_3\) (Fig. 5B, compare lanes 2 and 6). Strikingly, however, addition of 10 mM MoO\(_3\) to the gradient rendered formation of the DNA binding complex strictly ligand-dependent (Fig. 5, compare lanes 4 and 8).

**DISCUSSION**

The process of activation of the dioxin receptor from a cytoplasmic latent form to a nuclear transcriptionally active form includes release of hsp90 (12, 13). We have shown previously that, in addition to ligand, Arnt facilitates disruption of the dioxin receptor-hsp90 complex (29). In contrast to our results, Fukunaga et al. (39) have observed partial release of hsp90 from the dioxin receptor in the absence of Arnt upon addition of dioxin *in vitro*. The reason behind these discrepancies in results is presently unclear but may reflect different experimental conditions, most notably very significant dilution of the samples in the immunoprecipitation protocol used by Fukunaga et al. (39). Dilution is known to destabilize steroid hormone receptor-hsp90 complexes (25). In the present study treatment of cytosolic extracts from Hepa-1C4 cells expressing a mutant form of Arnt with 10 nM dioxin prior to fractionation through a sucrose density gradient yielded only one fraction of dioxin receptor sedimtating at the position (9 S) of the receptor-hsp90 complex, indicative of a failure to induce a significant
release of hsp90 under these conditions. Although it remains unclear whether hsp90 is released from the receptor prior or subsequent to nuclear translocation, the receptor has been observed to be associated with hsp90 upon extraction from purified nuclei (12). Moreover, the receptor shows ligand-dependent nuclear translocation in Hepa-1C4 cells (40) where we failed to produce ligand-induced release of hsp90 in vitro. Taken together, these data indicate that the receptor may be imported into the nucleus in association with hsp90. Obviously, it will be important to further investigate this issue.

Fractionation of the dioxin receptor through a sucrose density gradient resulted in a loss of the requirement of ligand to generate an XRE binding receptor form, whereas the interaction between hsp90 and the dioxin receptor was not disrupted by this procedure. Moreover, consistent with the ligand-independence in activation of DNA binding activity, addition of Arnt to the sucrose gradient fraction containing the receptor-hsp90 complex resulted in release of hsp90 in the absence of ligand. We interpret these results to indicate that factor(s) involved in stabilization of the latent form of the receptor may have been dissociated following sucrose gradient centrifugation. In an effort to identify such a putative factor we have examined the role of p23 in dioxin receptor activation. p23 is a protein that has been found to be associated with hsp90 (24, 41, 42). In the case of the progesterone receptor and the glucocorticoid receptors, p23 has been found to be associated with the high affinity ligand binding conformations of these receptors (23). It has been shown that formation of the ultimate ligand binding forms of these receptors represents a well ordered process where a number of different protein are involved, e.g. p23, hsp90, hsp70, and Hop (p60) (25, 43, 44).

Here we demonstrate that p23 is associated with the ligand binding form of the dioxin receptor. Moreover, as outlined in the model in Fig. 6, p23 was not displaced upon occupation of the ligand binding domain of the receptor by ligand but remains together with hsp90 associated with the receptor in an intermediary complex prior to dimerization of the receptor with Arnt and release of hsp90. Whereas certain steroid receptors show low ligand binding affinity in the absence of p23 (27), dissociation of p23 from the dioxin receptor by fractionation of the receptor on sucrose gradients did not appear to affect its ligand binding activity. Remarkably, however, in the absence of p23, release of hsp90 by Arnt and the formation of an XRE binding form did not require ligand. It is therefore possible that the role of ligand may be to counteract the function of p23 by binding to the dioxin receptor and possibly inducing a conformational change that will enable the receptor to dimerize with Arnt and subsequently release hsp90. This interpretation is strengthened by the reconstitution of ligand-dependent XRE binding activity of the dioxin receptor by addition of molybdate.

Molybdate is a widely used compound that stabilizes the interaction between, for example, the glucocorticoid receptor and hsp90 (41). In our system molybdate may supplement the functions of unknown factors that in nonfractionated cytosolic extracts stabilize the interaction between the dioxin receptor and hsp90. Alternatively, molybdate may mimic the function of other, hsp90-p23 stabilizing factors. The nature of these putative factor(s) stabilizing the interaction between p23 and hsp90 is presently unknown, but it may be a protein, possibly a chaperone, or a different type of agent such as the glucocorticoid receptor stabilizing factor modulator (45–47). Clearly, the precise role of ligand in dioxin receptor activation and the possible involvement of novel factors in this process needs to be further elucidated. As schematically represented in the model in Fig. 6, a candidate factor is p23, the role of which may be to stabilize an intermediary complex that contains the ligand-occupied hsp90-receptor complex. In summary, we provide evidence suggesting that the role of ligand in dioxin receptor activation may be to overcome the inhibitory effects of hsp90 associated factors such as the co-chaperone p23 and thereby facilitating release of hsp90 by Arnt, resulting in generation of the DNA binding form.

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**FIG. 6. Model of distinct steps in the dioxin receptor activation pathway.** p23 is associated with the ligand binding hsp90-dioxin receptor complex. Upon binding of ligand a stable intermediary complex exists consisting of ligand-occupied dioxin receptor, hsp90 and p23. This form of receptor requires Arnt for release of hsp90 (and possible corelease of p23) and formation of the DNA binding receptor-Arnt heterodimeric complex. See text for details.
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