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A Cellular Factor Stimulates Ligand-Dependent Release of hsp90 from the Basic Helix-Loop-Helix Dioxin Receptor

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In response to dioxin, the nuclear basic helix-loop-helix (bHLH) dioxin receptor forms a complex with the bHLH partner factor Arnt that regulates target gene transcription by binding to dioxin-responsive sequence motifs. Previously, we have demonstrated that the latent form of dioxin receptor present in extracts from untreated cells is stably associated with molecular chaperone protein hsp90, and Arnt is not a component of this complex. Here, we used a coimmunoprecipitation assay to demonstrate that the in vitro-translated dioxin receptor, but not Arnt, is stably associated with hsp90. Although it showed ligand-binding activity, the in vitro-translated dioxin receptor failed to dissociate from hsp90 upon exposure to ligand. Addition of a specific fraction from wild-type hepatoma cells, however, to the in vitro-expressed receptor promoted dioxin-dependent release of hsp90. This stimulatory effect was mediated via the bHLH dimerization and DNA-binding motif of the receptor. Moreover, ligand-dependent release of hsp90 from the receptor was not promoted by fractionated cytosolic extracts from mutant hepatoma cells which are deficient in the function of bHLH dioxin receptor partner factor Arnt. Thus, our results provide a novel model for regulation of bHLH factor activity and suggest that derepression of the dioxin receptor by ligand-induced release of hsp90 may require bHLH-mediated concomitant recruitment of an additional cellular factor, possibly the structurally related bHLH dimerization partner factor Arnt. In support of this model, addition of in vitro-expressed wild-type Arnt, but not a mutated form of Arnt lacking the bHLH motif, promoted release of hsp90 from the dioxin receptor in the presence of dioxin.

The nuclear dioxin receptor (also termed the aryl hydrocarbon receptor) mediates signal transduction by dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin). Like other nuclear receptors, the dioxin receptor is a ligand-inducible transcriptional regulator that directly binds to cognate response elements within regulated genes (for a recent review, see reference 37). In contrast to members of the steroid receptor superfamily, however, the dioxin receptor harbors a basic helix-loop-helix (bHLH) motif (5, 17) that represents a dimerization and DNA-binding surface (18) of a broad class of gene regulatory proteins including Myc, its positive and negative regulators Max and Mad (1 and references therein), lymphoid transcription factors, and muscle-differentiating factors such as MyoD and myogenin (for a recent review, see reference 27).

Dioxin and other receptor ligands (i.e., structurally related environmental pollutants) strictly regulate dioxin receptor function by modulating its DNA-binding activity in vivo (14, 19, 24, 32) and in vitro (6). A physiological ligand, if any, of the dioxin receptor has not been identified (for a review, see reference 37). Although the detailed mechanism of ligand-induced activation of the dioxin receptor into a functional form remains unclear, this process involves several distinct steps. Most notably, the latent, non-DNA-binding form of dioxin receptor has been recovered in cytosolic extracts from non-stimulated cells as an ~300-kDa heteromeric complex associated with the molecular chaperone hsp90 (references 10, 33, and 52 and references therein). In contrast, the nuclear, DNA-binding form of the receptor is an ~200-kDa heterodimeric complex (24) consisting of the ligand-occupied receptor and the structurally related bHLH partner factor Arnt (41, 51). The receptor-Arnt heterodimer binds target DNA, whereas neither Arnt nor the receptor exhibits any DNA-binding activity individually (51). Thus, activation of the dioxin receptor appears to be achieved by the ligand-induced release of hsp90 and the subsequent dimerization with the Arnt partner factor. In support of this model, Arnt fails to interact physically with the dioxin receptor in the absence of the ligand (51). In analogy to the activation mechanism proposed for the dioxin receptor, the glucocorticoid receptor is activated by hormone into a DNA-binding species upon release of hsp90 (12; for a review, see reference 40). Consequently, protein-protein interaction with hsp90 results in negative regulation of both the dioxin and glucocorticoid receptors. In addition, hsp90 appears to chaperone a ligand-binding conformation of both receptors since hsp90-free forms of the receptors exhibit significantly reduced affinity for their cognate ligands (4, 31, 39).

To understand further the activation pathway of the dioxin receptor, we demonstrated in this study that the in vitro-expressed dioxin receptor, but not in vitro-expressed Arnt, is associated with hsp90. The hsp90-associated form of dioxin receptor exhibited ligand-binding activity. Remarkably, however, receptor-hsp90 interaction was very stable and it was not possible to induce release of hsp90 from the in vitro-translated receptor upon exposure to ligand in vitro. To determine whether additional factors are required for this ligand-dependent response, we coincubated the in vitro-expressed receptor with a crude or fractionated cytosolic extract from hepatoma cells. By this analysis, we identified a fraction that promoted

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release of hsp90 from the receptor in the presence of dioxin. In the absence of ligand, this fraction did not perturb dioxin receptor-hsp90 interaction. Moreover, the effect of this fraction appeared to be mediated by the N-terminal bHLH motif of the receptor since the fraction did not stimulate dioxin-dependent release of hsp90 from a receptor construct lacking the bHLH domain. Taken together, these data argue that release of hsp90 and activation of the dioxin receptor by dioxin are not only induced by a mere conformational change of the receptor but may also require bHLH-mediated concomitant recruitment of an additional cellular factor, possibly the bHLH dimerization partner Arnt. In support of this model, ligand-dependent release of hsp90 from the receptor was not promoted by fractionated cytosolic extracts from Hepa-C4 cells, which are deficient in Arnt function. Moreover, in a reconstituted assay using in vitro-expressed proteins, wild-type Arnt, but not an Arnt deletion mutant lacking the bHLH motif, promoted release of hsp90 from the dioxin receptor in the presence of dioxin.

MATERIALS AND METHODS

Plasmid constructions. pDR/BS was obtained by subcloning cDNA of the murine dioxin receptor (5, 17) from pSportAhR (containing the full-length dioxin receptor and generously provided by Christopher Bradfield, Northwestern University Medical School, Chicago, Ill.) into pBluescript (Stratagene). A 1-kb cDNA fragment of the murine dioxin receptor was primed from the beginning of the coding region by 20 cycles (1 min at 94°C, 1 min at 55°C, 3 min at 72°C) of PCR (Perkin-Elmer Cetus). The primer corresponding to the N terminus of the coding region was modified to incorporate a consensus Kozak (CCGCCACCATGG) start sequence (28) and an upstream *Cla*I restriction site. The amplified cDNA was digested with *Cla*I and *Cel*II and subcloned into *Cla*I- and *Cel*II-digested pDR/BS to give pDR/ATG/BS. pτDBD/83-805/Gem (here designated pGR/DR) contains cDNA corresponding to the first 500 amino acids of the human glucocorticoid receptor fused to the cDNA encompassing codons 83 to 805 of the murine dioxin receptor (50). To create the very narrow bHLH deletion mutant pArntΔHLH, a 240-bp *Bcl*I fragment was excised from pGemArnt (51), and the plasmid was religated.

Cells, extract preparation, and fractionation on sucrose density gradients. Wild-type, dioxin-responsive mouse hepatoma Hepa-1c1c7 (Hepa-1) cells and the mutant, dioxin-resistant Hepa-1-C4 cell line derived from it (23) were grown in minimum essential medium supplemented with 10% fetal calf serum, 100 U of penicillin, and 100 μg of streptomycin (GIBCO-BRL) per ml as previously described (52). When cell growth was near confluency, cells were harvested by centrifugation and homogenized in ETG buffer (20 mM Tris-HCl [pH 7.4], 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol). The preparation was centrifuged at 120,000 × *g* for 45 min, and the resulting supernatant was collected and used as a cytosolic extract (typical protein concentration, ~5 mg/ml). The cytosolic fraction was either used immediately or stored at −70°C until required. In fractionation experiments, 250 μl of cytosol was layered onto 10 to 40% (wt/vol) linear sucrose gradients prepared in ETG buffer containing 50 mM NaCl. The gradients were centrifuged at 300,000 × *g* to a cumulative centrifugal effect of 1.7×10^{12} rad^2/s in a Beckman L8-60 ultracentrifuge. Fractions were collected by gravity flow starting from the bottom of the gradients and either used immediately or quickly frozen in liquid nitrogen and then stored at −70°C until required. External sedimentation marker proteins were run on separate gradients.

In vitro expression of receptors and immunoprecipitation experiments with anti-hsp90 antibodies. The radiolabeled dioxin receptor, Arnt, the glucocorticoid receptor, and the chimeric glucocorticoid-dioxin receptor were generated for immunoprecipitation experiments by in vitro transcription-translation of plasmids pDR/ATG/BS, pGemArnt (51), pT3,1118 (42), and pτDBD/83-805/Gem (50), respectively, in the presence of [^{35}S]methionine (New England Nuclear) in rabbit reticulocyte lysate (Promega) in accordance with the manufacturer's recommendations. For immunoprecipitation experiments, goat anti-mouse immunoglobulin M (IgM; Sigma) was coupled to CNBr-activated Sepharose 4B (Pharmacia). A 5-μl sample of monoclonal anti-hsp90 IgM antibody 3G3 (34; purchased from Affinity Bioreagents) or an equal concentration of control mouse IgM antibody TEPC 183 (Sigma) was then incubated with 160 μl of a 1:4 suspension of the goat anti-mouse antibody coupled to Sepharose in MENG buffer (25 mM MOPS [morpholinepropanesulfonic acid; pH 7.5], 1 mM EDTA, 0.02% NaN_3, 10% glycerol) on ice for 90 min. This Sepharose-adsorbed material was then pelleted and washed successively with 1 ml of MENG buffer containing 500 mM NaCl, MENG buffer alone, and finally supplemented MENG buffer (containing 20 mM molybdate, 2 mM dithiothreitol, and 2.5% [wt/vol] bovine serum albumin). In immunoprecipitation experiments, the pellets were resuspended in supplemented MENG buffer to which in vitro translation reaction mixtures diluted fivefold in supplemented MENG buffer minus bovine serum albumin were added. The reaction mixtures were incubated on ice for 90 min, after which Sepharose beads were pelleted by centrifugation and washed three times with MENG plus molybdate and dithiothreitol. Immunoprecipitated proteins were thereafter eluted by boiling in sodium dodecyl sulfate (SDS) buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and the gels were fixed in 7% acetic acid, soaked in Amplify (Amersham) for 30 min, dried under a vacuum, and subjected to autoradiography.

Ligand-binding assay. pDR/ATG/BS was translated in reticulocyte lysate in the presence of unlabeled methionine. Ligand-binding activity was assayed by a hydroxylapatite adsorption assay (20, 38). In vitro translation reaction mixtures (10 μl) were incubated with 10 μl of ETG buffer containing 1 mM phenylmethylsulfonyl fluoride, 5 μg of aprotinin per ml, and [^{3}H]dioxin (40 Ci/mmol; Chemsyn, Lenexa, Kans.) at a final concentration of 2.5 nM. After incubation for 90 min at room temperature, a 50-μl suspension of 50% (vol/vol) hydroxylapatite slurry in ETG buffer was added, and the mixtures were shaken on ice for 30 min. After rapid centrifugation, the supernatants were discarded, and the pellets were washed four times with 500 μl of ETG buffer containing 0.1% Tween 20. The pellets were eluted by being washed twice with ethanol, and the resulting supernatants were analyzed by scintillation counting.

Arnt antiserum and immunoblot analysis. Polyclonal Arnt antibodies were produced in chicken egg yolk, as previously described (21), against a synthetic peptide spanning amino acids 39 to 58 of human Arnt (25) coupled via an additional C-terminal cysteine residue to bovine serum albumin. Chicken immunoglobulins were purified to near homogeneity as previously described (21). For immunoblot analysis, proteins were separated by SDS–7% polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose filters. Immunodetection was achieved by incubation of the filters with anti-Arnt immunoglobulins followed by reaction with horseradish peroxidase-conjugated rabbit anti-chicken immunoglobulins (Sigma).

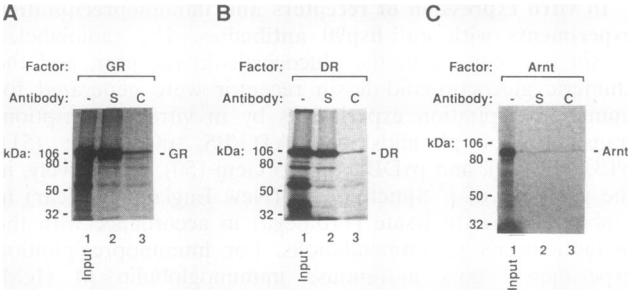

FIG. 1. Coimmunoprecipitation of the glucocorticoid receptor (A), the dioxin receptor (B), and Arnt (C) with hsp90-specific antibodies. The [^{35}S]methionine-labeled glucocorticoid receptor (GR), dioxin receptor (DR), or Arnt was expressed in vitro in reticulocyte lysate, coimmunoprecipitated with hsp90-specific antibodies as described in Materials and Methods, and analyzed by SDS-PAGE and subsequent fluorography. In each panel, lane 1 represents analysis of the total input translation products before immunoprecipitation, lane 2 represents proteins coimmunoprecipitated by the hsp90-specific antibody (S), and lane 3 represents proteins coimmunoprecipitated by the control IgM antibody (C). The positions of precipitated proteins and of molecular mass standard proteins are indicated.

RESULTS

The dioxin receptor, but not the bHLH partner factor Arnt, is associated with hsp90. In the absence of ligand, hsp90 forms distinct oligomeric complexes, both in vivo and in vitro, with certain steroid hormone receptors, most notably, the glucocorticoid receptor (for recent reviews, see references 40 and 47). Attempts to generate the hsp90-glucocorticoid receptor complex by incubating the purified components in solution have not been successful (40), suggesting that receptor conformation is altered upon release of hsp90 such that reassociation cannot occur. In cell-free model systems, reassociation between hsp90 and the purified glucocorticoid receptor is, however, promoted by rabbit reticulocyte lysate (43). Moreover, as soon as it is translated in reticulocyte lysate, the glucocorticoid receptor becomes associated with hsp90 (7, 13). To demonstrate glucocorticoid receptor-hsp90 interaction, we employed in coimmunoprecipitation experiments a monoclonal IgM antibody capable of recognizing both free hsp90 and hsp90 complexed with other proteins (34). As expected, immunoprecipitation of the [^{35}S]methionine-labeled, in vitro-expressed glucocorticoid receptor by these monoclonal anti-hsp90 antibodies revealed that the labeled, full-length ~95-kDa receptor coprecipitated with hsp90. In contrast, only low background levels of the glucocorticoid receptor were precipitated by control IgM antibodies (Fig. 1A, compare lanes 2 and 3), demonstrating the specificity of the coimmunoprecipitation assay.

We next wanted to examine whether in vitro-translated dioxin receptor was able to bind hsp90. In vitro translation of dioxin receptor mRNA in the presence of [^{35}S]methionine produced predominantly an ~95-kDa labeled protein, as assessed by SDS-PAGE (Fig. 1B, lane 1). The anti-hsp90 antibodies showed strong selectivity for the dioxin receptor, compared with control antibodies, in the coimmunoprecipitation assay (Fig. 1B, compare lanes 2 and 3), indicating that de novo-synthesized, ligand-free dioxin receptor, in analogy to the glucocorticoid receptor, forms a stable complex with hsp90 in solution. Association with hsp90 was not observed, however, upon in vitro translation of the ~90-kDa dioxin receptor partner protein Arnt, since only low levels of nonspecific interaction with both specific and control antibodies were detected in coimmunoprecipitation experiments (Fig. 1C, lanes 2 and 3). Thus, although Arnt and the dioxin receptor are structurally related and share, in addition to the bHLH motif, the ~270-amino-acid motif termed the PAS region (29, 48), they show very clear differences in their abilities to bind hsp90. Finally, we also expressed the full-length dioxin receptor by in vitro translation in wheat germ lysate but did not detect any formation of receptor-hsp90 complexes in coimmunoprecipitation experiments (data not shown), consistent with the inability of the glucocorticoid receptor to become associated with the wheat germ homolog of mammalian hsp90 (8).

Failure to reconstitute ligand-dependent release of hsp90 from the in vitro-expressed dioxin receptor. We have previously observed that the dioxin receptor shows no detectable dioxin-binding activity following dissociation of hsp90 (39), suggesting that hsp90 maintains and stabilizes the dioxin receptor in a ligand-binding conformation. To characterize further the dioxin receptor expressed under cell-free conditions in reticulocyte lysate, we therefore performed in vitro ligand-binding experiments with [^{3}H]dioxin as the specific ligand and a hydroxylapatite adsorption assay. Unprogrammed reticulocyte lysate showed low background levels of [^{3}H]dioxin-binding activity (Fig. 2A). In contrast, the in vitro-translated dioxin receptor exhibited high levels of [^{3}H]dioxin-binding activity. In control competition experiments, binding of [^{3}H]dioxin by the in vitro-translated dioxin receptor was inhibited in the presence of a 150-fold molar excess of the high-affinity dioxin analog 2,3,7,8-tetrachlorodibenzofuran but not in the presence of an identical excess of the high-affinity glucocorticoid receptor ligand dexamethasone (Fig. 2A), demonstrating specific labeling of the dioxin receptor.

We next investigated whether exposure to the ligand would induce dissociation of the hsp90-dioxin receptor complex generated in reticulocyte lysate. We performed hsp90 coimmunoprecipitation experiments following incubation of the in vitro-translated dioxin receptor with increasing concentrations of dioxin under conditions which readily activate the receptor into a DNA-binding form in cytosolic extracts of target cells (6). Remarkably, we were unable to detect any differences with regard to the amount of hsp90 bound to the receptor after this treatment with either dioxin or the vehicle, dimethyl sulfoxide, alone. Even upon addition of high concentrations (10 to 100 nM) of the ligand, it was not possible to dissociate hsp90 from the in vitro-synthesized receptor (Fig. 2B, compare lanes 1 to 5).

Given the background that exposure of the ligand-free dioxin receptor in cytosolic cell extracts to high ionic strength (0.5 M KCl) can induce a significant, albeit not total, disruption of the hsp90-receptor complex (reference 39 and references therein), we treated the in vitro-translated dioxin receptor with increasing concentrations of salt in the presence or absence of dioxin. This in vitro manipulation did not appear to facilitate ligand-induced release of hsp90 from the receptor but rather dramatically reduced the total amount of the dioxin receptor-hsp90 complex precipitated by the anti-hsp90 antibody either in the presence or in the absence of ligand (Fig. 2C). Increasing the temperature during ligand treatment or increasing the length of time during which the receptor was exposed to ligand also did not promote ligand-dependent dissociation of the receptor-hsp90 complex (data not shown). We therefore conclude that the de novo-synthesized dioxin receptor forms a very stable complex with hsp90 in solution. Although the in vitro-translated dioxin receptor showed high ligand-binding activity, we failed to reconstitute in reticulocyte lysate ligand-dependent activation of the receptor to its apparently hsp90-free form. It is therefore possible that this process

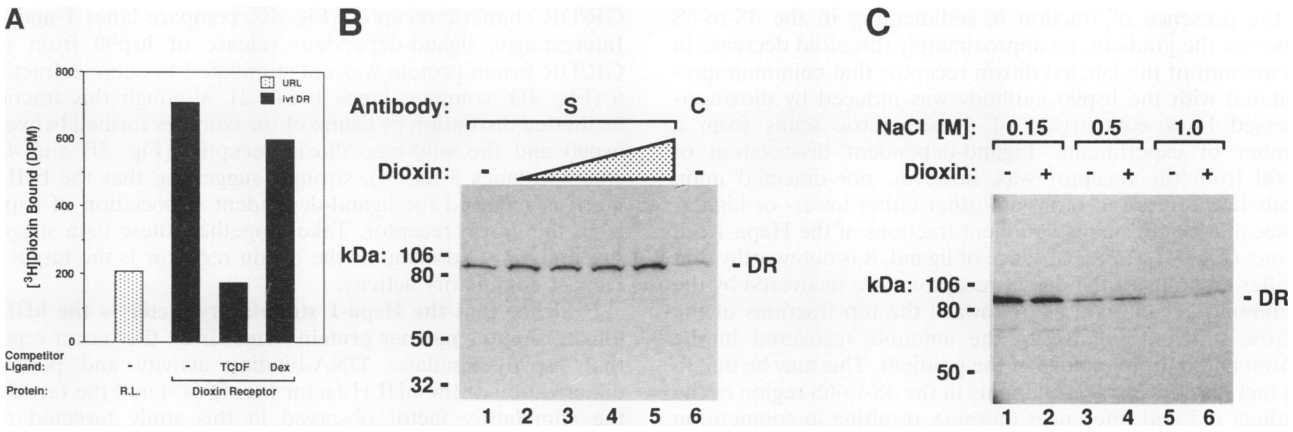

FIG. 2. Failure to reconstitute ligand-dependent release of hsp90 with the in vitro-translated dioxin receptor. (A) Ligand-binding experiments. Unprogrammed reticulocyte lysate (URL) or reticulocyte lysate (R.L.) containing the in vitro-translated dioxin receptor (ivt DR) was incubated with 2.5 nM [^{3}H]dioxin for 90 min at 25°C and then adsorbed to hydroxylapatite at 4°C for 30 min. After extensive washing, bound [^{3}H]dioxin was determined by scintillation counting. In control experiments, the specificity of the binding reaction was assessed by performing the reaction in the presence of a 150-fold molar excess of tetrachlorodibenzofuran (TCDF) or dexamethasone (Dex). Results of a representative experiment are shown. (B) Effect of ligand on release of hsp90. The [^{35}S]methionine-labeled, in vitro-translated dioxin receptor was incubated without (lane 1) or with (lanes 2 to 5) increasing concentrations (0.1, 1, 10, and 100 nM) dioxin at a final concentration of 150 mM NaCl for 3 h at 25°C. The total reaction volumes were then coimmunoprecipitated with specific anti-hsp90 antibodies (S), and the products were analyzed as outlined in the legend to Fig. 1. Lane 6 represents nonspecific immunoprecipitation of a reaction identical to that in lane 1 by control antibodies (C). (C) The dioxin receptor translated in vitro as described above was incubated with increasing concentrations of salt without or with 10 nM dioxin, as indicated, for 3 h at 25°C prior to coimmunoprecipitation analysis with hsp90 antibodies as described above.

requires a critical cofactor that is either not present or present only in insufficient amounts in reticulocyte lysate.

Ligand-dependent release of hsp90 from the dioxin receptor is stimulated by a cellular factor. In cytosolic extracts from a number of tissues and cell lines, including dioxin-responsive Hepa-1 hepatoma cells, it is possible to reconstitute ligand-dependent derepression of the dioxin receptor in vitro by treatment with ligand, resulting in induction of DNA-binding activity by the dioxin receptor-Arnt heterodimer (6, 15, 19, 30, 51). To investigate whether factors present in extracts from Hepa-1 cells promote ligand-dependent dissociation of hsp90 from the dioxin receptor, we initially added a crude cytosolic extract from nontreated cells to the in vitro-expressed [^{35}S]methionine-labeled dioxin receptor. These reactions were incubated at 25°C at a moderate salt concentration (150 mM NaCl) in the absence or presence of 10 nM dioxin and then analyzed by the coimmunoprecipitation assay with monoclonal anti-hsp90 antibodies. As shown in Fig. 3A, addition of the crude Hepa-1 extract resulted in a dioxin-dependent, significant reduction in the levels of the receptor that were coimmunoprecipitated by the anti-hsp90 antibody (compare lanes 1 and 2). We next fractionated this cytosolic extract on sucrose gradients. By using the resulting individual fractions, we observed that incubation of the in vitro-translated receptor with fraction 6 gave a significant decrease in the amount of the dioxin receptor that coimmunoprecipitated with the monoclonal antibodies in the presence of dioxin, in comparison with the amount of the receptor that was coimmunoprecipitated in the absence of ligand (Fig. 3B, compare lanes 11 and 12). This effect was also produced by exposing the receptor to fraction 5.

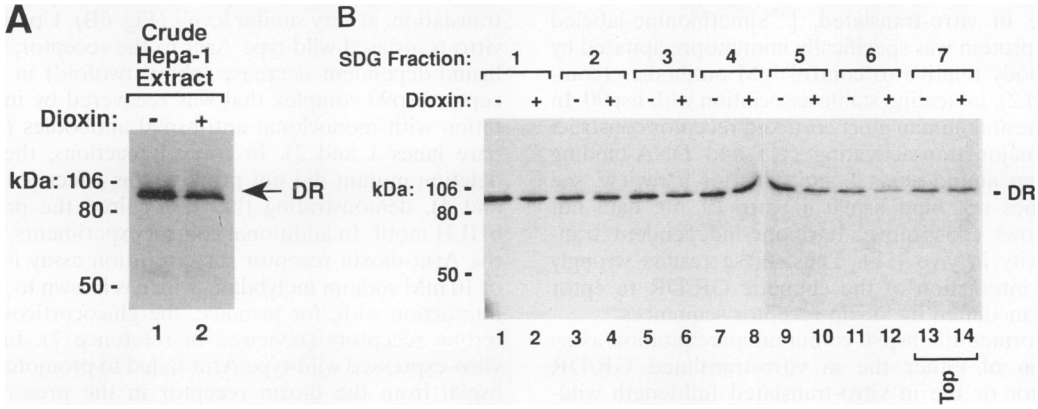

FIG. 3. Identification of a cytosolic Hepa-1 factor(s) that promotes ligand-dependent release of hsp90 from the dioxin receptor (DR). Aliquots (20 μl) of a crude (A) or sucrose density gradient (SDG)-fractionated (B) cytosolic extract from untreated wild-type Hepa-1 cells were incubated with 5 μl of the [^{35}S]methionine-labeled, in vitro-translated dioxin receptor either in the presence (even-numbered lanes) or in the absence (odd-numbered lanes) of 10 nM dioxin at 150 mM NaCl for 3 h at 25°C. The total reactions were then subjected to coimmunoprecipitation with hsp90-specific antibodies, and the products were analyzed as described in the legend to Fig. 1.

In the presence of fraction 6, sedimenting in the 4S-to-6S region of the gradient, an approximately threefold decrease in the amount of the labeled dioxin receptor that coimmunoprecipitated with the hsp90 antibody was induced by dioxin, as assessed by a comparison of densitometric scans from a number of experiments. Ligand-dependent dissociation of hsp90 from the receptor was, however, not detected upon incubation of the receptor with other either lower- or higher-molecular-weight sucrose gradient fractions of the Hepa-1 cell extract (Fig. 3B). In the absence of ligand, it is noteworthy that smaller amounts of the dioxin receptor were recovered by the coimmunoprecipitation assay toward the top fractions of the sucrose gradient relative to the amounts recovered in the central and bottom regions of the gradient. This may be due to the fact that free hsp90 sediments in the 4S-to-6S region of the gradient (13 and references therein), resulting in competition with the hsp90-dioxin receptor complex in the antibody-binding reaction and therefore, possibly, underestimation of the effect produced by ligand on the release of hsp90.

Stimulation of ligand-dependent dissociation of hsp90 by the Hepa-1 factor is mediated by the bHLH motif of the dioxin receptor. We conclude from the experiments described above that cytosolic extracts from Hepa-1 cells appear to contain a high-molecular-weight (~4S-to-7S) factor(s) that increases the dissociation of hsp90 from the dioxin receptor in the presence of the ligand. Interestingly, we have recently observed (50) that the ligand- and hsp90-binding activities of the dioxin receptor are colocalized within a region of the receptor (termed the PAS region; see the schematic drawing in Fig. 4A) that is conserved in the bHLH partner factor Arnt and the Drosophila factors Per and Sim (for a review, see reference 48). However, ligand-dependent activation of the DNA-binding activity of the dioxin receptor requires bHLH motif-mediated dimerization with the partner factor Arnt (50, 51). We therefore tested the effect of the Hepa-1 stimulatory activity on a chimeric glucocorticoid-dioxin receptor construct, GR/DR (shown schematically in Fig. 4A), in which the C-terminal hormone-binding domain of the glucocorticoid receptor has been substituted with the ligand-binding and C-terminal transactivation domains of the dioxin receptor (50). Importantly, this chimeric receptor lacks the very N-terminal bHLH motif of the dioxin receptor.

In initial experiments, we translated the chimeric GR/DR receptor in vitro to examine its ability to form a complex with hsp90 in coimmunoprecipitation experiments with the monoclonal anti-hsp90 antibody. The autoradiograph in Fig. 4B shows that the in vitro-translated, [^{35}S]methionine-labeled GR/DR fusion protein was specifically immunoprecipitated by the hsp90 antibody relative to control IgM antibodies (compare lanes 1 and 2), indicating stable association with hsp90. In contrast, the parental human glucocorticoid receptor construct spanning the major transactivating (τ1) and DNA-binding domains between amino acids 1 and 500 (for a review, see reference 2) does not bind hsp90 in vitro (9, 50; data not shown) and shows constitutive, hormone-independent transcriptional activity in vivo (22). Thus, these results strongly argue that the interaction of the chimeric GR/DR receptor with hsp90 was mediated by dioxin receptor sequences.

We next performed the hsp90 coimmunoprecipitation assay upon incubation of either the in vitro-translated GR/DR chimeric receptor or the in vitro-translated full-length wild-type dioxin receptor with sucrose gradient fraction 2. This fraction contained very high-molecular-weight (>13S) material of the Hepa-1 cytosolic extract that did not stimulate ligand-dependent dissociation from either the full-length dioxin receptor (Fig. 3B and 4C, compare lanes 3 and 4) or the GR/DR chimeric receptor (Fig. 4C, compare lanes 1 and 2). Interestingly, ligand-dependent release of hsp90 from the GR/DR fusion protein was not stimulated by sucrose fraction 6 (Fig. 4D, compare lanes 1 and 2), although this fraction facilitated disruption by ligand of the complex formed between hsp90 and the wild-type dioxin receptor (Fig. 3B and 4D, compare lanes 3 and 4), strongly suggesting that the bHLH motif is required for ligand-dependent dissociation of hsp90 from the dioxin receptor. Taken together, these data suggest that the bHLH domain of the dioxin receptor is the target of Hepa-1 stimulatory activity.

Evidence that the Hepa-1 stimulatory factor is the bHLH dioxin receptor partner protein Arnt. Given the recent report that hsp90 stimulates DNA-binding activity and possibly dimerization of the bHLH factor MyoD (45) and the fact that the stimulatory factor observed in this study targeted the bHLH motif of the dioxin receptor, it is very plausible that the Hepa-1 stimulatory activity represents the dioxin receptor bHLH dimerization partner factor Arnt. In support of this model, we detected by immunoblot analysis with specific Arnt antibodies the ~90-kDa Arnt protein predominantly in sucrose gradient fraction 6 but also in fraction 5 (Fig. 5A), where we had observed the stimulatory activity (compare Fig. 3B and 5A). In addition, an ~60-kDa degradation product of Arnt was also evenly distributed in fractions 5 and 6 of the sucrose gradient.

To test the possible role of Arnt, if any, in ligand-induced release of hsp90 from the dioxin receptor, we fractionated in an identical manner on sucrose gradients cytosolic extracts from wild-type Hepa-1 and mutant dioxin-resistant Hepa-1-C4 cells which are defective in the Arnt function (25 and references therein). As expected, sucrose gradient fraction 6 of the wild-type Hepa-1 extract promoted dioxin-dependent dissociation of hsp90 from the dioxin receptor in hsp90 coimmunoprecipitation experiments (Fig. 5B, compare lanes 1 and 2). In contrast, addition of the identical sucrose fraction of the mutant, Arnt-deficient Hepa-1-C4 extract to the in vitro-translated dioxin receptor did not promote this effect (Fig. 5B, compare lanes 3 and 4), strongly suggesting a role of Arnt in release of hsp90.

Given this background, we attempted to reconstitute ligand-dependent dissociation of hsp90 by using both in vitro-translated Arnt and the dioxin receptor. In these experiments, we expressed in vitro both wild-type Arnt and an Arnt deletion mutant, ArntΔbHLH, lacking the bHLH motif (schematically drawn in Fig. 6A). Both proteins were expressed by in vitro translation, at very similar levels (Fig. 6B). Upon addition of in vitro-translated wild-type Arnt to the receptor, we observed a ligand-dependent decrease (about twofold) in the dioxin receptor-hsp90 complex that was recovered by immunoprecipitation with monoclonal anti-hsp90 antibodies (Fig. 6C, compare lanes 1 and 2). In control reactions, the ArntΔbHLH deletion mutant did not produce this effect (compare lanes 3 and 4), demonstrating that it required the presence of the bHLH motif. In additional control experiments, we performed the Arnt-dioxin receptor reconstitution assay in the presence of 10 mM sodium molybdate, which is known to stabilize hsp90 interaction with, for instance, the glucocorticoid and progesterone receptors (reviewed in reference 7). Interestingly, in vitro-expressed wild-type Arnt failed to promote any release of hsp90 from the dioxin receptor in the presence of sodium molybdate, and under these conditions, Arnt and the ArntΔbHLH deletion mutant were equally inert in the reconstitution assay (Fig. 6D, compare lanes 1 to 4). Taken together, these results lend support to the hypothesis that Arnt, at least in part, relates to the stimulatory activity observed in wild-type

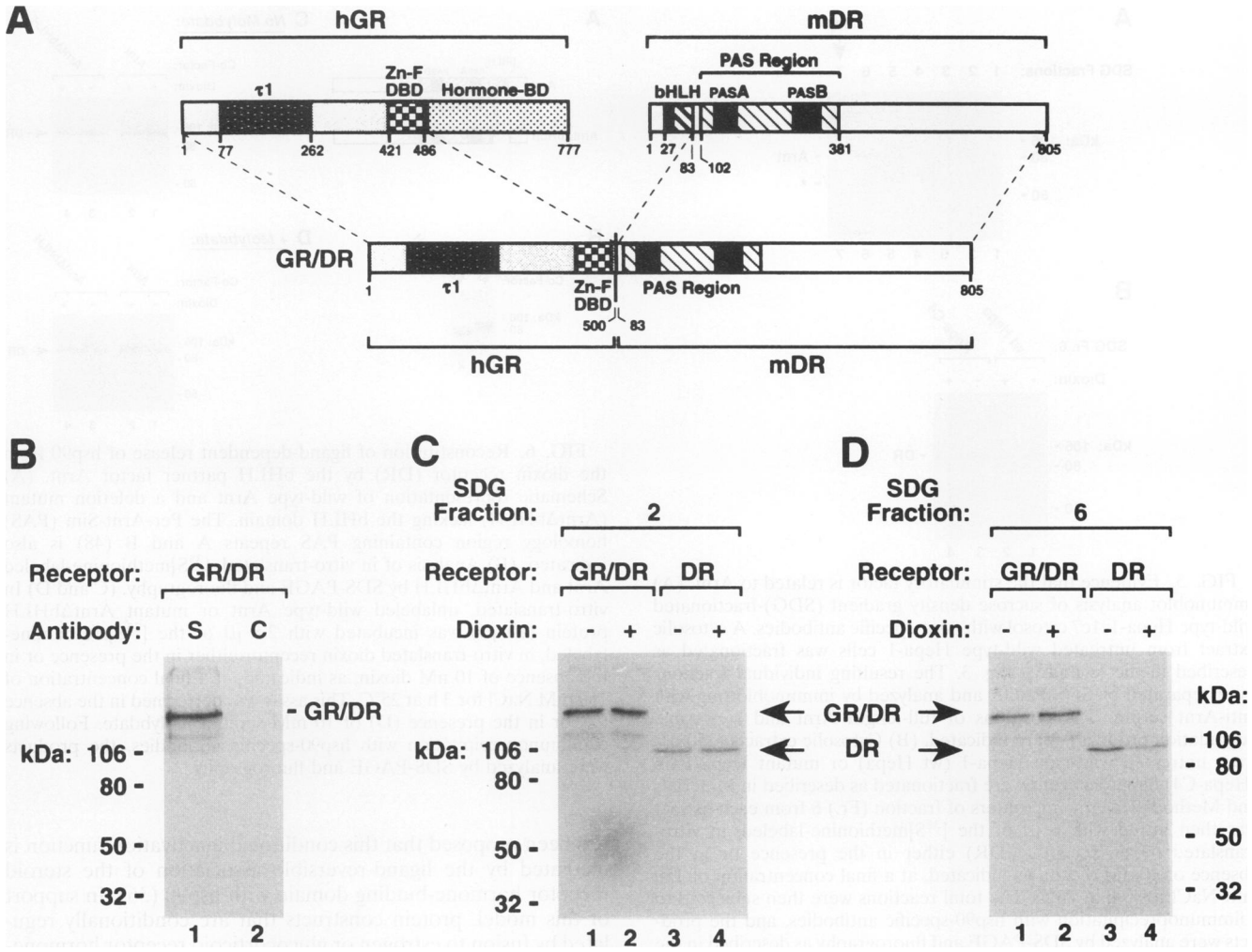

FIG. 4. The bHLH motif of the dioxin receptor (DR) is the target of regulation by the Hepa-1 stimulatory factor. (A) Schematic representation of structural motifs within the human glucocorticoid receptor (hGR), the mouse dioxin receptor (mDR), and the chimeric receptor construct (GR/DR) in which the bHLH domain of the dioxin receptor has been replaced with the N-terminal 500 amino acids of the human glucocorticoid receptor. The Per-Arnt-Sim (PAS) homology region of the dioxin receptor containing PAS repeats A and B (48) is also indicated. (B) The GR/DR chimeric receptor construct was translated in vitro in the presence of [^{35}S]methionine and analyzed by the coimmunoprecipitation assay with hsp90-specific antibodies (S; lane 1) or an equal concentration of control IgM antibodies (C; lane 2) as described in the legend to Fig. 1. (C and D) Hepa-1 cytosol was fractionated as described in the legend to Fig. 3. Similar amounts of the [^{35}S]methionine-labeled, in vitro-translated dioxin receptor (lanes 3 and 4 in panels C and D) or the GR/DR chimeric receptor (lanes 1 and 2 in both panels C and D) were mixed with 20 μl of sucrose gradient fraction 2 (C) or sucrose gradient fraction 6 (D) in the presence or absence of 10 nM dioxin, as indicated. The incubation was performed in the presence of 150 mM NaCl for 3 h at 25°C and was followed by coimmunoprecipitation with hsp90-specific antibodies and analysis by SDS-PAGE and fluorography.

Hepa-1 extracts. It is possible that the relatively low potency of in vitro-translated Arnt as a promoter of this stimulatory effect may be due to the low concentrations of protein generated in this system or, alternatively, the absence or insufficient levels of posttranslational modification of Arnt that may be required for efficient dimerization with the dioxin receptor. Earlier studies have indicated that protein kinase C-mediated phosphorylation of Arnt may be required for dimerization with the ligand-activated dioxin receptor (3). Finally, determination of whether the Hepa-1 stimulatory activity represents factors in addition to Arnt awaits further purification of this activity.

DISCUSSION

In the present study, we used an in vitro model system to identify a cellular factor that stimulated release of hsp90 from the dioxin receptor upon ligand treatment. This stimulatory activity was present in cytosolic extracts from dioxin-responsive wild-type Hepa-1 cells, whereas it was not detected in cytosolic extracts from Arnt-deficient, dioxin-resistant mutant Hepa-1C-4 cells, favoring the hypothesis that the stimulatory activity represents the bHLH dioxin receptor dimerization partner Arnt. In strong support of this model, in vitro-translated Arnt could substitute, albeit at a lower efficiency, for Hepa-1 activity in stimulating dissociation of hsp90 from the receptor. Consistent with this model, the Hepa-1 stimulatory factor promoted ligand-dependent release of hsp90 from the dioxin receptor via the bHLH domain of the receptor, and conversely, Arnt required its bHLH motif to induce dissociation of hsp90 in the reconstitution assay. It is also noteworthy that Arnt failed to stimulate dioxin-dependent release of hsp90 from the dioxin

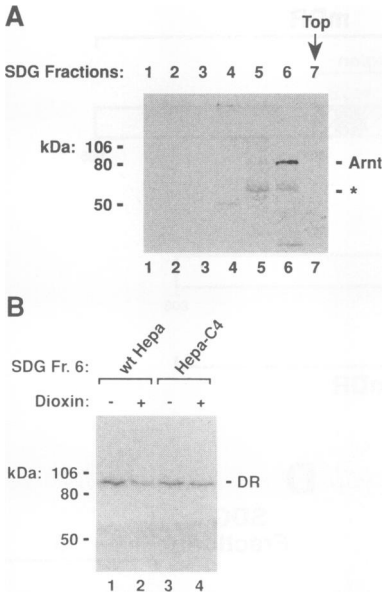

FIG. 5. Evidence that the stimulatory factor is related to Arnt. (A) Immunoblot analysis of sucrose density gradient (SDG)-fractionated wild-type Hepa-1c1c7 cytosol with Arnt-specific antibodies. A cytosolic extract from untreated wild-type Hepa-1 cells was fractionated as described in the legend to Fig. 3. The resulting individual fractions were separated by SDS-PAGE and analyzed by immunoblotting with anti-Arnt serum. The positions of full-length Arnt and a possible degradation product (*) are indicated. (B) Cytosolic extracts (250 μl) from untreated wild-type Hepa-1 (wt Hepa) or mutant Hepa-1-C4 (Hepa-C4) hepatoma cells were fractionated as described in Materials and Methods. Twenty microliters of fraction (Fr.) 6 from each extract was then mixed with 5 μl of the [^{35}S]methionine-labeled, in vitro-translated dioxin receptor (DR) either in the presence or in the absence of 10 nM dioxin, as indicated, at a final concentration of 150 mM NaCl for 3 h at 25°C. The total reactions were then subjected to coimmunoprecipitation with hsp90-specific antibodies, and the products were analyzed by SDS-PAGE and fluorography as described in the legend to Fig. 1.

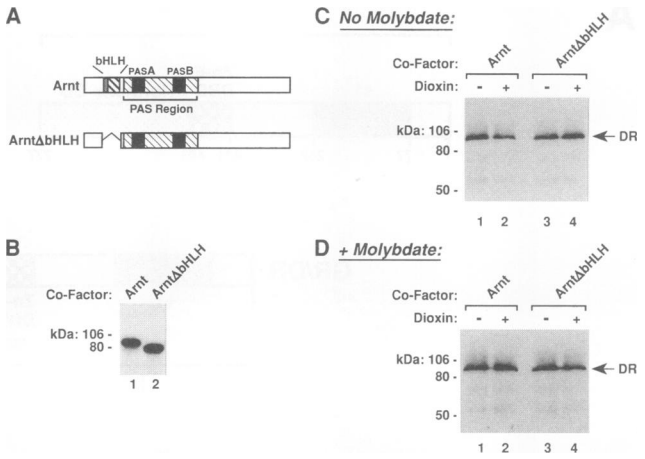

FIG. 6. Reconstitution of ligand-dependent release of hsp90 from the dioxin receptor (DR) by the bHLH partner factor Arnt. (A) Schematic representation of wild-type Arnt and a deletion mutant (ArntΔbHLH) lacking the bHLH domain. The Per-Arnt-Sim (PAS) homology region containing PAS repeats A and B (48) is also indicated. (B) Analysis of in vitro-translated, [^{35}S]methionine-labeled Arnt and ArntΔbHLH by SDS-PAGE and fluorography. (C and D) In vitro-translated, unlabeled wild-type Arnt or mutant ArntΔbHLH protein (10 μl) was incubated with 2.5 μl of the [^{35}S]methionine-labeled, in vitro-translated dioxin receptor either in the presence or in the absence of 10 nM dioxin, as indicated, at a final concentration of 150 mM NaCl for 3 h at 25°C. This assay was performed in the absence (C) or in the presence (D) of 10 mM sodium molybdate. Following coimmunoprecipitation with hsp90-specific antibodies, the products were analyzed by SDS-PAGE and fluorography.

receptor in the presence of sodium molybdate, a compound known to stabilize hsp90 interaction with a number of steroid hormone receptors (7). Finally, the Hepa-1 stimulatory activity cosedimented on sucrose gradients with Arnt immunoreactivity. It is unclear whether the relative difference between in vitro-expressed Arnt and the Hepa-1 stimulatory factor in the ability to promote hsp90 release is due to lack of cofactors, posttranslational modification of Arnt, or simply the concentration of Arnt versus that of the receptor. Interestingly, immunoblot analyses indicate that Arnt is expressed at significantly more abundant levels than the dioxin receptor in Hepa-1 cells (46). The ratio between Arnt and the receptor may therefore be an important parameter influencing the efficiency in Arnt-receptor heterodimerization.

Role of hsp90 in modulating dioxin receptor function. What is the functional significance of dioxin receptor-hsp90 interaction? In addition to the dioxin receptor, certain steroid hormone receptors, including the glucocorticoid and estrogen receptors, have been shown to associate with hsp90 in the ligand-free state (for recent reviews, see references 40 and 47). The hormone-binding domains of the glucocorticoid and estrogen receptors mediate association with hsp90 (9, 44), and these receptor domains can confer negative regulation on heterologous proteins in a fashion that can be reversed by hormone (references 16, 26, and 36 and references therein). It has been proposed that this conditional inactivation function is mediated by the ligand-reversible association of the steroid receptor hormone-binding domain with hsp90 (36). In support of this model, protein constructs that are conditionally regulated by fusion to estrogen or glucocorticoid receptor hormone-binding domains show hormone-regulated association with hsp90 in vitro (44), and hormone induction of specific DNA-binding activity by the glucocorticoid receptor is seen only following release of hsp90 (12). By analogy, we have recently observed that the ligand-binding domain of the dioxin receptor can confer negative regulation on the glucocorticoid receptor in fusion protein constructs (50), and we found in this study that such a construct is stably associated with hsp90. Moreover, ligand-induced DNA-binding activity by the dioxin receptor has been observed only following release of hsp90 (52).

Taken together, these data suggest that hsp90, in the absence of the ligand, may repress the functions of both the dioxin receptor and certain steroid hormone receptors by formation of a stable complex with the corresponding receptor. In addition, hsp90 appears to chaperone a ligand-binding conformation of both the dioxin and glucocorticoid receptors, since both receptors lose affinity for their cognate ligands following disruption of the hsp90-receptor complex (4, 31, 39). In support of this model, decreased hormone responsiveness of the glucocorticoid receptor has been observed in mutant yeast strains which express reduced levels of hsp90 (35).

Possible mechanism of activation of the dioxin receptor. As outlined above, ligand-dependent activation of the dioxin receptor to a functional form appears to be a very complex process involving several distinct, critical steps: (i) binding of the ligand, (ii) release of hsp90, and (iii) subsequent dimerization with Arnt, which promotes the DNA-binding function of the receptor (51). The present data indicate that Arnt, in fact,

may facilitate dissociation of hsp90 by its bHLH-mediated dimerization with the receptor. Thus, these two critical events in the receptor activation pathway appear to be interdependent and seem to occur concomitantly. The fact that the hsp90-free form of the dioxin receptor shows a dramatically reduced affinity for dioxin (39) strongly argues that the conformation of the receptor is altered upon release of hsp90. It is therefore possible that this altered receptor conformation is critical for efficient dimerization with Arnt.

Although Arnt is structurally related to the dioxin receptor, it does not appear to associate stably with hsp90 and it is not a component of the latent hsp90-dioxin receptor complex (51). It is still unclear, however, whether Arnt is a homodimer or forms heterodimeric complexes with as yet unidentified bHLH partner factors in nonstimulated target cells. We report here that a cellular factor, possibly Arnt, stimulates ligand-dependent release of hsp90 from the dioxin receptor, adding further complexity to the dioxin receptor activation pathway. In this context, it is noteworthy that specific factors might control the DNA-binding properties of bHLH factors MyoD and E47 and that this regulatory activity targets the bHLH domain of these factors (49). In fact, recently reported data suggest that hsp90 stimulates the DNA-binding activity of MyoD by modulating the conformation of the bHLH motif (45). Unlike the dioxin receptor, however, MyoD appears not to form a stable complex with hsp90, as assessed by UV cross-linking experiments, and it has therefore been proposed that the MyoD activation process involves a more transient interaction between hsp90 and MyoD (45). This observation suggests the intriguing and testable possibility that hsp90 also regulates dioxin receptor-Arnt dimerization by conformational activation of the bHLH domain of Arnt, although a putative hsp90-Arnt complex eluded detection in our immunoprecipitation assay, possibly because of its transitory nature. In any case, the present model system for activation of the dioxin receptor should provide insight into the mechanism by which the ligand controls dioxin receptor function. Purification of the stimulatory activity will permit unambiguous identification of this factor(s) and further studies on the complex functional interplay between the dioxin receptor and its bHLH partner factor, Arnt.

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