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Heat shock protein hsp90 regulates dioxin receptor function *in vivo*

(gene expression/yeast/protein folding)

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ABSTRACT **The dioxin (aryl hydrocarbon) receptor is a ligand-dependent basic helix–loop–helix (bHLH) factor that binds to xenobiotic response elements of target promoters upon heterodimerization with the bHLH partner factor Arnt. Here we have replaced the bHLH motif of the dioxin receptor with a heterologous DNA-binding domain to create fusion proteins that mediate ligand-dependent transcriptional enhancement in yeast (*Saccharomyces cerevisiae*). Previously, our experiments indicated that the ligand-free dioxin receptor is stably associated with the 90-kDa heat shock protein, hsp90. To investigate the role of hsp90 in dioxin signaling we have studied receptor function in a yeast strain where hsp90 expression can be down-regulated to about 5% relative to wild-type levels. At low levels of hsp90, ligand-dependent activation of the chimeric dioxin receptor construct was almost completely inhibited, whereas the activity of a similar chimeric construct containing the structurally related Arnt factor was not affected. Moreover, a chimeric dioxin receptor construct lacking the central ligand- and hsp90-binding region of the receptor showed constitutive transcriptional activity in yeast that was not impaired upon down-regulation of hsp90 expression levels. Thus, these data suggest that hsp90 is a critical determinant of conditional regulation of dioxin receptor function *in vivo* via the ligand-binding domain.**

Signal transduction by the environmental pollutant dioxin is mediated by the intracellular dioxin receptor, also termed the aryl hydrocarbon receptor. The receptor is a ubiquitous basic helix–loop–helix (bHLH) factor which, analogously to the structurally unrelated family of steroid hormone receptors, functions as a ligand-activated transcriptional regulator by directly recognizing cognate response elements within regulated genes (for a recent review see ref. 1). In addition to dioxin, the receptor is activated by a variety of planar aromatic ligands, including polychlorinated, polyaromatic, and polyheterocyclic hydrocarbons (reviewed in ref. 2). These ligands induce nuclear translocation of the receptor (ref. 3 and references therein) and regulate dimerization with the bHLH partner factor Arnt (1), enabling both proteins to specifically recognize xenobiotic response elements of target promoters (4–6) that are distinct from the twofold symmetric E box motif recognized by the large majority of bHLH or bHLH/leucine zipper factors (7).

In the absence of ligand, the dioxin receptor is recovered in an inducible cytoplasmic form that is characterized by stable association with the 90-kDa heat shock protein (hsp90) (8–13). Both ligand- (5, 14) and hsp90- (14) binding activities of the dioxin receptor are colocalized within the same domain within the C-terminal half of the PAS domain, a region of homology to Arnt and the *Drosophila* factors Per and Sim that is contiguous with the bHLH motif of the receptor (reviewed in ref. 15). While hsp90 is the only molecular chaperone so far demonstrated to bind the dioxin receptor, its role in receptor signaling remains unclear. The hsp90-associated form of receptor fails to dimerize with the bHLH/PAS partner factor Arnt (4), indicating that hsp90 may be important for repression of receptor function. Moreover, the hsp90-free form of receptor shows no detectable dioxin-binding activity (16), consistent with a role of hsp90 in chaperoning a high-affinity ligand-binding conformation of the receptor. In analogy to this model, certain steroid hormone receptors have been shown to be associated with hsp90, and this interaction has been postulated to be important for ligand-binding activity and/or induction of conformational changes necessary for transcriptional activation (reviewed in refs. 17 and 18).

To understand the mechanism of activation of the dioxin receptor and to examine the role of hsp90 in receptor signal transduction we have reconstituted ligand-dependent activation of the dioxin receptor in *Saccharomyces cerevisiae* by using chimeric receptor constructs. These constructs contained the DNA-binding domain of the glucocorticoid receptor fused to dioxin receptor fragments lacking their very N-terminal bHLH dimerization and DNA-binding domain, and they mediated ligand-dependent activation of a reporter gene construct in yeast. Ligand responsiveness of these chimeric receptors was severely hampered in a yeast strain in which hsp90 expression levels were down-regulated to about 5% of wild-type levels, indicating that hsp90 may be critical for folding of a functional receptor form. In contrast, the activity of a chimeric construct containing the structurally related factor Arnt was not affected at low hsp90 expression levels, consistent with the absence of any biochemical evidence demonstrating interaction between hsp90 and Arnt.

MATERIALS AND METHODS

Plasmid Constructions. To construct yeast expression vectors for dioxin receptor- or Arnt-glucocorticoid receptor chimeras, relevant fragments were digested from CMV4, GEM, or MT vectors (14, 19) and ligated into p2HG (20) by standard subcloning techniques, providing p2HG/GRDBD/mDR83-805, p2HG/GRDBD/mDR340-805, p2HG/GRDBD/mDR521-805, p2HG/GRDBD/Arnt128-774, and p2HG/GRDBD. Residues 3′ to codon 84 of the human dioxin receptor were obtained by PCR from phuAHR (21), using an upstream primer containing an *Xho* I site flanking codon 84 and a downstream T7 primer. The *Xho* I site was used to fuse human dioxin receptor sequences to GRDBD (glucocorticoid receptor DNA-binding domain) codons, and then PCR-

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Abbreviations: dioxin, 2,3,7,8-tetracholorodibenzo-*p*-dioxin; hsp90, 90-kDa heat shock protein; bHLH, basic helix–loop–helix; BNF, β-naphthoflavone (5,6-benzoflavone).

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derived dioxin receptor sequence was replaced with a native *Stu* I/*Nhe* I fragment from phuAHR, creating a final chimeric construct that was subcloned into p2HG to give p2HG/GRDBD/hDR84-848. A *Sal* I fragment was obtained from pτDBD/DR83-805/CMV4 (14) and subcloned into *Sal* I-digested pKVNX (22) to yield pτDBD/mDR83-805/KVNX. *CYC1* promoter-driven β-galactosidase reporter plasmids pLGZ-TAT (23) and pUCΔSS26X (20) containing a single or three tandem glucocorticoid response elements, respectively, have been described previously.

Yeast Strains and Transformations. Yeast strain W303-1A was transformed with pτDBD/mDR83-805/KVNX and mated with strain K396-11B containing reporter pLGZ-TAT, creating strain W303-1A/K396-11B, as described (22, 23). The yeast strain GRS4 has been described (20). Yeast transformations in GRS4 with p2HG/GRDBD/DR or p2HG/GRDBD/Arnt expression vectors together with reporter plasmid pUCΔSS26X were carried out by using a modification of the lithium acetate method (24).

β-Galactosidase Assays. Transactivation assays for τDBD/mDR83-805 were performed in strain W303-1A/K396-11B as described (22, 23). Briefly, yeast were grown through several generations in 3% glycerol/1% ethanol growth medium before 6 h of growth at 30°C in 10 ml of 2% galactose medium. Cells were pelleted and extracts were assayed for protein concentration and β-galactosidase activity as previously described (25). For quantitation of β-galactosidase reporter gene activities in GRS4 transformants, single colonies were grown to saturation in selective medium containing either 2% glucose or 2% galactose at 25°C. Cultures were diluted 1:6 in fresh medium and grown overnight in the presence or absence of specific ligand. Yeast cells were collected from the overnight culture by centrifugation and assayed for enzyme activity as previously described (25, 26).

Immunoblot Analysis. Yeast cells were grown at 25°C in appropriate selection media containing either 2% glucose or 2% galactose. Extracts were prepared as previously described (27). For assaying expression levels of glucocorticoid/dioxin receptor fusion constructs, a rabbit polyclonal antiserum raised against an N-terminal peptide of the human glucocorticoid receptor was used (PA1-511, Affinity Bioreagents, Neshanic Station, NJ), and for analysis of hsp90 expression levels a rat monoclonal antibody specific for hsp90 that cross-reacts with yeast hsp82 was used (SPA-845, StressGen Biotechnologies, Victoria, BC, Canada). Samples containing 20 μg and 5 μg of total protein extract were assayed for expression levels of fusion proteins and hsp90, respectively.

RESULTS

Ligand-Dependent Regulation of Dioxin Receptor Function in Yeast. To map ligand binding and transactivation domains of the dioxin receptor, we previously constructed chimeric receptors where the very N-terminal bHLH motif (amino acids 1–82) of the mouse dioxin receptor has been replaced by the N-terminal τ1 transactivation and zinc-finger DNA-binding domains (amino acids 1–500; here termed τDBD) of the human glucocorticoid receptor (14, 19). This N-terminal portion of the glucocorticoid receptor does not bind hsp90 (ref. 14; see ref. 17 for review). The resulting chimeric receptor, τDBD/mDR83-805 (Fig. 1*A*) shows distinct dioxin inducibility when cotransfected into mammalian cells with a glucocorticoid response element-driven reporter gene construct (14, 19). Importantly, this chimeric dioxin receptor construct functions in a dioxin-responsive manner upon transient transfection into mutant Arnt-deficient hepatoma cells and is, thus, functionally uncoupled from Arnt. We therefore wanted to examine ligand-dependent regulation of this chimeric receptor in *S. cerevisiae.*

To examine function of the dioxin receptor chimera in yeast we monitored the activity of β-galactosidase reporter genes

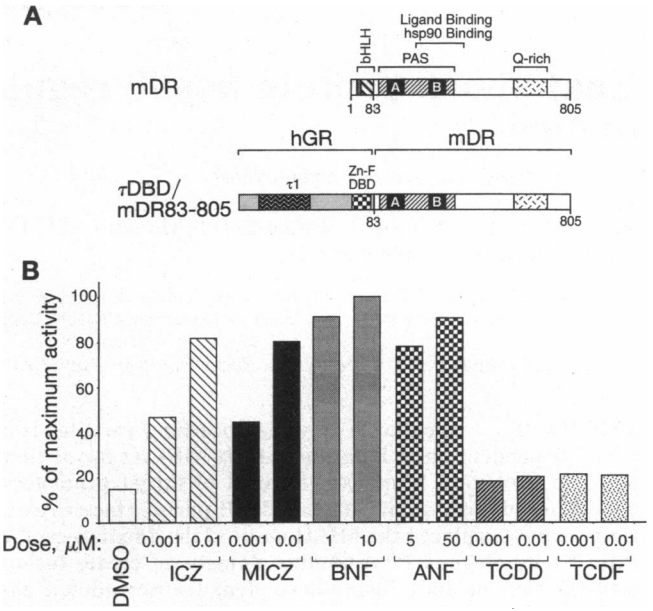

FIG. 1. Ligand-induced activation of a glucocorticoid–dioxin receptor chimera in yeast. (*A*) Structural motifs within the native mouse dioxin receptor (mDR) and in the chimeric human glucocorticoid receptor (hGR)/mouse dioxin receptor fusion protein. Zn-F DBD, zinc-finger DNA-binding domain; τ1, transactivation domain; PAS, Per/Arnt/Sim homology region; Q-rich, a motif rich in glutamine residues. (*B*) Yeast cultures (W303-1A/K396-11B) transformed with τDBD/mDR83-805 and the reporter gene pLGZ-TAT were grown in a medium containing 2% galactose and the indicated concentrations of ligands indolo[3,2-*b*]carbazole (ICZ), 5,11-dimethylindolo[3,2-*b*]-carbazole (MICZ), β-naphthoflavone (BNF), α-naphthoflavone (ANF), dioxin (TCDD; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin), 2,3,7,8-tetrachlorodibenzofuran (TCDF), or vehicle alone [0.1% dimethyl sulfoxide (DMSO)] and assayed after 6 h for β-galactosidase activity.

driven by the yeast *CYC1* promoter linked to single or multimerized motifs of the glucocorticoid response element of the tyrosine aminotransferase gene. Dose-dependent induction of the reporter gene by the τDBD/mDR83-805 chimeric receptor was observed when yeast cells were treated with 1–10 nM indole derivatives indolo[3,2-*b*]carbazole or 5,11-dimethylindolo[3,2-*b*]carbazole, two high-affinity dioxin receptor ligands (ref. 28 and references therein). Considerably higher doses (1–10 μM) of the weaker dioxin receptor agonist BNF were required to achieve similar levels of induction. Moreover, even higher doses of the partial dioxin receptor agonist α-naphthoflavone were required to produce this response (Fig. 1*B*). The rank order of potencies of these classes of ligands in yeast is in good agreement with that observed in mammalian cells (refs. 29 and 30 and references therein). Thus, the chimeric dioxin receptor construct reconstituted ligand-dependent regulation of dioxin receptor function in yeast. In contrast to mammalian cells (14), the τDBD/mDR83-805 fusion protein was not activated by exposure to polychlorinated hydrocarbons—e.g., dioxin (TCDD) or 2,3,7,8-tetrachlorodibenzofuran—when expressed in yeast (Fig. 1*B*), possibly due to the pronounced hydrophobicity of this class of ligands, which might impair uptake into the cells. It is formally possible, however, that other mechanisms underlie the observed difference in relative potency of the different receptor ligands in yeast. By analogy, two potent glucocorticoid receptor agonists in mammalian cells, dexamethasone and triamcinolone acetonide, show very poor agonist activity in yeast (22, 25, 26).

Reduced Levels of hsp90 Impair the Dioxin Receptor Activation Response. Given ligand-dependent regulation of dioxin receptor activity in yeast, we next wanted to assess ligand (BNF) responsiveness of chimeric glucocorticoid-dioxin re-

ceptor constructs in a yeast strain, GRS4, which lacks both chromosomal genes encoding the yeast homologues of mammalian hsp90 (*HSP82* and *HSC82*, respectively) and carries a low-copy *HSP82* plasmid under the control of the galactose-inducible *GAL1* promoter (20). hsp82 (63% identical to human hsp90) specifically interacts with the dioxin receptor as does mammalian hsp90 (J.M., unpublished results), and for simplicity therefore will subsequently be referred to as hsp90. In the presence of galactose GRS4 cells express wild-type levels of yeast hsp90, whereas the expression levels are down-regulated about 20-fold in glucose, as assessed by immunoblot analysis (ref. 20; Fig. 2*A*).

Since the human dioxin receptor has been reported to bind dioxin with an affinity about 1/10 that of the mouse dioxin receptor (30, 31) we initially examined ligand responsiveness in GRS4 cells of either mouse chimera GRDBD/mDR83-805 or a chimera containing the corresponding fragment of the human dioxin receptor linked to the DNA-binding domain (GRDBD) of the glucocorticoid receptor, GRDBD/hDR84-848 (Fig. 3*A*). These simplified chimeras lack the glucocorticoid receptor τ1 transactivation domain, omitted here to ensure that any observed transactivation functions would be derived from the dioxin receptor portion of the chimeras. In the presence of galactose, GRDBD/mDR83-805 activity was induced by BNF in a dose-dependent manner (Fig. 3*B*). Thus, our chimeric receptor model system faithfully reconstituted ligand responsiveness in yeast of the dioxin receptors. Interestingly, full induction of reporter gene activity by the human dioxin receptor chimera GRDBD/hDR84-848 required higher doses of BNF (Fig. 3*B*), suggesting a difference in affinity for BNF of about one order of magnitude between the mouse and human dioxin receptors.

Surprisingly, at low levels of hsp90 expression in glucose we failed to induce any significant levels of reporter gene activity at any of the concentrations of BNF tested. At the highest dose of BNF tested (10 μM), maximally a 2-fold induction response was observed in the presence of glucose (Fig. 3*B*). It was not possible to significantly increase the dose of BNF, since these concentrations of ligand appeared to be toxic to the cells (data not shown). Although the cells expressing low levels of hsp90 (Fig. 2*A*) failed to promote an induction response, they did not display any differences in expression of the fusion protein construct versus the cells expressing wild-type levels of hsp90, as assessed by immunoblotting (Fig. 2*B*). In conclusion, hsp90 expression levels constituted a critical parameter for determining ligand responsiveness of the dioxin receptor in yeast cells.

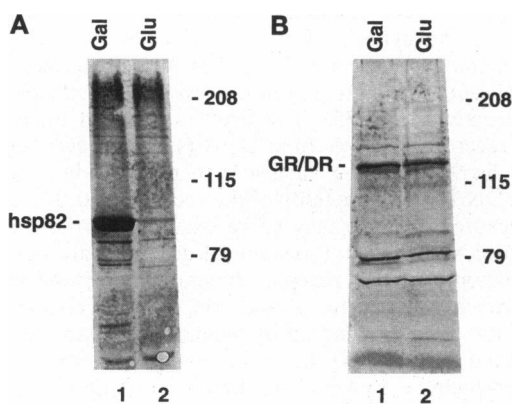

FIG. 2. Expression of yeast hsp90 but not a glucocorticoid–dioxin receptor chimera is reduced in glucose growth medium. GRS4 cells expressing the GRDBD/hDR84-848 fusion protein were grown in either galactose (Gal) or glucose (Glu) medium, as indicated, and whole cell extracts were analyzed by immunoblotting to monitor either yeast hsp90 (hsp82) (*A*) or GRDBD/hDR84-848 (GR/DR) (*B*) expression levels. Positions of molecular mass markers are given in kDa.

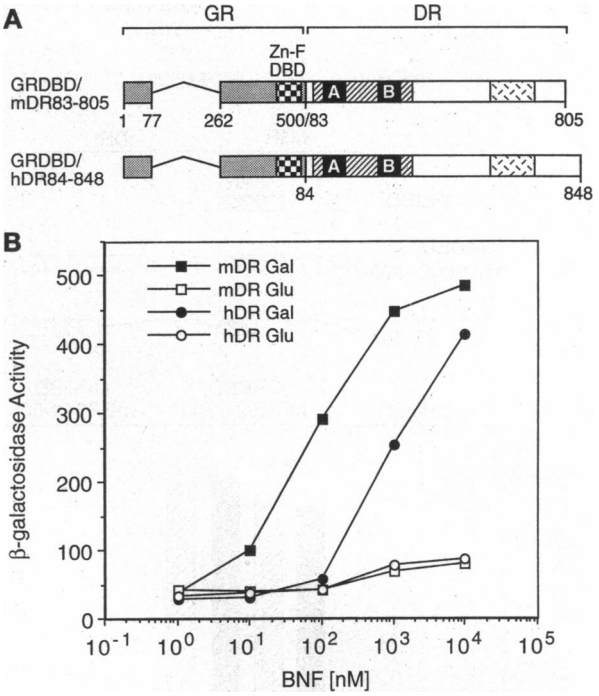

FIG. 3. Reduced levels of hsp90 abrogate dioxin receptor activation. (*A*) Schematic representation of the chimera between the glucocorticoid receptor (GR) and mouse or human dioxin receptors (DR). GRDBD, DNA-binding domain of the glucocorticoid receptor. (*B*) BNF dose–response curves of GRDBD/mDR83-805 (squares) and GRDBD/hDR84-848 (circles) expressed in GRS4 under conditions of normal (filled symbols) or low hsp90 levels (open symbols). Cultures were grown overnight with the indicated carbon source (galactose, Gal; or glucose, Glu) and BNF concentration before being assayed for β-galactosidase reporter gene activity. Data represent average values from two samples assayed in a given experiment.

The Activity of the C-Terminal Transactivation Domain of the Dioxin Receptor Is Not Affected by Down-Regulation of hsp90 Expression. The dioxin receptor contains a strong transactivation domain within its C terminus (19). For instance, fusion of a mouse dioxin receptor fragment spanning amino acids 521–805 to the glucocorticoid receptor DNA-binding domain produces a potent, constitutively active transcription factor upon transient expression in mammalian cells (19). In GRS4 yeast cells grown in galactose this chimeric receptor, GRDBD/DR521-805 (Fig. 4*A*), also showed strong constitutive activity (Fig. 4*B*). In contrast to dioxin receptor derivatives containing the ligand-binding domain, the activity of GRDBD/DR521-805 was not down-regulated by growing the cells in glucose (Fig. 4*B*). As a reference, the glucocorticoid receptor portion of this chimera, GRDBD, showed only minimal background activity, whether grown in glucose or galactose, and was not affected by ligand (Fig. 4*B*). These results demonstrate that the C-terminal transactivation domain of the dioxin receptor stimulates transcription with equal efficacy in cells expressing low or wild-type levels of hsp90, strongly arguing that this domain is not a target for regulation by hsp90.

We next examined the activity of a chimeric receptor construct, GRDBD/DR340-805, that contains only a part of the minimal ligand-binding domain located between amino acids 230 and 421 (Fig. 4*A*). The dioxin receptor portion of this chimera fails to bind ligand but forms a stable complex with hsp90 *in vitro* (19). Consistent with these biochemical characteristics, this chimera is irreversibly repressed upon expression in mammalian cells (19). Similarly, GRDBD/DR340-805 showed low levels of constitutive activity relative to the GRDBD fragment alone when expressed in GRS4 cells in the presence of galactose (Fig. 4*B*). A simple model for repression

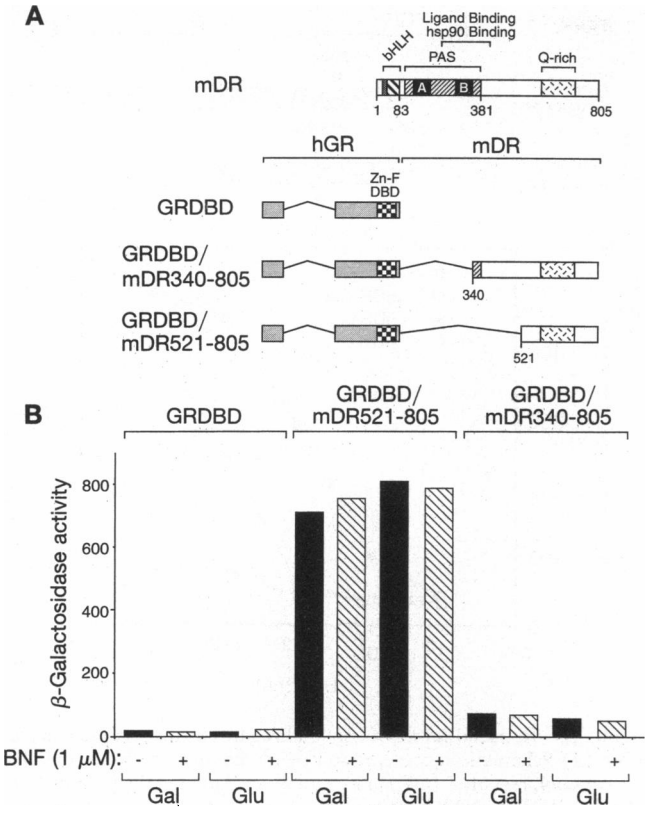

FIG. 4. Activity of the dioxin receptor C-terminal transactivation domain is unaltered under conditions of low hsp90 expression. (*A*) Schematic representation of GRDBD fused to C-terminal fragments of the mouse dioxin receptor. (*B*) GRDBD and mouse dioxin receptor chimeras GRDBD/mDR340-805 and GRDBD/mDR521-805 were expressed under conditions of normal (Gal) or low (Glu) hsp90 levels in GRS4, in the presence or absence of 1 μM BNF, and assayed for β-galactosidase activity. Data represent average values from two samples assayed in a given experiment.

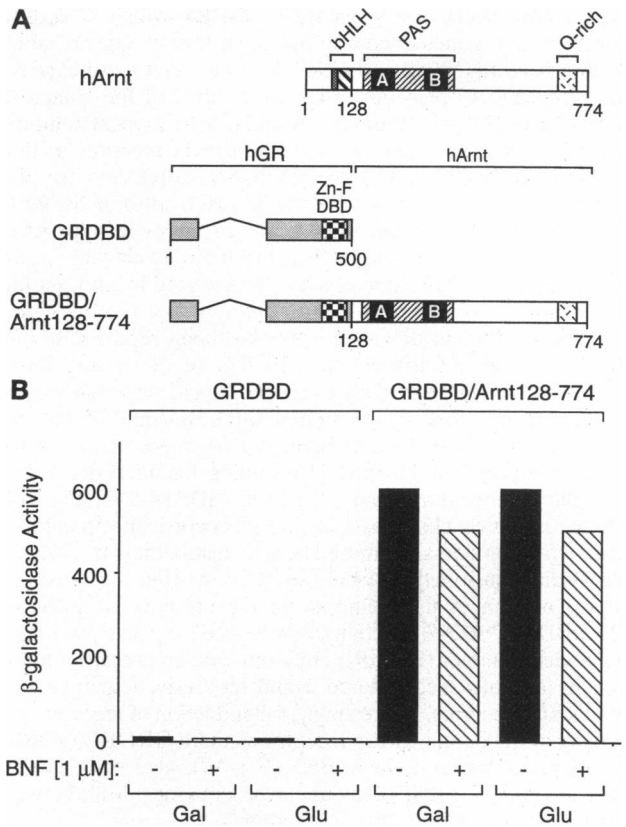

FIG. 5. Activity of the bHLH/PAS dioxin receptor partner factor Arnt is unaffected by reduced levels of hsp90. (*A*) Schematic representation of a glucocorticoid receptor-Arnt chimera lacking the N-terminal bHLH region of Arnt. (*B*) β-Galactosidase activities of GRDBD and chimera GRDBD/Arnt128-774 expressed in GRS4 in normal (Gal) or low (Glu) hsp90 environments, in the presence or absence of 1 μM BNF. Data represent average values from two samples assayed in a given experiment.

would be that hsp90 is constitutively associated with the remaining portion of the ligand-binding domain and, in contrast to the intact ligand-binding domain, ligand treatment fails to induce release of hsp90. In the presence of glucose, however, the activity of GRDBD/DR340-805 remained unaltered (Fig. 4*B*), demonstrating that down-regulation of hsp90 did not result in derepression of this chimera. Thus, in contrast to the constitutively active GRDBD/DR521-805 chimera, this dioxin receptor derivative is functionally inert at low levels of hsp90, arguing that the remaining region of the ligand binding domain possibly confers malfolding to structures located further from the C terminus.

hsp90 Is Not Important for Function of the bHLH/PAS Dioxin Receptor Partner Factor Arnt. As schematically represented in Fig. 1*A*, the ligand- and hsp90-binding domain of the dioxin receptor is contained within the C-terminal half of the PAS motif. As in the dioxin receptor the Arnt partner factor also contains a PAS motif contiguous with the bHLH domain (Fig. 5*A*). However, this factor does not interact *in vitro* with hsp90, as assessed by coimmunoprecipitation assays (11, 32). Fusion of the glucocorticoid receptor DNA-binding domain (GRDBD) to an Arnt fragment lacking the N-terminal bHLH domain creates a constitutively active transcriptional regulator, GRDBD/Arnt128-774, the activity of which is not affected by treatment of mammalian cells with dioxin receptor ligands (19). In a similar fashion GRDBD/Arnt128-774 activity was not altered by BNF treatment when expressed in GRS4 cells in the presence of galactose (Fig. 5*B*). Moreover, down-regulation of hsp90 expression in the presence of glucose did not affect the activity of this fusion protein. Thus, in agreement with the failure to observe any association of Arnt with hsp90 *in vitro*, hsp90 does not appear to play any role in modulating functional activity of the bHLH/PAS factor Arnt.

DISCUSSION

hsp90 is the most abundant constitutively expressed stress protein in the cytosol of eukaryotic cells (see ref. 33 for review), and it has recently been shown to chaperone protein folding *in vitro*, preventing protein aggregation and modulating protein activity (34, 35). The hsp90-associated form of the dioxin receptor does not bind DNA (13). However, dissociation of hsp90 *in vitro* is sufficient to unmask the previously cryptic DNA-binding activity of the receptor (10, 16), arguing that receptor function may be repressed by protein–protein interaction with hsp90. Biochemical data indicate that hsp90 does not only repress receptor function by a possible steric interference mechanism. In addition, hsp90 seems to be required for efficient binding of ligand. Once the receptor is dissociated from hsp90, it is not possible to form a stable receptor–ligand complex (16). These observations are consistent with the model that hsp90 acts as a molecular chaperone on the receptor, determining the ability of the protein to assume and/or maintain a ligand-binding conformation and thus facilitating subsequent functional responses of the receptor to the extracellular signal.

In the present study, dioxin receptor function is inhibited at low levels of hsp90 expression, suggesting that the receptor may be generally malfolded. The activity of dioxin receptor

constructs lacking the ligand-binding domain but containing the C-terminal transactivation domain was not affected by reduced levels of hsp90, suggesting that a central portion of the dioxin receptor spanning the ligand-binding domain is the target for regulation by hsp90. Thus, these data provide *in vivo* evidence that hsp90 is required for signaling by the dioxin receptor. This function of hsp90 is specific for the dioxin receptor, since the structurally related bHLH/PAS partner factor Arnt did not require hsp90 for activation of transcription.

As in the dioxin receptor system (16), release of hsp90 from the glucocorticoid receptor *in vitro* is accompanied by a loss of high-affinity ligand-binding activity (reviewed in refs. 17 and 18). It appears, therefore, that hsp90 serves as a cellular chaperone molecule which, in addition to repressing receptor activity in nonstimulated cells, directly determines signal responsiveness of distinct nuclear receptors (including the dioxin and glucocorticoid receptors) by mediating correct folding of the ligand-binding domains of these proteins into a high-affinity binding conformation. In support of this model, the glucocorticoid receptor exhibits a decreased hormone responsiveness when expressed in the GRS4 yeast strain at reduced levels of hsp90 (20). Despite hsp90 showing similar regulatory activities on the dioxin and glucocorticoid receptors, no obvious sequence similarities exist between their hsp90 binding regions. In a fashion similar to the dioxin receptor, agonist ligands induce nuclear import of the glucocorticoid receptor (reviewed in ref. 17). It will be of interest to determine whether the dioxin and glucocorticoid receptors gain constitutive entry into the nucleus in the low-hsp90 environment. Alternatively, ligand binding at wild-type levels of hsp90 may constitute an essential trigger for the nuclear translocation event. In this context it will also be important to determine whether ligand binding induces a conformational change that is required for efficient release of hsp90 and nuclear transport and to address the issue whether this conformational change is necessary for transcriptional activation. A yeast model system has recently been used to isolate hsp90 mutants which decrease the function of a number of steroid receptors, including the glucocorticoid receptor. Similar to the results obtained with reduced levels of hsp90, such hsp90 mutants negatively regulated the ability of the glucocorticoid receptor to be activated in response to hormone treatment (27). It will now be important to examine whether these hsp90 mutants also affect signal transduction by the dioxin receptor or whether these mutants specifically affect glucocorticoid receptor function.

Given the dramatic effects on dioxin receptor function that were observed at low levels of hsp90 in GRS4 cells, the present yeast model system should provide a useful tool for investigating mechanistic aspects in regulation of dioxin receptor signaling by the interesting molecular chaperone hsp90. The yeast model system will be particularly useful in a further mutational and functional analysis of the ligand-binding domain of the dioxin receptor. Finally, it will be a critical tool to examine whether the mechanisms of conditional regulation of the two structurally distinct ligand-inducible transcription factors, the dioxin and glucocorticoid receptors, are related.

Note Added in Proof. While this manuscript was in review, Carver *et al.* (36) also demonstrated that hsp90 is required for dioxin receptor signaling in yeast.

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