The intracellular dioxin (aryl hydrocarbon) receptor is a ligand-activated transcription factor that mediates the adaptive and toxic responses to environmental pollutants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin and structurally related congeners. Whereas the ligand-free receptor is characterized by its association with the molecular chaperone hsp90, exposure to ligand initiates a multistep activation process involving nuclear translocation, dissociation from the hsp90 complex, and dimerization with its partner protein Arnt. In this study, we have characterized a dioxin receptor deletion mutant lacking the minimal ligand-binding domain of the receptor. This mutant did not bind ligand and localized constitutively to the nucleus. However, this protein was functionally inert since it failed to dimerize with Arnt and to bind DNA. In contrast, a dioxin receptor deletion mutant lacking the minimal PAS B motif but maintaining the N-terminal half of the ligand-binding domain showed constitutive dimerization with Arnt, bound DNA, and activated transcription in a ligand-independent manner. Interestingly, this mutant showed a more potent functional activity than the dioxin-activated wild-type receptor in several different cell lines. In conclusion, the constitutively active dioxin receptor may provide an important mechanistic tool to investigate receptor-mediated regulatory pathways in closer detail.

The intracellular dioxin receptor also known as the aryl hydrocarbon receptor, is a ligand-dependent transcription factor that mediates the biological effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), commonly known as dioxin (1). Although disruption of the dioxin receptor gene in mice has not yielded conclusive results, it remains a possible scenario that physiological mechanisms may exist for activation of receptor function, e.g., during critical stages of vertebrate development (2–5). However, an endogenous ligand for the receptor has not yet been identified, suggesting that alternative pathways for receptor activation may exist.

In the absence of ligand, the dioxin receptor is generally found in the cytoplasm associated in high molecular weight complexes comprising a dimer of the molecular chaperone hsp90, an immunophilin homolog known as XAP2 (hepatitis B virus X-associated protein-2)/AIP (aryl hydrocarbon receptor-interacting protein)/ARA9 (aryl hydrocarbon receptor-associated protein-9), and the co-chaperone p23 (6–11). In the presence of dioxin, the receptor is converted to a functional DNA-binding species in a multistep process involving nuclear translocation, dissociation from the hsp90 complex, and dimerization with its partner protein Arnt (Ah receptor nuclear translocator). Formation of the dioxin receptor/Arnt heterodimer is a prerequisite for DNA binding and promotes transcription of target genes including those encoding xenobiotic-metabolizing enzymes such as CYP1A1 (1). Both the dioxin receptor and Arnt are members of a distinct subclass of the basic helix-loop-helix (bHLH) family of transcriptional regulators known as bHLH/PAS proteins. Members of this potent subfamily mediate diverse biological processes, including response to hypoxia, circadian rhythmicity, and development of the central nervous system (1, 12, 13). Contiguous to the amino-terminal bHLH DNA-binding and dimerization motifs, members of this subfamily are identified on the basis of a second region of homology, the PAS (Per-Arnt-Sim) domain, originally identified in the Drosophila proteins PER and SIM, and Arnt. The PAS domain encompasses a region of ~250–300 amino acids harboring two degenerate repeat sequences of 44 amino acids termed PAS A and PAS B, respectively, and has been shown to constitute an additional dimerization interface that can function independently of the bHLH motif (14, 15). More recently, additional roles in the regulation of heterodimerization and DNA binding specificities have been attributed to the PAS domain (16, 17). In the case of the dioxin receptor, the carboxyl-terminal half of the PAS domain, spanning the hydrophobic PAS B repeat motif, has been shown to harbor the core ligand-binding activity of the receptor (18, 19). In addition to binding ligand, this region has also been shown to mediate association with the molecular chaperone hsp90 (19), consistent with a role for hsp90 in regulating signal responsiveness by folding the ligand-binding domain (LBD) into...
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a high affinity ligand binding conformation (20–22). Taken together, the PAS domain appears to be a complex structure harboring a number of distinct functional activities.

In our efforts to understand further the complex interplay between structure and function of the dioxin receptor, we have examined the functional activity of a dioxin receptor deletion mutant lacking the core LBD. Although this protein is constitutively localized to the nucleus, it was functionally inert on a xenobiotic response element (XRE)-driven reporter gene both in the presence and absence of ligand. In contrast, deletion of amino acids 288–421 encompassing the minimal PAS B motif generated an activated form of the receptor that stimulated transcription in the absence of ligand, demonstrating that this mutant functions as a constitutively active regulatory protein. Interestingly, this mutant showed a more potent functional activity than the dioxin-activated wild-type receptor, possibly indicating that exposure to ligand does not induce maximal activation of the receptor.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—The plasmids used for in vitro translation of the full-length dioxin receptor (mDR/H9262) and for wild-type (Arnt/H11032) and mutant (Arnt/H9004) mDR constructs have been described previously (23, 24). For construction of the dioxin receptor deletion mutants DRαLBD and DRαPASB, pSPORTAhR containing full-length murine dioxin receptor cDNA (18) was amplified by PCR using primers designed to yield fragments of the dioxin receptor encoding codons 1–229 and 1–287, respectively. Primers were designed to carry restriction sites for cloning into the yeast expression plasmid pGFP-mDR/CMX (25). The resulting fragment was then inserted to provide the final construct pGFP-mDR/CMX (previously referred to as pGRDBDmDR83). Transformation of GR4 transformants was carried out as described previously (25). Transformation of GR4 with expression plasmids for the indicated glucocorticoid receptor/dioxin receptor fusion proteins together with the reporter plasmid pUC4SS26X was carried out using a modification of the lithium acetate method (30). Quantitation of β-galactosidase reporter gene activity in GR4 transformants was carried out as described previously (25).

**Subcellular Localization Assay Using GFP Fusion Proteins**—CHO cells were grown on poly-L-lysine-coated sterile coverslips in 35-mm diameter dishes and transfectionally transfected with 1 μg of each expression plasmid for GFP fusion proteins using 2 μl of FuGENE 6 transfection reagent (Roche Molecular Biochemicals) per dish. After a 6-h incubation, the medium was replaced with fresh medium, and incubation was continued for a further 24 h. The cells were then treated with 10 nm TCDD (Cambridge Isotope Laboratories) or with an equivalent volume of vehicle (Me2SO) alone. Following incubation for 48 h, cells were collected and washed with PBS; extracts were prepared; and luciferase activity was measured. Experiments were carried out in duplicate, and extracts were normalized for protein concentration. For immunoblot analysis, transfected CHO cells were collected, washed with PBS, and lysed in 50 μl of whole cell extract buffer (20 μM Hesper, pH 7.9, 1.5 mM MgSO4, 0.2 mM EDTA, 0.42 mM NaCl, 0.5% (v/v) Nonidet P-40, 25% (v/v) glycerol, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) on ice for 30 min. Following centrifugation, the resulting supernatants were used as whole cell extracts. The sample containing 5 μg of protein was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto nitrocellulose membranes. Immunodetection was achieved by incubation with mouse monoclonal anti-FLAG antibodies (Sigma), followed by chemiluminescence using the ECL detection system (Amersham Pharmacia Biotech).

**Yeast Strains, Transformations, and β-Galactosidase Assays**—The yeast strain GS4 has been described previously (29). Transformation of GS4 with expression plasmids for the indicated glucocorticoid receptor/dioxin receptor fusion proteins together with the reporter plasmid pUC4SS26X was carried out using a modification of the lithium acetate method (30). Quantitation of β-galactosidase reporter gene activity in GS4 transformants was carried out as described previously (29).

**In Vitro Translated and Co-immunoprecipitation Experiments**—The wild-type dioxin receptor, the dioxin receptor deletion constructs DRαLBD and DRαPASB, and Arnt were translated in vitro in the presence or absence of [35S]methionine (Amerham Pharmacia Biotech) in rabbit reticulocyte lysate (Promega) according to the manufacturer’s recommendations. For Arnt co-immunoprecipitation experiments, equal concentrations of the indicated in vitro translated, [35S]methionine-labeled proteins were incubated with in vitro translated, unlabeled Arnt (5 μl) in TEG buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% (w/v) glycerol, and 1 mM dithiothreitol) containing Complete™ mini protease inhibitors (Roche Molecular Biochemicals) in the presence or absence of 10 nm TCDD (dioxin) either for 2 h at 25°C or overnight at 4°C in a final volume of 10 μl. Protein mixtures were then precleared by incubation on ice with 10 μl of preimmune serum for 15 min by an additional 5-min digestion with 50 μl of protein A-Sepharose in TEG buffer supplemented with 150 μM NaCl, 0.2% Triton X-100, and 2 mg/ml bovine serum albumin. Following rapid centrifugation, the resulting supernatants were incubated with 10 μl of either anti-Arnt antisemur (24) or preimmune serum for 1 h at room temperature. Protein A-Sepharose was then added (50 μl of a 50% slurry in supplemented TEG buffer), and the samples were
incubated on ice for a further 45 min. After brief centrifugation, Sepha-
rose pellets were washed three times with 500 μl of supplemented TEG
buffer, followed by a final wash with TEG buffer alone. Co-immunopre-
cipitated proteins were eluted by boiling in SDS sample buffer and
analyzed by SDS-PAGE and chemiluminescence.

**DNA Binding Assay**—DNA binding experiments were performed
with in vitro translated, unlabeled Arnt together with the wild-type
dioxin receptor or the dioxin receptor deletion mutants DRΔLBD and
DRΔLBD as described previously (31). Briefly, equivalent concentra-
tions of the indicated dioxin receptor mutants fused to GFP. Twenty-four hours after transfection, the cells were treated with 10 nM
TCDD or vehicle alone (0.1% Me2SO) for 2 h as indicated. Cells were then washed with PBS and fixed with 4% paraformal-
dehyde as described under “Experimental Procedures.” Subcel-
ular localization of the expressed GFP fusion proteins was
observed using a Leica DMRXA fluorescent microscope with a GFP filter set. Ap-
proximately 200 cells were observed, and representative images are shown. The ex-
periments were repeated at least three times for each GFP fusion construct with
similar results. Original magnification was ×400. C, CHO cells were transiently trans-
sected with the XRE-driven luciferase reporter gene pTX.DIR together with
either the wild-type dioxin receptor or the dioxin receptor deletion mutant DRΔLBD
and Arnt. Following transfection, cells were treated with either 10 nM
TCDD (black bars) or vehicle alone (0.1% Me2SO; shaded bars). The control lanes (CTRL) represent activity from the
reporter alone and empty expression vector. Data are from one experiment performed in duplicate and are representative of three independent experiments.

**Fig. 1.** A dioxin receptor deletion mutant lacking the minimal LBD is
constitutively localized to the nu-
cleus, but is functionally inert on an
XRE-driven reporter gene in CHO
cells. A, shown is schematic representa-
tion of the structural motifs within the
full-length mouse dioxin receptor (mDR)
and the dioxin receptor deletion mutant
DRΔLBD. B, shown is the subcellular
distribution of GFP-dioxin receptor fusion
proteins. CHO cells grown on glass covers-
slips were transiently transfected with 1
μg of each expression plasmid for the
indicated dioxin receptor mutants fused to
GFP. Twenty-four hours after transfec-
tion, the cells were treated with 10 nM
TCDD or vehicle alone (0.1% Me2SO) for
2 h as indicated. Cells were then washed
with PBS and fixed with 4% paraformal-
dehyde as described under “Experimental
Procedures.” Subcellular localization of
the expressed GFP fusion proteins was
observed using a Leica DMRXA fluorescent
microscope with a GFP filter set. Ap-
approximately 200 cells were observed, and
representative images are shown. The ex-
periments were repeated at least three
times for each GFP fusion construct with
similar results. Original magnification
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sected with the XRE-driven luciferase
reporter gene pTX.DIR together with
either the wild-type dioxin receptor or the
dioxin receptor deletion mutant DRΔLBD
and Arnt. Following transfection, cells
were treated with either 10 nM
TCDD (black bars) or vehicle alone (0.1% 
Me2SO; shaded bars). The control lanes
(CTRL) represent activity from the
reporter alone and empty expression vector. Data are from one experiment performed in duplicate and are representative of three independent experiments.
translated, unlabeled wild-type dioxin receptor or the indicated dioxin receptor deletion mutants were made up to a final volume of 10 μl with blank reticulocyte lysate translation mixture, followed by dilution with 3 volumes of TEG buffer containing 2 mM dithiothreitol, 5 μg/ml protease inhibitor mixture (aprotinin, leupeptin, and pepstatin A), and 1 mM phenylmethylsulfonyl fluoride. The reaction mixtures were then incubated with 1 nM [3H]TCDD (40 Ci/mmol; Chemsyn, Lenexa, KS) in the presence or absence of a 150-fold molar excess of the specific competitor tetrachlorodibenzo-furan at room temperature for 90 min. Following incubation, the reaction mixture were treated with 50 μl of a 10% slurry of dextran-coated charcoal in TEG buffer for 5 min on ice, followed by centrifugation. The resulting supernatants were then incubated with 50 μl of a 50% slurry of hydroxylapatite in TEG buffer on ice for 30 min. Following rapid centrifugation, the supernatants were discarded, and the remaining pellets were washed four times with 500 μl of ice-cold TEG buffer containing 0.1% Tween 20. The pellets were eluted by incubating twice with 500 μl of ethanol, and the pooled supernatants were analyzed by scintillation counting.

RESULTS

A Dioxin Receptor Deletion Mutant Lacking the Minimal LBD Is Constitutively Localized to the Nucleus, but Is Functionally Inert—In the absence of ligand, the dioxin receptor exists in the cytoplasm in a latent, non-DNA-binding form characterized by association with the heat shock protein hsp90. Exposure to ligand results in rapid nuclear accumulation of the receptor and conversion to a heterodimeric complex with Arnt, a form that is now competent to bind DNA and to initiate the transcription of target genes (1). Thus, the initial step in the activation of dioxin receptor function is binding of ligand. In addition to being a critical determinant of ligand-binding activity, the LBD of the dioxin receptor has also been postulated to be involved in the repression of a number of receptor activities that map outside the LBD itself such as dimerization with Arnt, DNA binding, and transcriptional activity (19, 26, 33). It has not yet been determined how the ligand-binding domain modulates this repressive activity. However, since hsp90 binding has been shown to colocalize within this region, it has been suggested that hsp90 itself may function as the agent of repression either by steric interference or by misfolding of adjacent structures (19, 22). We were interested to determine whether deletion of the minimal region of the dioxin receptor harboring the ligand- and hsp90-binding activities of the receptor would result in a protein that was uncoupled from regulation by dioxin. To this end, we have examined the functional activities, both in vitro and in vivo, of a dioxin receptor deletion mutant (DRΔLBD) lacking the core-delineated LBD that is located between amino acids 230 and 421 (19, 21) and that spans the C-terminal half of the PAS domain of the mouse dioxin receptor (Fig. 1A). In control experiments, in vitro translation of DRΔLBD in reticulocyte lysates resulted in a protein that had lost the ability to bind ligand, consistent with deletion of the minimal domain required for high affinity ligand binding of the dioxin receptor (data not shown). Furthermore, in a specific co-immunoprecipitation assay using monoclonal anti-hsp90 antibodies, this protein showed only low levels of interaction with hsp90 (data not shown) that were attributed to non-LBD interactions via the bHLH motif (31).

A bipartite nuclear localization signal (NLS) has been identified in the N terminus of the dioxin receptor, incorporating basic residues within the DNA-binding domain of the bHLH motif. This single N-terminal NLS motif has been shown to be sufficient to mediate ligand-inducible nuclear import of the
dioxin receptor in the context of the full-length protein (34). Using expression vectors carrying GFP fused in frame with either the full-length mouse dioxin receptor or the dioxin receptor deletion mutant DRALBD, we examined the effect of deletion of the minimal LBD on intracellular localization of the dioxin receptor in living cells. In control experiments, transient transfection of CHO cells with the parental GFP construct alone revealed a uniform distribution of fluorescence throughout the cell that was unaffected by the presence of ligand (Fig. 1B). Transient expression of the GFP-dioxin receptor fusion construct resulted in a similar distribution as the parental GFP vector alone in untreated cells. As expected, upon exposure to ligand, fluorescence rapidly accumulated in the nuclear compartment of the cell (Fig. 1B) (34–36). In contrast, however, expression of the GFP-DRAALBD fusion construct showed constitutive nuclear localization in CHO cells even in the absence of ligand (Fig. 1B). Thus, deletion of the minimal LBD was sufficient to unmask the potent constitutive NLS activity contained within the remaining structure of the receptor. Furthermore, expression of a GFP fusion construct containing the minimal LBD of the dioxin receptor encompassing amino acids 230–421 resulted in constitutive cytoplasmic fluorescence that was unaffected by the addition of ligand (Fig. 1B). Taken together, the results indicate that the region encompassing the C-terminal portion of the PAS domain harbors a structure(s) capable of repressing nuclear import of the receptor. Since this region directly coincides with the core LBD of the receptor, the results are consistent with a model whereby, in the context of the full-length receptor, the ligand-binding domain is capable of mediating conditional repression on a distant NLS.

To determine whether the nuclear localized, LBD-deficient mutant of the dioxin receptor was transcriptionally active, we next analyzed reporter gene activity by cotransfection with an XRE-driven luciferase reporter gene in CHO cells (Fig. 1C). In control experiments, coexpression of the wild-type dioxin receptor together with Arnt resulted in dioxin-dependent activation of reporter gene activity. To our surprise, however, coexpression of Arnt together with the dioxin receptor deletion mutant DRALBD failed to stimulate reporter gene activity in the presence or absence of dioxin. Thus, whereas deletion of the minimal ligand/hsp90-binding region of the dioxin receptor was sufficient to unmask or derepress the constitutive NLS activity in the bHLH domain of the receptor, the resulting nuclear localized protein failed to function as a ligand-independent transcriptional activator.

DRALBD Is Impaired in Its Ability to Interact with Arnt and Fails to Recognize the XRE Sequence Motif—The C terminus of the dioxin receptor harbors potent transactivation domains that, when attached to a heterologous DNA-binding domain, can function autonomously, displaying constitutive transcrip-
Specificity of DNA-binding complexes was analyzed by incubation with receptor-specific (PASB). The positions of protein mass markers are shown on the left. C, equal concentrations of the in vitro translated, [35S]methionine labeled wild-type dioxin receptor or the dioxin receptor deletion mutant DRAPASB were incubated with an equal concentration of unlabeled Arnt in the presence or absence of 10 nM TCDD as indicated. Co-immunoprecipitation was carried out with Arnt-specific antiserum, and the resulting co-immunoprecipitated products were visualized by SDS-PAGE and fluorography. D, in vitro translated, unlabeled DRAPASB (lane 1) or DRAPASB together with an equal concentration of unlabeled Arnt (lanes 2–6) was incubated for 2 h at 25 °C in the absence (lanes 1, 2, and 4–6) or presence (lane 3) of specific ligand. DNA-binding activity was assessed by a gel mobility shift assay using a 32P-labeled XRE probe. Specificity of DNA-binding complexes was analyzed by incubation with receptor-specific (aDR; lane 4), Arnt-specific (aArnt; lane 5), or preimmune (P.I.S.; lane 6) serum. The positions of free probe (Free), the dioxin receptor deletion mutant-Arnt complex (DRAPASB/Arnt), and a supershifted complex (SS) are indicated.

Fig. 4. DRAPASB shows both constitutive dimerization activity with Arnt and constitutive interaction with the XRE sequence motif. A, shown is a schematic representation of the full-length mouse dioxin receptor (mDR) and the dioxin receptor deletion mutant lacking the C-terminal half of the minimal LBD (termed DRAPASB). B, shown are the results from the analysis of the in vitro translated, [35S]methionine-labeled wild-type dioxin receptor or the dioxin receptor deletion mutant DRAPASB. The positions of protein mass markers are shown on the left. C, equal concentrations of the in vitro translated, [35S]methionine dioxin receptor or DRAPASB were incubated with an equal concentration of unlabeled Arnt in the presence or absence of 10 nM TCDD as indicated. Co-immunoprecipitation was carried out with Arnt-specific antiserum, and the resulting co-immunoprecipitated products were visualized by SDS-PAGE and fluorography. D, in vitro translated, unlabeled DRAPASB (lane 1) or DRAPASB together with an equal concentration of unlabeled Arnt (lanes 2–6) was incubated for 2 h at 25 °C in the absence (lanes 1, 2, and 4–6) or presence (lane 3) of specific ligand. DNA-binding activity was assessed by a gel mobility shift assay using a 32P-labeled XRE probe. Specificity of DNA-binding complexes was analyzed by incubation with receptor-specific (aDR; lane 4), Arnt-specific (aArnt; lane 5), or preimmune (P.I.S.; lane 6) serum. The positions of free probe (Free), the dioxin receptor deletion mutant-Arnt complex (DRAPASB/Arnt), and a supershifted complex (SS) are indicated.

Constitutive dioxin receptor mutant

In the natural structural context of the intact receptor, however, transcriptional activity requires the presence of ligand, suggesting an additional role for the LBD in regulation of the distal transactivation function (26). Consequently, we wanted to determine whether deletion of the minimal LBD in DR-LBD had been sufficient to derepress the potent transcriptional activity inherent in the C terminus of the receptor. To this end, we constructed a fusion protein in which we replaced the N-terminal bHLH motif of DR-LBD with the glucocorticoid receptor zinc finger DNA-binding domain (GRDBD/DR-LBD). Whereas a reference chimeric receptor, GRDBD/DR (26), containing an intact LBD showed strictly ligand-dependent stimulation of a glucocorticoid response element, GRDBD/DR-LBD displayed constitutive transcriptional activity that was unaffected by the presence of ligand (Fig. 2A).

A number of biochemical experiments suggest that hsp90 regulates dioxin receptor function by folding the receptor in a high affinity ligand binding conformation (21). This model is strongly supported by yeast (Saccharomyces cerevisiae) genetic experiments that demonstrated that down-regulation of hsp90 expression levels results in a ligand-non-responsive form of the receptor (20, 22). Importantly, consistent with the deletion of the hsp90-binding region overlapping with the ligand binding function of the dioxin receptor, the transcriptional activity of the chimeric GRDBD/DR-LBD protein was unaffected by changes in the level of hsp90 in yeast cells (Fig. 2B). Taken together, our results indicate that deletion of the minimal domain harboring both the ligand- and hsp90-binding activities of the receptor efficiently derepresses the potent constitutive C-terminal transactivation function in DR-LBD and renders the transactivation function of the dioxin receptor independent of regulation by hsp90.

Given the inability of DR-LBD to function on an XRE-driven reporter gene, we next examined whether the protein was deficient in its ability to dimerize with Arnt or to bind DNA. To this end, we expressed the wild-type dioxin receptor, DR-LBD, and Arnt by in vitro translation in rabbit reticulocyte lysate. In control experiments, we monitored wild-type dioxin receptor/Arnt dimerization activity by incubating the [35S]methionine-labeled dioxin receptor with an equal concentration of unlabeled Arnt in the presence or absence of ligand, followed by immunoprecipitation using polyclonal anti-Arnt antibodies. As expected, we observed ligand-dependent interaction between the wild-type receptor and Arnt (Fig. 3B, compare lanes 2 and 3). In contrast, however, DR-LBD failed to form a stable complex with Arnt in the presence or absence of ligand since only low levels of nonspecific interaction were demonstrated by the co-immunoprecipitation assay (Fig. 3B, compare lanes 5 and 6). In excellent agreement with its impaired ability to interact with Arnt, DR-LBD failed to bind the XRE sequence motif in gel mobility shift experiments (Fig. 3C). In conclusion, these results suggest that deletion of the minimal LBD of the dioxin receptor strongly impairs the ability of the receptor to recruit Arnt and thus form a DNA-binding complex, possibly due to induction of conformational changes within the deletion mutant.
**Definition of a Dioxin Receptor Deletion Mutant That Shows Both Constitutive Dimerization Activity with Arnt and Constitutive Interaction with the XRE Sequence Motif**—In addition to harboring the minimal ligand- and hsp90-binding activities, the PAS domain of the dioxin receptor has also been shown to mediate a number of additional receptor activities. We have previously demonstrated that the PAS domain of the dioxin receptor can function as a dimerization interface independently of the bHLH domain in a hybrid-protein interaction assay in mammalian cells (15). Furthermore, the C-terminal half of the PAS domain located between amino acids 230 and 421 and containing the minimal LBD spanning the PAS B motif has been shown to be essential for the PAS-mediated interaction with Arnt (15). These results demonstrate that additional sequences N-terminal of the minimal PAS B motif incorporating the N-terminal half of the LBD are sufficient to reconstitute dimerization and DNA-binding activity between the dioxin receptor and Arnt. Since, in contrast to the wild-type dioxin receptor, DRA\textsubscript{PASB} was able to bind Arnt in a ligand-independent manner, the results also suggest that deletion of the minimal PAS B motif may be sufficient to generate a conformational change in the bHLH domain of the receptor to form a surface competent of interacting with Arnt independently of ligand binding.

**Potent Constitutive Functional Activity of the Dioxin Receptor Deletion Mutant Lacking the Minimal PAS B Motif**—We next examined the functional activity of DRA\textsubscript{PASB} together with Arnt in transient transfection assays using the XRE-driven luciferase reporter gene construct. Although DRA\textsubscript{PASB} contained an N-terminal portion of the ligand/hsp90-binding domain, in vitro translated DR\textsubscript{PASB} was unable to bind ligand in vitro (Fig. 5A). In addition, DRA\textsubscript{PASB} maintained the ability to transactivate in a ligand- and hsp90-independent manner as a GRDBD chimera in yeast cells (Fig. 5B), indicating that extension of the PAS domain to incorporate the N-terminal half of the minimal LBD did not impair the functional activity of the C-terminal transactivation domain. Moreover, it did not repress the ability of a GFP-DRA\textsubscript{PASB} fusion protein to localize constitutively to the nucleus (Fig. 5C) (36).
with 10 nM TCDD (PASB and Arnt. Following transfection, cells were treated with the either wild-type dioxin receptor or the dioxin receptor deletion mutant DR/H9004 in the absence of ligand. Values represent the mean ± S.E. of three independent experiments performed in duplicate. CTRL, CHO and HeLa cells, respectively, were transiently transfected with the XRE-driven luciferase reporter gene pTX.DIR together with the empty expression vector and reporter gene alone (control (CTRL)) in the absence of ligand. Data are presented as luciferase activity relative to the observed reporter gene activity was unaffected by the presence of ligand, demonstrating that this dioxin receptor deletion mutant functioned as a constitutively active regulatory protein.

In contrast to the wild-type dioxin receptor, the DRΔPASB strongly stimulated XRE-driven reporter gene activity upon coexpression with Arnt in CHO cells in the absence of ligand (Fig. 6A). Thus, in the natural structural context of the intact receptor, the PAS B motif is the critical determinant to maintain the receptor in a transcriptionally inactive state. Furthermore, the observed reporter gene activity was unaffected by the presence of ligand, demonstrating that this dioxin receptor deletion mutant functioned as a constitutively active regulatory protein. To our surprise, however, the constitutive activity of DRΔPASB was found to be more potent than that produced by the wild-type receptor exposed to a maximally inducing dose of dioxin (Fig. 6A). In these experiments, the wild-type dioxin receptor and the dioxin receptor deletion mutant were expressed at similar levels (Fig. 6B). Potent transactivation was observed in a number of different cell lines, including human cervical adenocarcinoma HeLa and mouse adrenal Y1 cells (Fig. 6C) (data not shown).

**DISCUSSION**

Transcription factors are generally acknowledged to have a modular structure. In the case of the dioxin receptor, the receptor is composed of a number of domains that harbor distinct separable functions, including N-terminal DNA-binding and dimerization domains and a C-terminal transactivation domain. Ligand binding by the dioxin receptor is an independent property of the centrally located LBD encompassing amino acids 230–421 and spanning the C-terminal half of the PAS domain. Moreover, this minimal LBD of the dioxin receptor has been shown to be transferable, retaining its activity when attached to other proteins, supporting the view that receptor LBDs are independent entities containing all the information necessary for ligand binding (19). In addition to being a critical determinant of ligand-binding activity, the LBD of the dioxin receptor has also been postulated to function in the repression of receptor function in the absence of ligand (19). Similarly, in the absence of specific agonist, the LBD of the glucocorticoid receptor functions in the repression of a number of receptor activities, including nuclear localization, dimerization, DNA binding, and transactivation.

The receptor was now constitutively localized to the nucleus. In striking contrast to steroid receptors, however, this nuclear localized, LBD-deficient mutant of the receptor was functionally inert in reporter gene assays in the presence and absence of ligand. Further analysis revealed that, although the C-terminal transactivation function had been efficiently derepressed, this receptor deletion mutant had lost the ability to dimerize with Arnt, thus rendering it incapable of forming an active DNA-binding complex. Inclusion of the N-terminal amino acids of the minimal LBD to the border of the PAS B motif reconstituted dimerization and DNA-binding activity with Arnt. Moreover, the presence of the N-terminal half of the LBD did not impair either the N-terminal NLS activity or the functional activity of the C-terminal transactivation domain of the receptor, indicating that this region is not required for repression of these receptor activities. Furthermore, the receptor was now converted into a constitutive transcriptional activator. Thus, whereas the entire LBD of the glucocorticoid receptor appears to regulate all of these receptor activities, our results suggest that the C-terminal half of the minimal LBD of the dioxin receptor incorporating the PAS B motif is both sufficient and required to maintain the receptor in a transcriptionally inactive state in the absence of ligand.

Previous studies have clearly defined the minimal sequence of the LBD of the dioxin receptor with wild-type ligand binding affinity as extending from amino acids 230 to 421 (21). Moreover, this region of the receptor has also been shown to modu-
late interaction with the molecular chaperone hsp90, consistent with its role in the folding of a high affinity ligand binding conformation of the receptor (20–22). In this study, we have observed that deletion of the C-terminal half of the LBD incorporating the PAS B motif abolished ligand binding, revealing the critical nature of the residues between amino acids 288 and 421 for ligand-binding activity. Interestingly, this dioxin receptor deletion mutant had also lost the ability to associate with hsp90 in vitro, suggesting that the region spanning the C-terminal half of the minimal LBD incorporating the PAS B motif is the target for regulation by hsp90. In support of this model, the activity of this dioxin receptor deletion mutant was unaffected by reduced levels of hsp90 in the yeast model system. We propose therefore that, in addition to modulating repression of receptor function in the absence of ligand, this region of the receptor may also be required for the folding of the entire delineated LBD to give the native tertiary structure that is optimal for high affinity ligand binding possibly by association with hsp90.

In summary, we have identified the domain of the dioxin receptor that determines repression of receptor function to be the C-terminal 134 amino acids of the LBD. In addition, our results indicate that this region of the receptor is the target for regulation by hsp90. Furthermore, deletion of this domain converts the receptor into a constitutive transcriptional activator. In conclusion, the constitutively active dioxin receptor may provide an important mechanistic tool to investigate receptor-mediated regulatory pathways in closer detail.

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