Definition of a Minimal Domain of the Dioxin Receptor That Is
Associated with Hsp90 and Maintains Wild Type Ligand Binding
Affinity and Specificity*

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Pascal Coumailleau†, Lorenz Poellinger‡, Jan-Ake Gustafsson§, and Murray L. Whelan†¶

From the Departments of Medical Nutrition, and Bioscience, Karolinska Institute, Huddinge University Hospital F-60, Novum, S-141 86 Huddinge, Sweden

The dioxin receptor is a cytoplasmic basic helix-loop-helix/Per-Arnt-Sim homology (bHLH/PAS) protein known to bind planar polycyclic ligands including polycyclic aromatic hydrocarbons, benzo[alpha]pyrene, benzo[a]pyrene, and halogenated aromatic hydrocarbons, e.g. dioxins. Ligand-induced activation of the dioxin receptor initiates a process whereby the receptor is transformed into a nuclear transcription factor complex with a specific bHLH/PAS partner protein, Arnt. In analogy to the glucocorticoid receptor, the latent dioxin receptor is found associated with the molecular chaperone hsp90. We have defined and isolated a minimal ligand binding domain of the dioxin receptor from the central PAS region, comprising of amino acids 230 to 421, and found this domain to interact with hsp90 in vitro. Expression of the minimal ligand binding domain in wheat germ lysates or bacteria, systems which harbor hsp90 homologs unable to interact with the glucocorticoid or dioxin receptors, resulted in non-ligand binding forms of this minimal 230 to 421 fragment. Importantly, affinity of the minimal ligand binding domain for dioxin was similar to the affinity inherent in the full-length dioxin receptor, and a profile of ligand structures which specifically bound the minimal ligand binding domain was found to be conserved between this domain and the native receptor. These experiments show that the minimal ligand binding domain maintains the quantitative and qualitative aspects of ligand binding exhibited by the full-length receptor, implying that the central ligand binding pocket may exist to accommodate all classes of specific dioxin receptor ligands, and that this pocket is critically dependent upon hsp90 for its ligand binding conformation.

Signal transduction by dioxins, related halogenated hydrocarbons, and polycyclic aromatic hydrocarbons is mediated by the dioxin receptor (also known as the aryl hydrocarbon receptor), an intracellular bHLH/PAS protein which, in response to exogenous ligands, forms a transcription factor complex with a specific bHLH/PAS co-factor, Arnt (for recent reviews, see Swanson and Bradfield (1993), Hankinson (1994), and Whitlock (1994)). The dioxin receptor and Arnt are distinguished from other members of the bHLH family of transcription factors by virtue of PAS (Per-Arnt-Sim) homology regions, segments of 250 to 300 amino acids juxtaposed to the bHLH motifs which share similarity with the bHLH/PAS transcription factor Sim (Nambu et al., 1991), and Drosophila PAS circadian oscillator Per (Huang et al., 1993). The ligand-activated dioxin receptor/Arnt heterodimer recognizes a specific core DNA sequence, the XRE (xenobiotic response element, TNGCGTG, Lusska et al. (1993)), which bears some resemblance to the E-box core (CA(C/G)(C/G)TG) recognized by the majority of bHLH proteins (Kadish, 1993). XRE sequences function as dioxin responsive enhancers and are present in upstream regions of several dioxin-regulated genes (e.g. cytochrome P450IA1, glutathione S-transferase, and various other genes encoding xenobiotic metabolizing enzymes; for a review, see Landers and Bunce (1991)). Recent studies designed to investigate the transcription activating capabilities of the dioxin receptor and Arnt subunits have revealed these proteins to harbor potent transactivation domains in their C termini (Whitelaw et al., 1994; Jain et al., 1994; Li et al., 1994). Furthermore, phosphorylation states of the dioxin receptor and Arnt may be a critical factor in dioxin signaling, as modulation of protein kinase activities has been demonstrated to inhibit dioxin induced XRE binding activities and responsiveness of XRE driven reporter genes (Okino et al., 1992; Berghard et al., 1993; Carrier et al., 1992; Gradin et al., 1994).

In untreated cells, the dioxin receptor is held in a latent cytoplasmic complex with the molecular chaperone hsp90 (Perdew, 1988; Denis et al., 1988) and a 43-kDa protein (Chen and Perdew, 1994). Ligand signaling initiates a transformation process whereby the receptor translocates to the nucleus (Pollenz et al., 1994), hsp90 is released, and dimerization with Arnt is achieved. As Arnt has been reported to be a nuclear protein (Pollenz et al., 1994; Hord and Perdew, 1994), and Arnt may play an active role in releasing hsp90 from the dioxin bound receptor (McGuire et al., 1994), disruption of the hsp90-dioxin receptor complex may be a nuclear rather than cytoplasmic event. Consistent with this hypothesis, dioxin receptor-hsp90 complexes have been isolated from nuclear extracts of ligand-treated cells (Wilhelmsson et al., 1990; Perdew, 1991). Hsp90 is an intrinsic component of the dioxin receptor ligand signaling mechanism, as ligand responsiveness of chimeric dioxin receptor constructs in a genetically manipulated strain of Saccharomyces cerevisiae has been abrogated in depleted hsp90 environments (Carver et al., 1994; Whitelaw et al., 1995). Multiple roles for hsp90 in maintaining a functional dioxin receptor form are suggested by in vitro studies, which have shown hsp90 to (i)
impose repression on the ligand free dioxin receptor, preventing premature interaction with Arnt (Matsushita et al., 1993; Probst et al., 1993; Whitelaw et al., 1993), (ii) to act as a "chaperoning" factor in formation of a receptor capable of binding ligand (Antonsson et al., 1995; Pongratz et al., 1992), and (iii) possibly also chaperone a DNA binding conformation of the ligand-activated receptor (Antonsson et al., 1995).

In a previous strategy to identify regions of the mouse dioxin receptor which could convey dioxin responsiveness onto heterologous transcription factors, a core ligand binding domain between amino acids 230 and 421 was found to bind dioxin in the context of a glucocorticoid receptor/dioxin receptor chimera (Whitelaw et al., 1993a). We have now isolated and studied this core ligand binding domain in detail and find that in vitro translation in reticulocyte lysate binding of dioxin and other specific ligands is quantitatively and qualitatively similar to that observed with the full-length native dioxin receptor. Importantly, this 192-amino acid region also binds hsp90 in vitro, strengthening initial implications that hsp90 binding localizes to the ligand binding domain to convey a repression upon the ligand free dioxin receptor (Whitelaw et al., 1994). In vitro translation of this ligand binding domain in wheat germ lysates, which contain an hsp90 homolog unable to interact with the dioxin receptor (Antonsson et al., 1995), failed to produce a protein with ligand binding activity, implying a role for hsp90 in chaperoning a functional ligand binding conformation of this domain. Consistent with this hypothesis, bacterial expression and purification of the ligand binding domain to homogeneity also produced a non-functional protein. These studies confirm the ligand binding domain of the dioxin receptor to reside between amino acids 230 and 421, and establish the interaction of this domain with hsp90 to be essential in formation of a functional ligand binding entity.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs—**Plasmids pDBD/GEM, pDBD/DBD/DR30–421/GEM, and pDBD/DBD/DR230–421/GEM have been previously described (Whitelaw et al., 1993a). Plasmids pDBD/DR30–421/GEM and pDBD/DBD/DR30–400/GEM were constructed by subcloning a PCR amplified sequence from pSportAhR (Burbach et al., 1992; generously provided by Dr. Christopher A. Bradfield, Northwestern University Medical School, Chicago, IL) into pDBD/GEM via XhoI restriction sites flanking the codon sequences of primers. The coding region for the minimal ligand binding domain of the dioxin receptor (codons 230 to 421) was amplified from pSportAhR by PCR (1 min at 95°C, 1 min at 55°C; 1 min and 50 s, 72°C) with an upstream primer designed to provide a HindIII restriction site followed by a Kozak consensus start site (5'-AATTCATCAATGTTCCCGCGATATTTCAAGGAGGAGG-3') and a downstream primer designed to provide an XhoI restriction sites at its terminus (5'-TACGAGGCCTACCTCCTAGTATACCT-3'). The PCR fragment was subcloned into the HindIII and XhoI sites of pGem7ZfI (Promega) in-frame with the stop codon from the XbaI site in the polylinker. HisHsAhLBD/pET19b was generated by subcloning into pET19b (Qiagen) a fragment encompassing tagged ligand binding domain (LB) codons. This DNA fragment was generated by PCR using an upstream primer containing an NdeI restriction site and bases coding for the hemagglutinin epitope (Kolodziej and Young, 1991) of the monoclonal antibody 12CA5 (Babc0; 5-GCTGGAGCATATGATCTTTATGATGTCTTCTTATGATTGATGATTAGGAGGAGG-3') and a downstream primer designed to provide an N-terminal myc epitope fusion (5'-GGTTCAGCTCAGCTATGATTGATGATTAGGAGGAGG-3'). PCR conditions were the same as above except that the annealing temperature was 60°C. PCR fragments were digested with NdeI and BamHI and subcloned into the NdeI and BamHI sites of pET19b in-frame with the N-terminal histidine tag. The protein expressed from this vector contained a stretch of 20 amino acids in the C terminus belonging to the pET19b vector. The HisHsAhLBD protein which does not contain the additional pET19b 20 amino acids in the C terminus but encompasses only the His tag, the hemagglutinin epitope, and the minimal ligand binding domain (amino acids 230–421) of the dioxin receptor was obtained by subcloning the NdeI/BglII fragment from HisHsAhLBD/pET19b and the BglII/SphI fragment from pLBD/Gem7ZfI into pSP72 (Promega), providing pHsHsAhLBD/SP72.
Minimal Ligand Binding Domain of the Dioxin Receptor

Delineation of the Core Ligand Binding Domain of the Dioxin Receptor—In previous studies designed to map regions of the dioxin receptor which bind dioxin and enable conversion of a constitutive transcription factor into a dioxin-dependent regulatory protein, we created fusion proteins between C-terminal constitutive transcription factor into a dioxin-dependent regulatory protein, we created fusion proteins between C-terminal DNA binding domain and a transactivation domain ("DBD") harbor a “zinc finger” DNA binding domain and a transactivation domain ("TAD"). A chimera where this DBD fragment replaced the very N-terminal bHLH motif of the dioxin receptor, DBD/DR83–805 (Fig. 1), revealed the dioxin receptor C terminus to confer repression upon the constitutive transactivating capacity of DBD in transient transfection assays, which was subsequently relieved in the presence of dioxin (Whitelaw et al., 1993a). Several such dioxin receptor chimeras were tested both for dioxin responsiveness in transient transfection assays and in vitro. [3H]dioxin binding activity, allowing an approximate demarcation of a region between amino acids 230 and 421 as harboring the ligand binding domain (Fig. 1, Whitelaw et al., 1993a).

To ascertain whether a smaller region within this 230 to 421 amino acid domain was capable of binding dioxin, we have now made further chimeras where the N-terminal and C-terminal boundaries of the 230 to 421 region were truncated, providing fusion proteins DBD/DR300–400 and DBD/DR280–421 (Fig. 1). As hydroxylapatite ligand binding assays with [3H]dioxin have previously shown that in vitro translated DBD does not bind ligand (Whitelaw et al., 1993a), we therefore performed this in vitro assay with DBD/DR300–400, DBD/DR280–421, DBD/DR230–421, and DBD/DR83–805 fusion proteins translated in reticulocyte lysates. Translation of chimeras in the presence of [35S]methionine gave radiolabeled fusion proteins (Fig. 2A), allowing direct comparison of [3H]dioxin retention to provide a crude approximation of binding affinities.

Binding of [3H]dioxin by DBD/DR83–805, a chimera lacking only the bHLH domain of the dioxin receptor, is similar to that of DBD/DR230–421 (Whitelaw et al., 1993a, Fig. 2B), verifying that the dioxin binding region is contained within residues 230 to 421. As expected, competition with an excess of TCDF, another dioxin receptor-specific ligand, displaced binding of [3H]dioxin by these chimeras to the background levels shown by unprogrammed lysates (Fig. 2B). Truncation of dioxin receptor residues from both the N-terminal and C-terminal ends of DBD/DR230–421 provided chimera DBD/DR300–400 (Fig. 1), a fusion protein devoid of dioxin binding activity (Fig. 2B). A second chimera, DBD/DR83–400, consisting of a 50 amino acid truncation from the N-terminal end of the core 230–421 dioxin receptor domain, produced a fusion protein with greatly reduced dioxin binding capability (Fig. 2B), as did a C terminally truncated chimera DBD/DR230–400 (data not shown). These results indicate that the region of the dioxin receptor between amino acids 230 and 421 provides an accurate demarcation of the core ligand binding domain and that this domain, exhibiting a ligand binding capability similar to that of DBD/DR83–805 (which contains 90% of dioxin receptor residues), very likely maintains most if not all of the ligand binding activity shown by the native dioxin receptor. The accuracy of these chimeras in estimating ligand binding capabilities of native dioxin receptor residues is illustrated by an in vitro translation of the isolated ligand binding domain, DR230–421 (Fig. 1), showing similar [3H]dioxin binding capacity to the chimera DBD/DR230–421 (Fig. 2B).

Interaction of the Ligand Binding Domain with Hsp90 Is Essential for Ligand Binding Activity—Previous experiments revealed that chimera DBD/DR230–421 was capable of interaction with hsp90 (Whitelaw et al., 1993a), leading to the proposal that ligand and hsp90 binding activities overlapped or co-localized within this region, and strengthening the postulation that Hsp90 was essential for chaperoning a ligand binding conformation of the receptor (Pongratz et al., 1992; Whitelaw et al., 1993a). To further test this hypothesis we performed an hsp90 co-immunoprecipitation assay with in vitro translation mixtures containing the isolated LBD, DR230–421. In vitro translation of the native dioxin receptor and DR230–421 in the presence of [35S]methionine gave radiolabeled proteins of the expected size when analyzed by SDS-PAGE (Fig. 3A). Immunoprecipitation experiments were performed with [35S]methionine-labeled translation mixtures containing either the LBD or the native dioxin receptor, and with either control antibodies or a specific hsp90 antibody. Both the native dioxin receptor (McGuire et al., 1994; Fig. 3B, compare lanes 1 and 2) and the LBD (Fig. 3B, compare lanes 3 and 4) were found to interact with hsp90 in vitro by this co-immunoprecipitation assay. To test for functional significance of this interaction, we in vitro translated the LBD in wheat germ lysates, a medium containing an hsp90 homolog which does not interact with members of the steroid hormone receptor superfamily (reviewed by Pratt et al., 1992) or the dioxin receptor (Antonsson et al., 1995), and performed a [3H]dioxin binding assay. The LBD was successfully expressed in wheat germ lysate (data not shown), although translation in this medium gave negligible ligand binding activity (Fig. 3C). Interaction of the ligand binding domain of the dioxin receptor with hsp90 therefore seems essential in formation of a functional ligand binding species. Consistent with this hypothesis, wheat germ translations of the native dioxin receptor have also proven to lack ligand binding activity (Antonsson et al., 1995), while non-ligand binding chimeric receptors DBD/DR280–421 and DBD/DR300–400 revealed a severely depleted hsp90 interaction when compared to DBD/DR230–421 (data not shown).

The Minimal Ligand Binding Domain of the Dioxin Receptor Has an Affinity for Dioxin Similar to That of the Native Dioxin Receptor—Having defined the coordinates of a functional dioxin receptor ligand binding domain, we sought to compare the relative affinities this domain and the full-length dioxin recep-
tor exhibited for dioxin. To create a ligand binding domain DNA construct that would provide versatility for overexpression and purification of the LBD, we fused sequences which encode a histidine tag and a hemagglutinin epitope to the N terminus of the DR230–421 cDNA, providing HisHaLBD (Fig. 1). Histidine sequences offer the possibility of rapid purification by binding to nickel-agarose resins, while the hemagglutinin epitope provides for ready detection and immunoprecipitation of tagged proteins by a commercially available antibody. Translation of HisHaLBD and a histidine-tagged version of the dioxin receptor (HisDR, Fig. 1) in reticulocyte lysates containing [35S]methionine produced radiolabeled bands of the expected molecular weight (Fig. 4A). The histidine and hemagglutinin epitope tags were not detrimental to hsp90 interaction with the LBD, as shown by the ability of hsp90 antibodies to specifically co-immunoprecipitate the HisHaLBD fusion protein from [35S]methionine radiolabeled in vitro translation mixtures (Fig. 4B). To verify that ligand binding was maintained in the presence of these amino acid tags we performed[^H]dioxin binding assays on in vitro translated proteins. Reticulocyte lysate translation mixtures containing HisDR were observed to specifically bind[^H]dioxin (Fig. 4C) in a manner similar to that of the wild-type receptor (Antonsson et al., 1995), while no difference was observed between HisHaLBD and untagged LBD in their abilities to bind[^H]dioxin in our in vitro ligand binding assay (Fig. 4C). In excellent agreement with these observations a tag of histidine residues has recently been reported to have no effect on the ability of the full-length dioxin receptor to bind ligand (Chan et al., 1994).

After establishing that the exogenous amino acid tags were
not detrimental to ligand binding, we performed a quantitative and comparative analysis of affinities the in vitro expressed minimal ligand binding domain and the full-length dioxin receptor exhibited for dioxin. Hydroxylapatite ligand binding assays were therefore carried out with in vitro translated HisDR and HisHaLBD proteins, using increasing concentrations of [3H]dioxin, providing reproducible saturation binding curves representatively shown in Fig. 4, D and E. Scatchard analysis of these binding curves revealed dissociation constant (K_D) values of 0.41 nM for the full-length dioxin receptor and 0.47 nM for the ligand binding domain, respectively. These values are in excellent agreement with K_D values previously calculated for the native dioxin receptor in crude cell extracts (Ema et al., 1994), verifying fidelity of the in vitro ligand binding assay and establishing that the core domain between amino acids 230 and 421 of the dioxin receptor mediates full ligand binding activity including wild type affinity for dioxin.

The Ligand Binding Domain Maintains the Ligand Binding Specificity of the Native Dioxin Receptor—Observation that the ligand binding domain and the full-length dioxin receptor share similar affinities for dioxin stimulated a further comparison in addressing the qualitative nature of the ligands which can be specifically bound by the minimal LBD. The native dioxin receptor is well characterized in binding a number of distinct classes of ligands in addition to dioxin. These classes of

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**Fig. 2.** In vitro ligand binding assays of rDBD-dioxin receptor fusion proteins and the DR230–421 minimal LBD. A, chimeric rDBD/dioxin receptor proteins were in vitro translated in reticulocyte lysates in the presence of [35S]methionine and aliquots (2 μl) analyzed by 7.5% SDS-PAGE and fluorography. Positions of the molecular mass standard proteins are indicated. B, hydroxylapatite ligand binding assays performed with unlabeled reticulocyte lysate (−) or translation mixtures containing the indicated chimeric protein or LBD using 2 nM [3H]TCDD as specific ligand and 250-fold μ excesses of TCDF as specific competitor.

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**Fig. 3.** Association of the dioxin receptor LBD with hsp90 is necessary for ligand binding activity. A, aliquots of the full-length dioxin receptor (lane 1) and the minimal ligand binding domain (lane 2) translated in reticulocyte lysates in the presence of [35S]methionine were analyzed by 10% SDS-PAGE and fluorography. B, aliquots (10 μl) of the [35S]methionine-labeled dioxin receptor (lanes 1 and 2) or LBD (lanes 3 and 4) translation mixtures were incubated with specific (lanes S) anti-hsp90 antibody 3G3 (5 μl) or control (lanes C) IgM which had been preadsorbed to a resin of goat anti-mouse IgM Sepharose. After extensive washing, immunoprecipitated proteins were analyzed by 10% SDS-PAGE and fluorography. The positions of the molecular mass standard proteins are indicated. C, aliquots (10 μl) of either unprogrammed (−) or LBD containing reticulocyte or wheat germ lysates were subjected to hydroxylapatite ligand binding assays with 2 nM [3H]TCDD in the presence or absence of a 100-fold μ excess of TCDF, as indicated.
Fig. 4. Saturation binding analysis of HisDR and HisHaLBD. A, HisHaLBD (lane 1) and HisDR (lane 2) were translated in reticulocyte lysates in the presence of [35S]methionine and aliquots (2 μl) were analyzed by 10% SDS-PAGE and fluorography. B, aliquots (10 μl) of the [35S]methionine-labeled dioxin receptor (lanes 1 and 2) or HisHaLBD (lanes 3 and 4) translation mixtures were incubated with specific (lanes S) anti-hsp90 antibody 3G3 (5 μl) or control (lanes C) IgM which had been preadsorbed to a resin of goat anti-mouse IgM-Sepharose. After extensive washing, immunoprecipitated proteins were analyzed by 10% SDS-PAGE and fluorography. C, hydroxyapatite ligand binding experiments were performed with the indicated in vitro translation mixtures using 2 nM [3H]TCDD in the presence (+) or absence (−) of 250-fold m excesses of TCDF. Panels D and E show specific saturation curves for HisDR and HisHaLBD, respectively, using in vitro translated proteins and increasing concentrations (0.2 to 2.5 nM) of [3H]TCDD. Nonspecific binding was determined by using a 250 μM excess of TCDF. Scatchard plots and K_D (dissociation constants) or r (linear regression coefficient) values are shown for each protein.
ligands include polycyclic aromatic hydrocarbons, heterocyclic amines, and flavones, in addition to dioxins (Gillner et al. (1993), and references therein). To investigate whether this ligand binding specificity was conserved within the 230–421 ligand binding domain, we performed in vitro [3H]dioxin binding assays in the presence of 100-fold excesses of specific competitors from different dioxin receptor ligand classes. Specific agonists TCDF, β-naphthoflavone, and indolo[3,2-b]carbazole, but not the glucocorticoid receptor agonist dexamethasone, were able to compete with [3H]dioxin for binding to the minimal LBD (Fig. 5). This result is consistent with the core region of the dioxin receptor between amino acids 230 and 421 containing residues capable of specifically recognizing several distinct classes of dioxin receptor ligands in similar fashion to the full-length receptor, and suggests that a single ligand binding pocket may exist to bind these classes of dioxin receptor ligands.

Bacterial Overexpression and Purification of the Ligand Binding Domain Yields a Protein Devoid of Dioxin Binding Activity—In an attempt to produce large quantities of the ligand binding domain for structural studies, we expressed the HisHaLBD construct in bacteria under control of the lac promoter. Using an antibody directed against the hemagglutinin epitope, we were able to detect specific expression of the LBD in bacteria induced with IPTG (Fig. 6A, lane 1), with a lack of any background antibody staining in uninduced, transformed bacteria (lane 3) or either uninduced or induced bacteria transformed with empty expression vector (lanes 2 and 4). Analysis of bacterial extracts showed the LBD to form insoluble aggregates which pelleted with cellular debris upon centrifugation (data not shown). Extraction of the pellet with urea allowed solubilization of the precipitated proteins, which were bound to nickel-agarose resin and eluted with increasing concentrations of imidazole. Immunodetection of column fractions revealed HisHaLBD to elute with concentrations of 100 and 150 mM imidazole (data not shown). Analysis by SDS-PAGE and silver staining showed the LBD in these fractions to be purified to near homogeneity (Fig. 6B).

Ligand binding assays on the bacterially expressed, purified
LBD showed a lack of any specific [3H]dioxin binding, with levels of total [3H]dioxin binding activity in the hydroxylapatite assay being identical in the absence or presence of specific competitor TCDF (Fig. 6C). Since we have demonstrated HisHaLBD to bind hsp90 upon in vitro expression in reticulocyte lysate (Fig. 4B), and this interaction is possibly essential for chaperoning a ligand binding conformation of the LBD (Figs. 3, B and C), the lack of ligand binding shown by the purified bacterially expressed LBD is likely to result from malfolding of the LBD in the absence of hsp90. Consistent with this notion, expression of the glucocorticoid receptor in bacteria produces a receptor which does not interact with the bacterial hsp90 homolog, and shows significantly attenuated affinity for ligand (Ohara-Nemoto et al., 1990). In attempts to renature the purified LBD in the presence of hsp90, we preincubated the purified LBD with purified hsp90, as described by Wied et al. (1992), before performing the [3H]dioxin binding assay. Total ligand binding activity following this procedure was not increased above that observed for the purified, untreated LBD (Fig. 6C). One possibility for this apparent lack of renaturation of the LBD is that other heat shock proteins or molecular chaperones may play critical roles in the postulated renaturation process. Consistent with this idea, reconstitution of the semipurified glucocorticoid receptor into a ligand binding oligomeric complex has been successful by incubation in reticulocyte lysates, with roles for several heat shock proteins being proposed as essential (for a review, see Pratt, 1993). Moreover, this reticulocyte lysate procedure has been successful in renaturing high affinity ligand binding conformations of bacterially expressed mineralocorticoid receptor (Caamaño et al., 1993). However, incubation of the purified LBD with reticulocyte lysate according to the steroid receptor protocols (Scherrer et al., 1990) failed to provide a receptor species exhibiting either ligand binding (Fig. 6C) or hsp90 binding (data not shown) activities, in direct contrast to the ligand and hsp90 binding seen when the LBD is translated in reticulocyte lysate (Fig. 4, B and C). These results are consistent with a need for the LBD to be chaperoned into a native conformation by hsp90 and perhaps other heat shock proteins, and indicate that once the LBD has become extensively denatured or misfolded, renaturation, if possible at all, may require several factors and/or reagents in a multi-faceted process.

DISCUSSION

The Ligand Binding Domain of the Dioxin Receptor Localizes to a Central Portion of the Dioxin Receptor and Encompasses PAS-B—The dioxin receptor is a bHLH/PAS transcription factor which is capable of regulating dioxin responsive genes when complexed with a specific bHLH/PAS partner protein, Arnt (Hoffman et al., 1991). Both the dioxin receptor and Arnt share a similar molecular architecture to a third bHLH/PAS protein, the Drosophila single-minded (Sim) factor, a crucial component for development of midline cells of the central nervous system (Nambu et al., 1991). All three proteins contain the DNA binding and dimerization bHLH motifs in the very N terminus, juxtaposed with the PAS domains, which in the case of the dioxin receptor and Arnt are thought to stabilize dimerization (Reisz-Porszasz et al., 1994) and affect target DNA sequence affinity and/or specificity (Antonsson et al., 1995). Transactivation domains are found exclusively in the C terminus of Arnt (Jain et al., 1994; Li et al., 1994; Whitelaw et al., 1994), the dioxin receptor (Jain et al., 1994; Whitelaw et al., 1994), and Sim (Franks and Crews, 1994). The dioxin receptor is distinct, however, in harboring a ligand binding domain, which we have now defined as a region encompassing PAS B. In contrast, the PAS region of Arnt appears to be free from any regulatory activity (Whitelaw et al., 1994), and while no function has been assigned to the Sim PAS domain, it is a critical region for Sim activity (Franks and Crews, 1994). Despite the Sim PAS region showing a greater similarity with the dioxin receptor LBD than any other protein segment (31% identity), Sim does not bind dioxin, although its interaction with hsp90 is perhaps indicative of Sim being an orphan ligand binding receptor. There is currently no established endogenous ligand, nor physiological activation mechanism, known for the dioxin receptor (Poel linger et al., 1992).

We have found the ligand binding domain of the mouse dioxin receptor to reside between amino acids 230 and 421, a segment of 192 amino acids which includes within its borders a region which was cross-linked with a radiolabeled photoaffinity dioxin ligand (amino acids 230 to 337; Burbach et al. (1992)) and a polymorphic residue (amino acid 375) which influences mouse strains to harbor low (DBA/2J) or high (C57BL) affinity receptor phenotypes (Ema et al., 1994). Attempts to define a shorter ligand binding domain, by deletion to the boundaries of amino acids 300 to 400, or to between amino acids 280 and 421, failed to provide fragments with significant dioxin binding activity. Our delineation between amino acids 230 and 421 therefore represents a close approximation to the concise limits which would define the minimal ligand binding domain. Definition of a minimal ligand binding domain has also been attempted for the structurally unrelated but functionally similar glucocorticoid receptor. A 16-kDa tryptic fragment, consisting of amino acids 537 to 673 of the rat glucocorticoid receptor, was found to bind in a profile of glucocorticoid receptor ligands with a similar qualitative specificity as the native receptor (Chakroborti and Simons, 1991). The affinity of this 16-kDa fragment for the prototypic dexamethasone agonist was, however, approximately 20-fold lower than that of the native receptor (Simons et al., 1989). In contrast, the ligand binding domain of the dioxin receptor not only maintains the qualitative ligand binding specificity shown by the native receptor, but also binds dioxin with an affinity similar to the full-length receptor. The 192-amino acid ligand binding domain of the dioxin receptor is therefore a more precisely defined entity than the broad 300 amino acid C terminus of the glucocorticoid receptor that has been found necessary to provide full ligand binding activity (Ohara-Nemoto et al., 1990, and references therein).

Hsp90 Is an Intrinsic Component of the Dioxin Receptor Ligand Binding Domain—In untreated cytosolic extracts (Pendew, 1988; Denis et al., 1988), or upon in vitro translation in rabbit reticulocyte lysates (McGuire et al., 1994), the dioxin receptor is found tightly complexed with the molecular chaperone hsp90. Artificial disruption of the cytosolic dioxin receptor-hsp90 complex, or translation in wheat germ lysates, which contain a homolog of hsp90 which does not complex with the receptor, lead to a dioxin receptor incapable of binding ligand (Pongratz et al., 1992; Antonsson et al., 1995). Furthermore, in a genetically engineered strain of S. cerevisiae where hsp90 expression can be manipulated, in vivo ligand responsiveness of the dioxin receptor is almost totally abrogated when hsp90 is lowered to around 5% of normal levels (Carver et al., 1994; Whitelaw et al., 1995). These observations establish hsp90 as an essential element of the dioxin receptor ligand signaling pathway, and are consistent with a proposed role for hsp90 in chaperoning a ligand binding conformation of the receptor (Pongratz et al., 1992). In support of this hypothesis, hsp90 has been found to have a chaperoning function by binding denatured proteins and enhancing refolding in vitro (Miyata and

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2 P. Coumailleau, unpublished observations.
3 J. McGuire, unpublished observations.
Minimal Ligand Binding Domain of the Dioxin Receptor

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Yahara, 1992; Wiech et al., 1992; Jacob and Buchner, 1994). In conclusion, our finding that the ligand binding domain of the dioxin receptor mediates interaction with hsp90, and that expression of the LBD in systems which fail to provide interaction with hsp90 lead to nondioxin binding entities, strongly infers that a major role of hsp90 in the dioxin receptor system is to chaperone a native conformation of the ligand binding domain. In analogy to the dioxin receptor, the glucocorticoid receptor is a ligand activated transcription factor which exists in a latent cytoplasmic complex with hsp90. Expression of the glucocorticoid receptor in yeast strains containing low hsp90 levels (Picard et al., 1990) or mutants of hsp90 (Bohen et al., 1993) produces a compromise in sensitivity of the receptor for ligand activation, while expression in bacteria, an organism where an hsp90 homolog (C62.5, Bardwell and Craig, 1987) shows only 42% identity to mammalian hsp90 and does not interact with the receptor, provides a form of the glucocorticoid receptor with a severely lowered affinity for ligand (Ohara-Nemoto et al., 1990). It therefore seems that interaction of hsp90 with the dioxin and glucocorticoid receptors may perform similar functions in the chaperoning of ligand binding domains. In support of this idea, the 16-kDa core ligand binding domain of the glucocorticoid receptor was also found to interact with hsp90 (Chakroborti and Simons, 1991). Attempts to further delineate the ligand and hsp90 binding domains within the glucocorticoid receptor have revealed a minimal hsp90 binding region to reside within the core steroid binding sequences (Howard et al., 1990; Dalman et al., 1991). Despite this striking mechanistic similarity between the bHLH/PAS dioxin receptor and zinc finger glucocorticoid receptor, no structural homology is evident when aligning their ligand/hsp90 binding domains. The need for hsp90 interaction to impart high affinity ligand binding activity, while observed for both the glucocorticoid (Ohara-Nemoto et al., 1990) and mineralocorticoid receptors (Caamano et al., 1993), does not seem to be general for the zinc finger superfamily of nuclear receptors. Bacterial expression of the large C-terminal progesterone (Eul et al., 1989) or retinoid X receptor (Cheng et al., 1994) ligand binding domains produce species which display wild type progesterone and 9-cis-retinoic acid binding activities, respectively, indicating no evident requirement for hsp90 to chaperone ligand binding conformations for these receptors. Recent studies of steroid hormone receptor activation in yeast model systems have found a homolog of the DnaJ chaperone, Ydj1, to be an essential component of hsp90-mediated signal transduction mechanisms (Kimura et al., 1995; Caplan et al., 1995). As these studies imply Ydj1 also exerts its affects via the ligand binding domains of steroid hormone receptors, it will now be important to investigate the potential influence of the Ydj1 chaperone on signaling through the dioxin receptor LBD.

While the ligand binding domains of the dioxin and glucocorticoid receptors are mechanistically similar in requiring interaction with hsp90 to enable ligand binding, these distinct sequences do not seem to be equally amenable to renaturation from a denatured state. When the glucocorticoid receptor was stripped of hsp90 and associated proteins, reassociation with hsp90 was achieved by incubation with rabbit reticulocyte lysate, a medium rich in heat shock protein complexes (Scherrer et al., 1990), or by incubation with a semipurified heat shock protein complex isolated from reticulocyte lysate (Hutchison et al., 1994). Reassociation with the hsp90-heat shock protein complex was accompanied by reconstitution of ligand binding, a phenomenon also observed with the bacterially expressed mineralocorticoid receptor, which was found to bind aldosterone with wild type affinity only after incubation with reticulocyte lysate (Caamano et al., 1993). Using these protocols, we have failed to renature the dioxin binding state of the bacterially expressed and purified dioxin receptor LBD. When expressed in bacteria, the dioxin receptor LBD is recovered from extracts in a particulate fraction, requiring high urea concentrations for solubilization. This process may be severely denaturing, resulting in a totally malformed protein fragment which resists renaturation by the mild reticulocyte lysis conditions used when renaturing glucocorticoid and mineralocorticoid receptors, which are recovered from the soluble fractions of bacterial extracts. We are currently developing expression systems which will enable the dioxin receptor ligand binding domain to be recovered in the soluble fraction of bacterial extracts, and may therefore prove more amenable to reconstitution with hsp90 and show subsequent recovery of ligand binding activity.

Role of the Ligand/Hsp90 Binding Domain in Activation and Repression of Dioxin Receptor Activity—Heat- and salt-induced removal of hsp90 from the dioxin receptor in crude cellular extracts converts the receptor into a form capable of binding the XRE recognition sequence (Pongratz et al., 1992), a form which by definition contains the cofactor Arnt. Interaction with hsp90 therefore performs an inhibitory role, preventing premature interaction with Arnt in the unliganded state. While we have not fully defined the residues necessary for hsp90 interaction, the hsp90 binding region seems to be coincident with, or at least lie within, the ligand binding domain, suggesting this ligand/hsp90 binding central portion of the receptor to form a critical region of repression. Consistent with this proposal, chimeras between residues C-terminal of the dioxin receptor LBD and a heterologous DNA binding domain show strong, ligand independent transcription activity, while including residues from the ligand/hsp90 binding domain in these chimeras represses this activity (Whitehat et al., 1994). Hsp90 does not interact with dioxin receptor residues C-terminal of the ligand binding domain in vitro (Whitehat et al., 1994) or in vivo (Whitehat et al., 1995), supporting a mechanism whereby hsp90 is the agent of repression.

Somewhat paradoxically, the interaction of hsp90 with the ligand binding domain allows the dioxin receptor to be activated, as translation in systems where the receptor does not interact with hsp90, either in vitro (wheat germ lysate) or in vivo (engineered S. cerevisiae), produces inert, dioxin nonresponsive proteins. The LBD-hsp90 interaction may therefore be inextricably entwined with both positive and negative regulatory functions of the dioxin receptor. Interestingly, there exists a very heterogeneous population of ligands for the dioxin receptor, seemingly all binding to the same central LBD core of the receptor. The nature of these ligand-receptor interactions is poorly understood, with planarity and size exclusion limits being the only common denominators employed in deriving a conceptual model to explain binding for the different ligand classes (Gillner et al., 1993), and references therein). Identification of the minimal LBD will facilitate exploration of the ligand binding pocket by reticulocyte protein mutagenesis, allowing a detailed definition of critical residues, or perhaps subsets of residues, which may confer differential specific binding to the dioxin, indole, flavone, and polycyclic aromatic hydrocarbon ligand classes.

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