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Definition of a novel ligand binding domain of a nuclear bHLH receptor: co-localization of ligand and hsp90 binding activities within the regulable inactivation domain of the dioxin receptor

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The dioxin receptor mediates signal transduction by dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) and binds to DNA target sequences as a heterodimer of the ~100 kDa ligand binding receptor and the ~85 kDa auxiliary factor, Arnt. Both of these factors encompass an N-terminal basic helix–loop–helix (bHLH) motif required for DNA binding and dimerization. In this study we describe the construction of glucocorticoid/dioxin receptor fusion proteins which allow the regulation of glucocorticoid receptor activity by dioxin in transient transfections of CHO and hepatoma cells. Thus, in the absence of dioxin, chimeric receptor constructs which contain large 500–720 amino acid C-terminal dioxin receptor fragments, but lack the N-terminal bHLH motif, confer repression upon the transcriptional activity of a glucocorticoid receptor derivative, τDBD, containing its N-terminal strong transactivating signal (τ) and its DNA binding domain (DBD). In the presence of dioxin, this repression is reversed. Importantly, these chimeric receptors did not require the bHLH Arnt co-factor for function. A considerably smaller region of the dioxin receptor, located between amino acids 230 and 421, showed specific dioxin binding activity *in vitro*. Moreover, dioxin binding *in vitro* correlated with the ability of receptor fragments to form stable complexes *in vitro* with the molecular chaperone hsp90. These findings support the notion that hsp90 may be important for folding of a dioxin binding configuration of the receptor. Finally, τDBD activity was constitutively repressed in a dioxin non-responsive manner by dioxin receptor fragments which failed to bind ligand but also failed to bind hsp90 *in vitro*, indicating that alternative mechanisms in addition to hsp90 binding may contribute to the inactivation function. In summary, the dioxin receptor system provides a novel and complex model of regulation of bHLH factors that may also give important insights into the mechanism of action of ligand-activated nuclear receptors.

Key words: basic helix–loop–helix factors/dioxin receptor/glucocorticoid receptor/ligand binding domain/negative regulation

Introduction

Dioxins are a set of environmental contaminants produced by minor side reactions during chemical manufacturing processes. The potent toxicity of dioxins is well established in animal models, typified by thymic wasting and immune suppression, severe epithelial disorders and tumor promotion (reviewed by Poland and Knutson, 1982). At the genetic level dioxins induce the expression of a battery of xenobiotic metabolizing gene products, such as cytochrome P450IA1, glutathione *S*-transferase Ya, aldehyde dehydrogenase and quinone oxidoreductase. These toxic and biochemical responses are mediated by the dioxin receptor (also called aryl hydrocarbon receptor), an intracellular protein which binds dioxin (TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) and its planar aromatic congeners with saturable high affinity (see Poellinger *et al.*, 1992 for review). The dioxin receptor has recently been shown to harbor a basic helix–loop–helix (bHLH) sequence motif (Burbach *et al.*, 1992; Ema *et al.*, 1992) that is common to the dimerization and DNA binding domains of a large group of gene regulatory factors that includes Myc/Max and MyoD (for a recent review see Kadesh, 1993). No endogenous ligand for this nuclear receptor is currently known. While our understanding of the events linking signal transduction of dioxins to their complex physiological phenomena is rudimentary, numerous studies have indicated a sequential process by which dioxin induces activation of the receptor to a functional form (reviewed by Poellinger *et al.*, 1992; Whitelaw *et al.*, 1993).

In non-stimulated target cells, a latent (non-DNA-binding) form of the receptor is maintained as an inert complex with the 90 kDa heat shock protein (hsp90), most probably in the cytoplasm of the cell (Perdew, 1988; Wilhelmsson *et al.*, 1990). Treatment with ligand initiates conversion of the receptor to a nuclear form, capable of binding specific dioxin-inducible transcriptional control sequences, termed xenobiotic response elements (XREs), upstream of dioxin responsive genes (Denison *et al.*, 1988; Fujisawa-Sehara *et al.*, 1988; Hapgood *et al.*, 1989; Neuhold *et al.*, 1989). During this activation process hsp90 is released from the receptor, while an auxiliary bHLH protein, Arnt, becomes complexed to produce a heterodimeric species. We and others have recently shown that Arnt, thought to be essential for nuclear translocation of the receptor (Hoffman *et al.*, 1991), is also part of the DNA-bound receptor complex (Reyes *et al.*, 1992; Whitelaw *et al.*, 1993). It appears that hsp90 modulates the functional interaction between the dioxin receptor and Arnt (Whitelaw *et al.*, 1993). Moreover, preliminary experiments indicate that this dimerization process may be under the control of a protein kinase C-dependent mechanism (Carrier *et al.*, 1992; Berghard *et al.*, 1993). The dioxin receptor system therefore provides an interesting model of a signal-controlled and highly regulated dimerization of bHLH factors.

Although the bHLH dioxin receptor is not structurally related to members of the steroid receptor superfamily (see Beato, 1989 for review), there are certain striking similarities in functional properties between these two nuclear receptor systems. Most notably, both the dioxin and glucocorticoid

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receptors form distinct complexes with hsp90. While it appears that hsp90 inhibits the DNA binding activity of both receptors (Denis *et al.*, 1988; Wilhelmsson *et al.*, 1990 and references therein), the precise role of hsp90 in the function of the glucocorticoid and dioxin receptors remains unclear. Since hsp90-free glucocorticoid and dioxin receptors exhibit low affinity for their corresponding target ligands (Bresnick *et al.*, 1989; Nemoto *et al.*, 1990; Pongratz *et al.*, 1992), it has been suggested that hsp90 may be critical for the ligand binding conformation of both receptors (reviewed by Pratt *et al.*, 1992). In support of this notion, hsp90 has recently been demonstrated to chaperone protein folding *in vitro* (Wiech *et al.*, 1992). Moreover, the glucocorticoid receptor expressed in yeast mutants expressing low levels of hsp90 is severely impaired in its hormone responsiveness (Picard *et al.*, 1990).

To investigate further the activation process of the dioxin receptor we have mapped the ligand binding region of the dioxin receptor. To this end we have constructed chimeric receptors containing the N-terminal transactivating and central DNA binding domains of the glucocorticoid receptor fused to a series of dioxin receptor fragments lacking the bHLH domain. In the present analysis we have combined transient transfection studies of receptor function *in vivo* with *in vitro* assays examining the ligand and hsp90 binding properties of the different receptor derivatives. Here we show that the ligand binding domain of the dioxin receptor mapped to a region between amino acids 230 and 421, and a concise correlation existed between the ability to bind ligand and the ability to form a stable complex with hsp90 *in vitro*. We also show that the constitutively active transactivation domain in the N-terminus of the glucocorticoid receptor was repressed in CHO and hepatoma cells by fusion of dioxin receptor fragments. For dioxin binding fragments this repression in both CHO and hepatoma cells could be relieved upon treatment with ligand. Interestingly, however, a larger region of the dioxin receptor was required in these cells for full de-repression by ligand *in vivo* than for binding of ligand *in vitro*, arguing that structures other than the concise ligand and hsp90 binding domains may be necessary to reverse the negative influence on receptor function. This seems to distinguish the dioxin receptor from the glucocorticoid receptor, where these two functions appear to be inseparable. Finally, a fragment of the dioxin receptor between amino acids 230 and 337 was identified that did not mediate hsp90 binding *in vitro* but mediated ligand-independent repression in CHO cells, indicating that mechanisms in addition to hsp90 interaction may be utilized to confer negative regulation on the remainder of the protein or on heterologous factors.

Results

Construction and analysis of dioxin receptor domain deletion mutants

Whereas members of the steroid hormone receptor superfamily are characterized by a highly conserved, centrally located DNA binding domain (the so-called zinc finger motif; Härd *et al.*, 1990; Luisi *et al.*, 1991 and references therein), the dioxin receptor contains a bHLH motif at the very N-terminus (shown schematically in Figure 1A) that is necessary for dimerization with the bHLH partner factor, Arnt, and subsequent DNA binding activity (Whitelaw *et al.*, 1993). To delineate a minimal region of the dioxin receptor essential for ligand responsiveness and binding, we constructed fusion genes in which a region of the human glucocorticoid receptor spanning its major transactivating ($\tau 1$, Giguère *et al.*, 1986; Hollenberg and Evans, 1988) and DNA binding domains was attached to dioxin receptor derivatives lacking the N-terminal bHLH motif (as detailed in Figure 1B). The activity of the resulting chimeric receptor constructs was initially monitored by transient transfection assays in hepatoma, CHO and COS cells by their ability to induce transcription from glucocorticoid response elements (GREs) in an MMTV promoter−placental alkaline phosphatase reporter gene, pMMTV-AF (Alksnis *et al.*, 1991; Göttlicher *et al.*, 1992). Based on this assay, the activation of the GRE-driven promoter by dioxin could be examined.

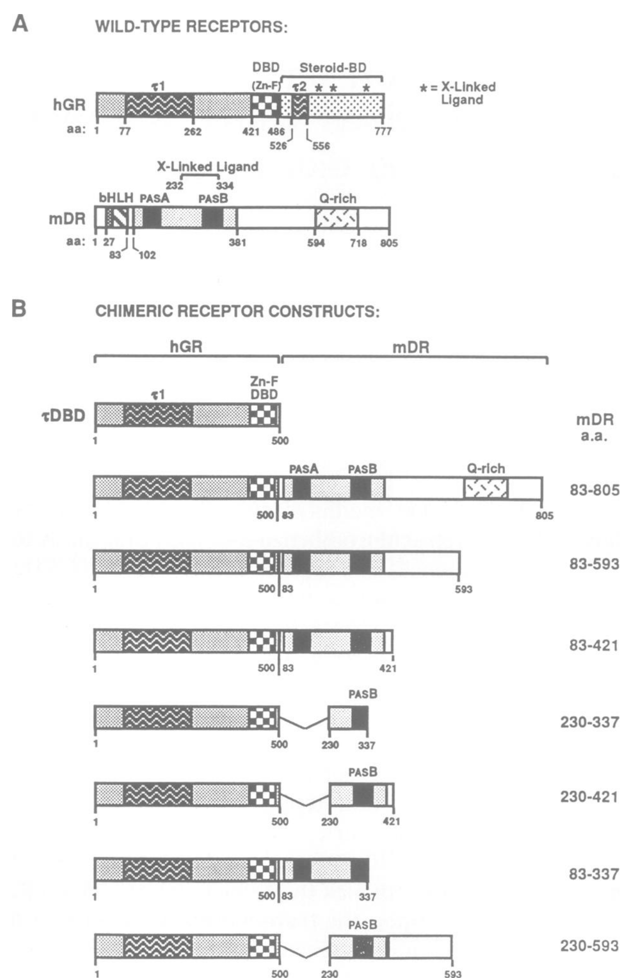

Fig. 1. Structural motifs within the dioxin and glucocorticoid receptors. **(A)** The structural organization of the wild-type human glucocorticoid (hGR) and mouse dioxin (mDR) receptors is schematically represented with functional domains of the glucocorticoid receptor indicated by $\tau 1$ and $\tau 2$ (transactivating regions 1 and 2), DBD [DNA binding domain containing the zinc finger (Zn-F) motif] and steroid binding domain (Steroid-BD). In the case of the dioxin receptor sequence motifs are indicated by bHLH (basic region helix−loop−helix), Per-Arnt-Sim (PAS) homology region (between amino acids 102 and 381) containing the two PAS repeats A and B, and a glutamine-rich sequence. Amino acids in the glucocorticoid receptor or a peptide fragment of the dioxin receptor identified by ligand crosslinking studies are indicated by stars and a bar, respectively. **(B)** Chimeric receptor constructs containing the N-terminal 500 amino acids of the human glucocorticoid receptor (termed τDBD) were constructed by fusion to varying C-terminal segments of the murine dioxin receptor.

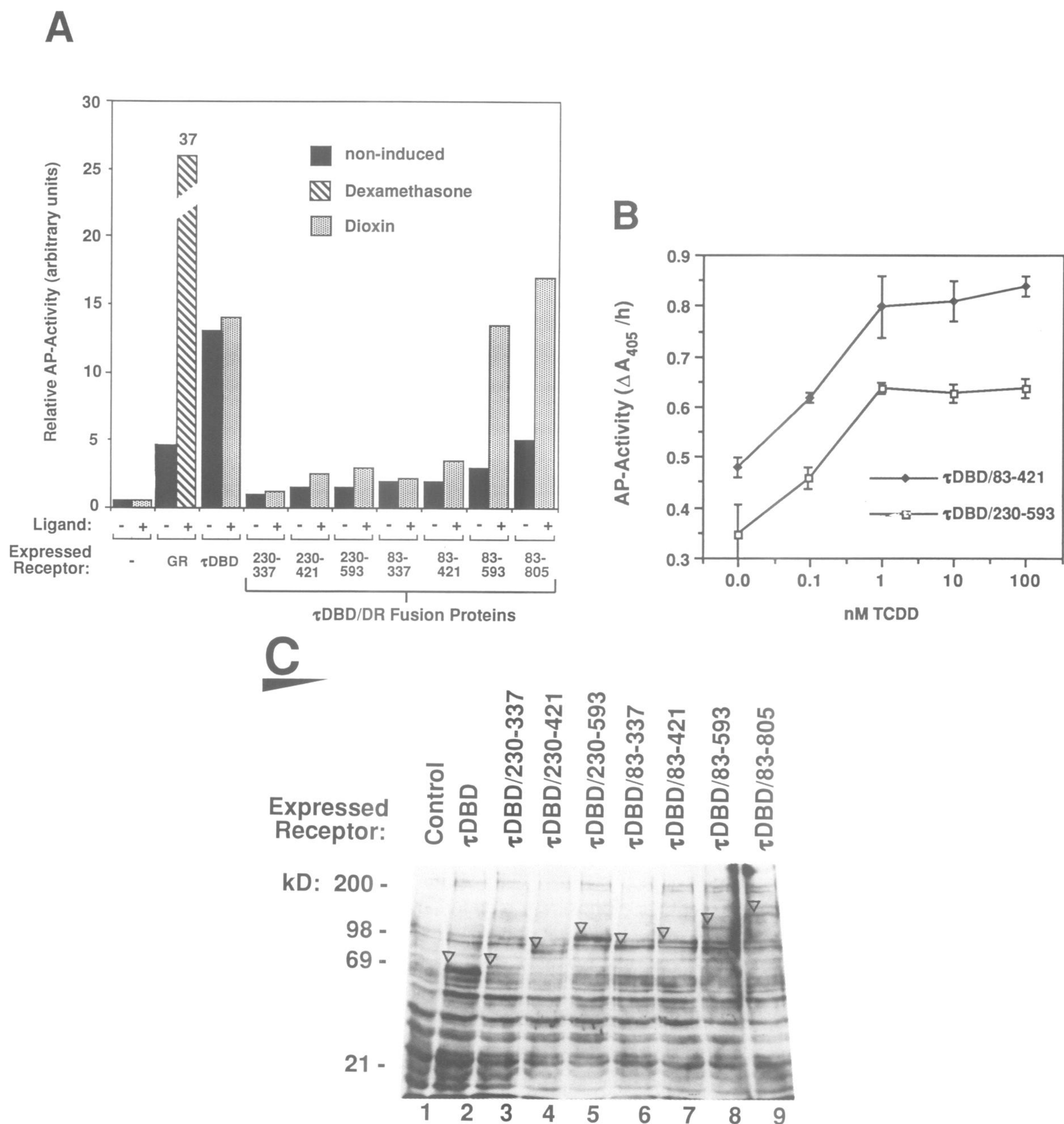

Fig. 2. Dioxin-induced activation of chimeric receptors in transient transfections of CHO cells. (**A**) The pMMTV-AF reporter gene was transfected with either blank MT vector, pMT-GR, pMT-τDBD or chimeric pMT-τDBD/dioxin receptor expression constructs. After 72 h of treatment with dexamethasone (5 μM) or dioxin (10 nM) as indicated, secreted alkaline phosphatase (AP) was measured by colorimetric assay. Results are representative data from at least three independent transfections. (**B**) Dose–response of dioxin-induced activation of pMT-τDBD/83–421 and pMT-τDBD/230–593 in CHO cells. The pMMTV-AF reporter gene was transfected with chimeric receptor constructs and treated with 0–100 nM dioxin. After 72 h secreted alkaline phosphatase (AP) was measured by colorimetric assay. Results are means (± standard deviation) of typical transfections. (**C**) Detection of τDBD/dioxin receptor chimeric proteins by Western blot. Whole cell extracts were prepared 48 h after CHO cells had been transfected with pτDBD/dioxin receptor/CMV4 constructs. Following SDS–PAGE and transfer to nitrocellulose, immunodetection with the monoclonal antibody 293 raised against the N-terminus of glucocorticoid receptor revealed full-length chimeric receptors, as indicated by the arrowheads.

The parental glucocorticoid receptor fragment (here termed τDBD, Figure 1B) harbors its transactivating and DNA binding functions but lacks the large, C-terminal portion of the protein where the hormone binding capacity of the receptor is contained. Furthermore, the deletion of the steroid binding domain gives rise to a constitutively active regulator which shows strong, hormone-independent activation of transcription of target genes (Godowski *et al.*, 1987; Hollenberg *et al.*, 1987; Figure 2A). Finally, when expressed in CHO (Figure 2A), hepatoma (Figure 5B) or COS (data not shown) cells, the transcriptional activity of the parental τDBD glucocorticoid receptor fragment was also not altered following exposure to dioxin.

Following deletion of the N-terminal bHLH motif of the dioxin receptor the remaining portion of the receptor located between amino acids 83 and 805 was fused C-terminally to the τDBD glucocorticoid receptor fragment. Transfection of this chimeric receptor (τDBD/83–805) into CHO (Figure 2A) or hepatoma cells (Figure 5B) only resulted in low levels of transcriptional stimulation of the GRE-driven

reporter gene. A strong activation response could, however, be observed following treatment of the cells with dioxin. CHO cells therefore provided a useful model system for further analysis of ligand-induced activation mechanisms by glucocorticoid/dioxin receptor chimeras.

Repression of glucocorticoid receptor activity by a minimal dioxin receptor fragment

In addition to the bHLH motif the dioxin receptor shares with the *Drosophila* protein Sim and the receptor co-regulator Arnt a distinct domain of similar sequence. This domain is also found in the *Drosophila* protein Per (which does not possess the bHLH motif) and is call PAS (for Per, Arnt, Sim; reviewed by Takahashi, 1992). The PAS domain encompasses ~280 amino acids (located between amino acids 102 and 381 of the dioxin receptor, Figure 1A) and contains two 51 amino acid direct repeat sequence motifs (PAS-A and PAS-B in Figure 1A). On the basis of the analysis of peptide fragments that were covalently crosslinked by a dioxin receptor photoaffinity ligand it has been proposed that the ligand binding domain of the mouse dioxin receptor is contained within a region of the receptor (between amino acids 232 and 334) that spans almost the entire PAS-B repeat motif (Burbach *et al.*, 1992). To examine directly the function of this putative ligand binding domain of the dioxin receptor, we fused a mouse dioxin receptor fragment spanning amino acids 230–337 to the C-terminus of the glucocorticoid receptor τDBD construct. Interestingly, the transcriptional activity of the resulting chimeric receptor (called τDBD/230–337) was strongly repressed in CHO cells (Figure 2A), implying that this region of the dioxin receptor harbors an inactivation function that can be conferred on the activity of a heterologous transactivator. Importantly, however, this mode of repression of glucocorticoid receptor activity was not reversed upon addition of dioxin. To investigate if ligand responsiveness lay N-terminally to the 230–337 segment, we extended this chimera to include the PAS-A domain, forming τDBD/83–337. Transfection into CHO cells gave a similar repression of the τ1 activity which was unaffected by dioxin treatment. Thus, we conclude that the regions spanning amino acids 230–337 and 83–337 of the dioxin receptor were not sufficient for ligand responsiveness.

Delineation of a domain that confers dioxin regulation upon the otherwise constitutively active τDBD glucocorticoid receptor fragment

We next examined the activity of chimeric receptor derivatives τDBD/230–421, containing the complete PAS-B domain of the dioxin receptor, and τDBD/83–421, spanning the entire PAS-A and PAS-B domains. These constructs also showed repression of transactivation in CHO cells, which could be relieved only slightly, but significantly (as detailed below), by dioxin treatment (Figure 2A). For completely dioxin-dependent regulation of τDBD activity it was necessary to fuse it to a rather large portion of the dioxin receptor encompassing amino acids 83–593. This observation implies that a region between amino acids 421 and 593 is important for regulation, although the τDBD/230–593 chimera, containing both this region and the minimal ligand-responsive domain, also failed to provide a totally regulable and reversible inactivation function.

While chimeric receptors containing amino acids 230–421, 230–593 or 83–421 of the dioxin receptor were unable to fully relieve repression upon exposure to ligand, they did show consistent, albeit small, levels of ligand responsiveness. Ligand-induced activation of these chimeras ranged from 1.5- to 2-fold, in contrast to chimeras containing the region 230–337 or 83–337, which were devoid of any ligand response (Figure 2A). To assess this ligand-dependent activation response in more detail, we performed a ligand dose–response experiment with the two receptor chimeras encompassing the C- and N-terminal boundaries of the minimal ligand response region of the dioxin receptor: τDBD/230–593 and τDBD/83–421. Figure 2B shows that activation of transcription by both these chimeras was already induced in CHO cells following exposure to 0.1 nM dioxin. Maximal levels of the activation response were produced by treatment of the cells with 1 nM dioxin. This induction response could not be further increased by addition of dioxin up to concentrations of 100 nM. These results correlate well with the K_d of ~1 nM for dioxin binding *in vitro* by the receptor (reviewed by Poland and Knutson, 1982) and the EC_{50} value of ~1–2 nM for dioxin-induced *in vitro* activation of the DNA binding activity of the receptor (Cuthill *et al.*, 1991). Thus, these results imply that the ligand binding domain may lie within the region of amino acids 230–421 of the dioxin receptor. Moreover, these results strongly argue that, although the τDBD/230–593 and τDBD/83–421 chimeric receptors do not show a total ligand-dependent relief of the inactivation function, they are not defective in their affinity for dioxin. The above results were obtained with both metallothionein promoter-driven (Figure 2A) and more powerful cytomegalovirus enhancer/promoter-driven expression vectors (data not shown). The low levels of ligand responsiveness observed for some of the shorter τDBD/dioxin receptor chimeras cannot be explained by variance in protein expression levels or DNA binding, as immunochemical staining of immunoblots indicated that the expression levels in CHO cells were similar for all tested chimeric receptors (Figure 2C), while specific DNA precipitation assays showed that *in vitro* translated chimeric receptors could recognize GRE sequences *in vitro* (data not shown). Thus, we conclude that the region spanning amino acids 230–421 is necessary but not sufficient for full total ligand-induced relief of the inactivation function of the dioxin receptor.

A comparison of the level of dioxin regulation of the τDBD/83–593 chimeric receptor with the rather subtle ligand response seen for the τDBD/230–593 derivative indicates that a region important for activation may lie between amino acids 83 and 230 of the dioxin receptor. However, shorter chimeras containing this region are either non-responsive to dioxin (τDBD/83–337) or, in the case of the τDBD/83–421 derivative, show only the low level of ligand responsiveness that was observed with the τDBD/230–593 chimera (Figure 2A). These data demonstrate that the region between amino acids 83 and 230 cannot independently reverse the inactivation function that is conferred on the τDBD derivative by the dioxin receptor fragment located between amino acids 230 and 337. Inclusion of the largest dioxin receptor fragment (83–805), spanning the intact C-terminus of the receptor, yielded levels of inducibility and transactivation in CHO cells that were comparable to those produced by the τDBD/83–593 chimeric receptor (Figure 2A). The τDBD/83–593 chimera

lacks the glutamine-rich region located close to the very C-terminus of the dioxin receptor (Burbach *et al.*, 1992). Glutamine-rich sequence motifs have been identified as transactivation domains of, for instance, the transcription factors SP1 (Courey and Tjan, 1988) and Oct-2 (Gerster *et al.*, 1990; Müller-Immerglück *et al.*, 1990). Interestingly, however, this region of the dioxin receptor did not greatly contribute to the overall transactivating potency of the τDBD construct when expressed in CHO cells since, upon stimulation by dioxin, very similar levels of transactivation were observed with the τDBD/83–805 chimeric receptor as compared with those obtained with τDBD protein alone (Figure 2A). The C-terminal glutamine-rich region clearly added transcriptional potency to the τDBD in COS (data not shown) and hepatoma cells (Figure 5B), indicating that a cell type-specific transactivation function is to be found within the very C-terminal domain of the dioxin receptor.

Mapping of the ligand binding domain of the dioxin receptor by direct ligand binding assays in vitro

To further define and characterize the primary determinants for ligand binding activity of the dioxin receptor we employed *in vitro* dioxin binding assays. To this end, the various chimeric receptor derivatives were expressed by *in vitro* translation of their corresponding mRNAs and subsequently used in hydroxylapatite adsorption ligand binding assays using [^{3}H]dioxin as specific ligand. The *in vitro* translated τDBD glucocorticoid receptor derivative alone showed no binding of [^{3}H]dioxin that surpassed the very low background levels that were observed with unprogrammed reticulocyte lysate (Figure 3). As expected from the functional transfection studies above, we also detected no binding of [^{3}H]dioxin by the chimeric receptor τDBD/230–337 bearing the minimal putative ligand binding motif of the dioxin receptor, as defined by ligand crosslinking studies (Burbach *et al.*, 1992). In addition, the construct containing dioxin receptor sequences from amino acids 83–337 was equally inert in the *in vitro* dioxin binding studies, ruling out the possibility that N-terminal residues were important for binding the ligand (Figure 3). However, the smallest chimera to show ligand responsiveness in transfection assays, τDBD/230–421, showed very high levels of dioxin binding activity (Figure 3). Specificity of the ligand binding reaction was established in competition experiments using a 150-fold molar excess of unlabeled ligand in addition to [^{3}H]dioxin. In these experiments, labeling of τDBD/83–805 by [^{3}H]dioxin was inhibited by an excess of the high affinity dioxin receptor ligand, 2,3,7,8-tetrachlorodibenzofuran (TCDF), whereas the [^{3}H]dioxin binding reaction was not affected by the presence of an excess of the high affinity glucocorticoid receptor ligand, dexamethasone (Figure 3). In conclusion, these *in vitro* assays define as the minimal ligand binding domain an identical region (between amino acids 230 and 421) of the receptor protein to that found as the minimal ligand-responsive domain *in vivo*. The minimal ligand binding region between amino acids 230 and 421 is also contained within the τDBD/83–421 and τDBD/230–597 chimeric receptors, which were found to produce minor ligand-responsiveness in CHO cells (Figure 2A and B). We interpret these results to indicate that the ligand binding structure of the dioxin receptor is necessary but not sufficient for a complete ligand-dependent de-repression of target factor

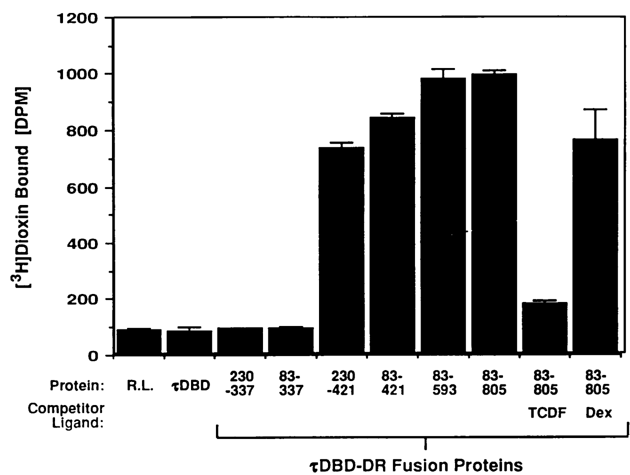

Fig. 3. Mapping of the dioxin receptor ligand binding domain by binding of [^{3}H]TCDD to τDBD/dioxin receptor chimeras. Unprogrammed reticulocyte lysate (lane 1) or reticulocyte lysates containing freshly translated chimeric receptor proteins (10 μl) were incubated with 2.5 nM [^{3}H]TCDD for 90 min at 25°C, followed by 30 min incubation with hydroxylapatite at 4°C. After extensive washing with TEG/0.1% Tween 20 buffer, bound [^{3}H]TCDD was measured by scintillation counting. The final two lanes show experiments in the presence of 150-fold molar excess of competitor ligands TCDF (tetrachlorodibenzofuran, specific competitor) and dexamethasone (Dex, non-specific competitor). Results are means ± standard error of typical experiments.

function. It appears rather that additional structural determinants within the receptor are required for transmission of the ligand-induced de-repression signal to DNA binding and/or transactivating surfaces of the receptor *in vivo*.

Correlation between ligand binding activity and association with the molecular chaperone hsp90

In analogy to the present results obtained with the dioxin receptor, the ligand binding domains of the glucocorticoid and estrogen receptors can be fused to a heterologous transcription factor, bringing its function under hormonal control (Picard *et al.*, 1988; Eilers *et al.*, 1989). Although the dioxin and steroid hormone receptors are different in primary structure, the dioxin, glucocorticoid and estrogen receptors are all known to form stable complexes with the molecular chaperone hsp90 (Perdew *et al.*, 1988; Beato, 1989; Wilhelmsson *et al.*, 1990 and references therein). While the role of hsp90 in steroid receptor function is not yet fully understood, hsp90 has been suggested to be important for folding of the glucocorticoid receptor in a hormone binding, repressed configuration (see Pratt *et al.*, 1992; Smith and Toft, 1993 for recent reviews). In the case of the dioxin receptor, hsp90 appears to repress the DNA binding activity of the receptor in the absence of ligand (Wilhelmsson *et al.*, 1990; Pongratz *et al.*, 1992), possibly by interference with the interaction between the receptor and the bHLH partner factor, Arnt (Whitelaw *et al.*, 1993).

To investigate the role of hsp90 in the ligand binding function of the dioxin receptor, we expressed the different τDBD-dioxin receptor derivatives by *in vitro* translation in reticulocyte lysates. Translation in the presence of [^{35}S]methionine produced single, labeled proteins of the expected molecular mass, as assessed by SDS–PAGE (Figure 4A). We next employed a unique monoclonal anti-hsp90 antibody capable of immunoprecipitating species of

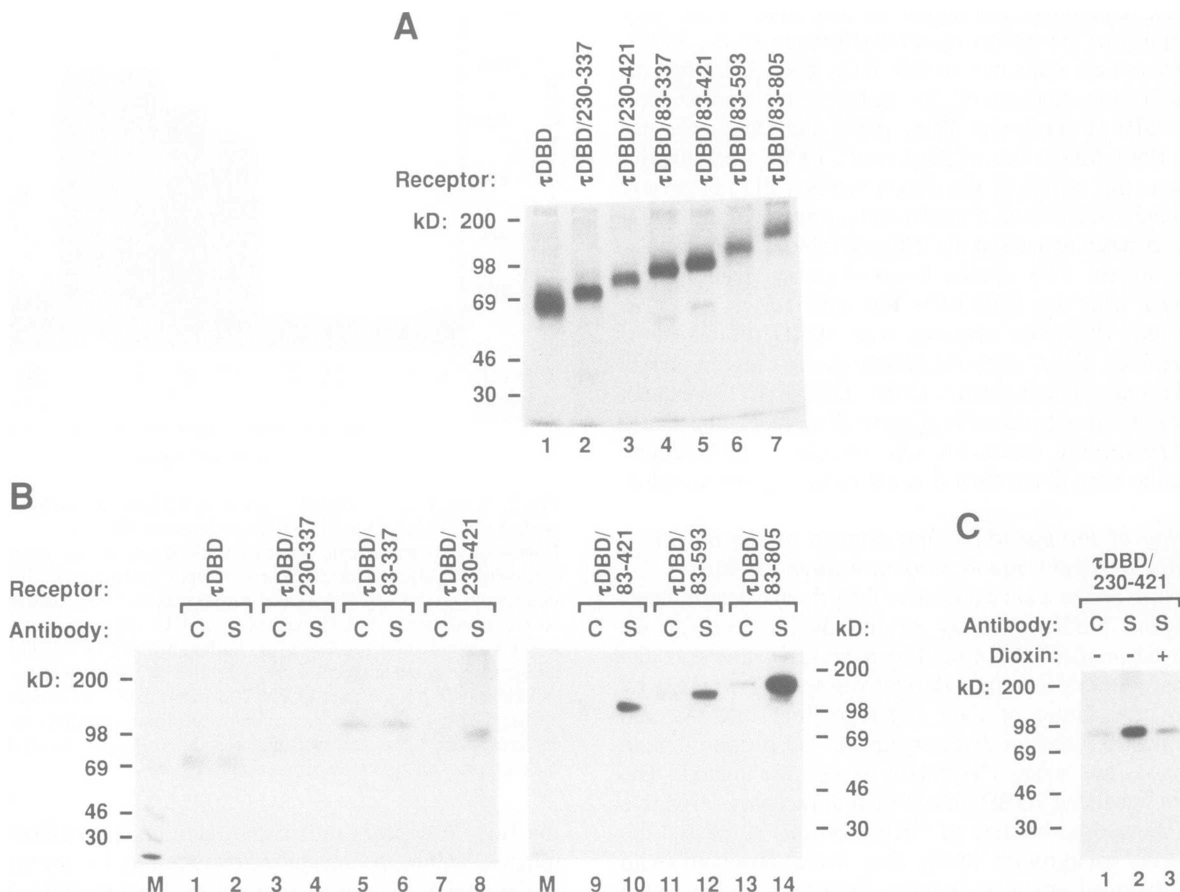

Fig. 4. The region mediating hsp90 interaction correlates with the ligand binding domain in τDBD/dioxin receptor chimeras. (**A**) Analysis of [^{35}S]methionine-labelled, *in vitro* translated τDBD/dioxin receptor fusion proteins by SDS–PAGE and fluorography. (**B**) Co-immunoprecipitation of τDBD/dioxin receptor chimeras with hsp90-specific antibodies. An anti-hsp90 antibody, 3G3 (5 μl), or control IgM ascites (2.5 μl) were preadsorbed to rabbit anti-mouse antibody–Sepharose. Reticulocyte lysates containing [^{35}S]methionine-labelled receptor fusion proteins (5 μl) were incubated with the antibody resin and washed extensively, and the immunoprecipitated proteins were analysed by SDS–PAGE and fluorography. Even numbered lanes contain proteins co-immunoprecipitated by the hsp90-specific antibody (S), while odd numbered lanes contain non-specific control immunoprecipitates (C). Lanes M contain molecular weight markers. (**C**) Reticulocyte lysate containing [^{35}S]methionine-labelled τDBD/230–421 was incubated with 10 nM dioxin (lane 3) or with vehicle alone (lanes 1 and 2) at 25°C for 3 h, followed by the immunoprecipitation protocol outlined for panel B. Lane 1 represents immunoprecipitation with control antibodies; lanes 2 and 3 show immunoprecipitation with the anti-hsp90 monoclonal antibody, 3G3.

hsp90 complexed with other proteins (Perdew and Whitelaw, 1991) to examine directly a possible association between the *in vitro* translated products and hsp90. The precipitated material was subsequently analyzed by SDS–PAGE and fluorography. By this procedure the anti-hsp90 antibody showed no selectivity for the τDBD protein alone as compared with a control IgM antibody (Figure 4B, compare lanes 1 and 2), in perfect agreement with the absence in the τDBD construct of the C-terminal region of the glucocorticoid receptor that mediates interaction with hsp90 (reviewed by Beato, 1989). In a similar fashion, only low levels of non-specific interaction with the anti-hsp90 antibody were observed in reactions containing the τDBD/230–337 and τDBD/83–337 chimeric receptors (Figure 4B, lanes 3–6). In contrast, the τDBD/230-421 chimeric receptor was selectively precipitated by the anti-hsp90 antibody (compare lanes 7 and 8), indicating that the region between amino acids 230 and 421 of the dioxin receptor mediated association with hsp90 in solution. Consistent with this interpretation, the larger chimeric receptors spanning additional N-terminal and C-terminal amino acids of the dioxin receptor (τDBD/83–421, τDBD/83–593 and τDBD/83–805) were preferentially precipitated by the anti-hsp90 antibody, whereas little or no reactivity was observed with the control antibody (Figure 4B, lanes 9–14). As the proposed mechanism of dioxin receptor activation entails the ligand induced release of hsp90, we reasoned that dioxin treatment of the hsp90-bound fusion proteins might invoke dissociation of hsp90. To test this hypothesis and to provide further evidence that the 230–421 amino acid segment of the dioxin receptor was sufficient to bind hsp90, we repeated the immunoprecipitation protocol after τDBD/230–421 had been incubated with 10 nM TCDD. As shown in Figure 4C, this provided a marked decrease in the amount of τDBD/230–421 co-immunoprecipitated by the hsp90 antibody. The above data suggest that a dioxin receptor core region of ~190 amino acids (amino acids 230–421) encompasses two elemental properties: ligand binding activity and stable association with hsp90. The correlation between these two receptor functions supports the notion that hsp90 may be an important determinant of the ligand binding conformation, possibly by chaperoning protein folding. We are currently trying to test this hypothesis in closer detail.

A dioxin-regulated τDBD chimeric receptor does not require the bHLH Arnt co-regulator for function

The ligand-activated, DNA binding form of dioxin receptor is a ~200 kDa heteromeric complex of the receptor and

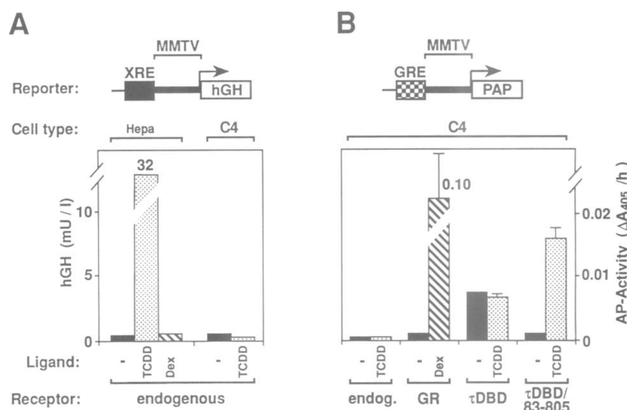

Fig. 5. Dioxin activation of a chimeric τDBD/dioxin receptor protein does not require the bHLH Arnt coregulator. **(A)** The native dioxin receptor is unresponsive to dioxin in Arnt-deficient C4 cells. Transfection of the reporter gene, XRE-MMTV-hGH, into wild-type Hepa 1c1c7 cells and Arnt-deficient C4 cells was followed by treatment with dioxin (TCDD; 5 nM) or dexamethasone (Dex; 5 μM) as indicated. Secreted growth hormone levels were measured by radioimmunoassay. **(B)** Arnt-deficient C4 cells were transfected with the pMMTV-AF reporter gene and either pMT-GR, pMT-τDBD or pMT-τDBD/83–805. Cells were treated with dioxin (TCDD; 10 nM) or dexamethasone (Dex; 5 μM) for 72 h as indicated. Secreted alkaline phosphatase (AP) was measured by colorimetric assay. Results are means ± standard error of typical experiments.

its ~85 kDa bHLH partner molecule Arnt (Reyes *et al.*, 1992; Whitelaw *et al.*, 1993). Neither the receptor nor Arnt bind DNA individually but they require one another for functional interaction with DNA target sequences (Whitelaw *et al.*, 1993). Thus, in Arnt-deficient hepatoma cell mutants (Hepa-1-C4), the dioxin receptor is non-functional, although it exhibits bona fide levels of ligand binding activity and is expressed at levels similar to those in wild-type Hepa-1 hepatoma cells (Hoffman *et al.*, 1991 and references therein). As illustrated in Figure 5A, transfection of wild-type Hepa-1 cells with an MMTV reporter gene construct containing a single XRE sequence motif in lieu of the endogenous MMTV GREs gave a rather dramatic induction response by the endogenous dioxin receptor upon addition of dioxin, whereas very low basal activity was observed in the absence of receptor ligand or following stimulation with dexamethasone. In contrast, this dioxin induction response was completely absent in the mutant Hepa-1-C4 cells, as assessed by transfection with the identical XRE-MMTV reporter gene construct. Immunochemical staining with Arnt-specific antibodies demonstrated expression of Arnt in both COS and CHO cell lines (M.Whitelaw and L.Poellinger, unpublished results), necessitating the use of the Arnt-deficient Hepa-1-C4 cells to establish if τDBD/dioxin receptor chimeras are able to activate the reporter gene transcription independently of the Arnt partner factor. In the absence of dioxin, transfection of the Hepa-1-C4 cells with the τDBD/83–805 chimeric receptor produced very low levels of activation of a co-transfected MMTV reporter gene containing endogenous GRE sequences. These levels were similar to those observed in Hepa-1-C4 transfected with the GRE-MMTV reporter alone (Figure 5B). Thus, the activity of the τDBD/83–805 receptor is also constitutively repressed in these cells, possibly to a higher degree than in CHO cells (compare Figures 2A and 5B). Interestingly, however, the activity of the τDBD/83–805 chimera was strongly stimulated by dioxin (Figure 5B), demonstrating that the function of this chimeric receptor is uncoupled from the Arnt co-regulator. Since the τDBD/83–805 factor lacks the N-terminal bHLH motif of the dioxin receptor, this observation is consistent with the model that the bHLH motif is the sole mediator of the functional interaction between the dioxin receptor and Arnt. Conversely, we have recently demonstrated that the bHLH motif of Arnt mediates interaction with the dioxin receptor (Whitelaw *et al.*, 1993). It is also interesting to note that the transcriptional activity of the dioxin-stimulated τDBD/83–805 chimeric receptor was higher in Hepa-1-C4 cells than the corresponding activity of the parental τDBD factor alone (Figure 5B), in a similar fashion to the higher activity of τDBD/83–805 than τDBD seen in COS cells (data not shown). Given the very similar activities of these two proteins in CHO cells (Figure 2A) it is therefore possible that cell type-specific co-factors determine the transcriptional potency of the dioxin receptor.

Discussion

Delineation of a ligand binding core region of the dioxin receptor

In our efforts to understand the mechanism of signal transduction by the dioxin receptor and to facilitate functional comparisons between the dioxin and steroid hormone receptor systems we have attempted to identify structural determinants of the ligand binding specificity of the dioxin receptor. The present results indicated that a core region between amino acids 230 and 421 is sufficient for specific dioxin binding activity *in vitro*. This region also provided very subtle but detectable levels of dioxin responsiveness *in vivo*. In contrast, steroid hormone receptors require considerably larger regions for ligand binding activity (reviewed by Beato, 1989). Although the three-dimensional structures of the ligand binding domains of steroid hormone receptors have not yet been determined, ligand crosslinking and mutational studies have indicated amino acids relevant for hormone binding. By this procedure Cys638, Met604 and Cys736 of the human glucocorticoid receptor (indicated by stars in Figure 1A) have been identified as amino acids which are either in close proximity to the ligand or directly involved in the ligand binding process (Strömstedt *et al.*, 1990; Chakraborti *et al.*, 1992). However, these residues are not conserved in the minimal ligand binding domain of the dioxin receptor, and we failed to detect any other conserved sequence motifs in a preliminary alignment of the ligand binding domains of the glucocorticoid, estrogen, progesterone and mineralocorticoid receptors with that of the dioxin receptor.

Function of the ligand binding domain of the dioxin receptor

The minimal ligand binding region appeared to be sufficient to confer dioxin regulation upon a heterologous transcription factor *in vivo* since the τDBD/230–421 chimeric receptor showed constitutive repression in CHO cells and was activated, albeit to a low degree, by dioxin treatment in these cells. All chimeras containing this minimal ligand binding region showed ligand responsiveness in CHO cells, while the two chimeric receptors devoid of this domain, τDBD/83–337 and τDBD/230–337, showed repression of τDBD activity but could not be activated by ligand.

Moreover, activation of chimeras containing the minimal ligand-responsive domain was maximal at ~1 nM dioxin, in excellent agreement with the K_d of ~1 nM (Poland and Knutson, 1982) for dioxin binding *in vitro* by the receptor. Strikingly, portions of the dioxin receptor significantly larger than the minimal ligand binding domain were necessary in CHO and hepatoma cells to obtain ligand-induced levels of activity that were equal to those produced by the parental τDBD fragment. It is presently difficult to envisage the specific functional and structural basis for the requirement of amino acids in addition to those determining ligand binding activity for full ligand-induced relief of the inactivation function. Although it is conceivable that a larger portion of the receptor may be required for aquiring a fully ligand-responsive conformation, it is equally possible that discrete ligand-dependent transactivation functions are harbored adjacent to the minimal ligand binding domain. Given the strong dioxin-induced activation response of the τDBD/83−593 chimera and the low response of the τDBD/83−421 and τDBD/230−593 chimeras, such inducible transactivation functions may be located between amino acids 83 and 230, and between 421 and 593. By analogy, the ligand binding domains of the glucocorticoid and estrogen receptors harbor a discrete, hormone-inducible transactivation function (called τ2 in Figure 1A; Giguère *et al.*, 1986; Hollenberg and Evans, 1988; Webster *et al.*, 1988). Alternatively, it is possible that the dioxin receptor regions between amino acids 83 and 230 and between 421 and 593 contain transactivation functions which synergize with each other and the more potent transactivation domain in the C-terminus, but cannot act independently. Consistent with this mechanism, a recent analysis of TEF-1, a transcription factor active on the SV40 enhancer, revealed the presence of at least three regions essential for transactivation which could not function independently (Hwang *et al.*, 1993). We are currently examining these two models for potentiation of the dioxin response in closer detail. Finally, the strong activity of the τDBD/83−805 chimera in COS and Hepa cells indicates that a glutamine-rich transactivation domain is located in the very C-terminus of the dioxin receptor between amino acids 593 and 805. This transactivation function does not appear to be similarly active in CHO cells. The transcriptional potency of the dioxin receptor could therefore be determined by cell type-specific coactivating factors interacting with the C-terminal glutamine-rich domain. Clearly, this property would increase the potential of the dioxin receptor to respond to regulatory signals in a cell-specific manner. In analogy to this model, the autonomous, constitutive, N-terminal activation domain of the estrogen receptor has been shown to exhibit cell type specificity (Tora *et al.*, 1989).

Functionally, the requirement of regions beyond the minimal ligand binding domain for full ligand responsiveness *in vivo* distinguishes the dioxin receptor from the glucocorticoid receptor. In the case of the glucocorticoid receptor, the ligand binding and regulable inactivation functions of the receptor appear to coincide (Picard *et al.*, 1988 and references therein). Generally, the ligand binding domains of steroid hormone receptors seem to be very complex structurally and functionally since they may also harbor dimerization, nuclear translocation and/or transactivation functions in addition to mediating hormone binding activity (see Green and Chambon, 1988; Beato, 1989 for reviews). Although it is formally possible that the dioxin receptor may require dimerization with as yet unidentified co-factors for function, it is noteworthy that the dimerization interface mediating the interaction with the Arnt co-factor seems to be strictly limited to the bHLH motif in the very N-terminus of the receptor. With regard to nuclear localization signals, it is striking that the dioxin receptor exhibits a nuclear translocation-deficient phenotype in Arnt-deficient, mutant hepatoma cells. This has been interpreted to indicate that the bHLH factor, Arnt, is the primary determinant of nuclear localization of the dioxin receptor (Hoffman *et al.*, 1991). If this is the case, the dioxin receptor may lack endogenous nuclear translocation signals, arguing that the dioxin receptor region mediating ligand-dependent regulation may be less complex than the corresponding region of the glucocorticoid receptor which also harbors a hormone-dependent nuclear translocation signal, in addition to a constitutive translocation signal coinciding with its DNA binding domain (Picard and Yamamoto, 1987). Finally, it is possible that protein configuration may be critical for dioxin-dependent derepression or activation *in vivo*, explaining why a rather large region of the protein is required for this effect to occur. In contrast, it has been argued that the hormone binding region of the glucocorticoid receptor operates rather independently of surrounding protein structure (Picard *et al.*, 1988).

Role of hsp90 in modulating dioxin receptor function

Our experiments show that the minimal ligand binding region of the dioxin receptor located between amino acids 230 and 421 also constituted the minimal domain required for formation of a stable complex *in vitro* with the molecular chaperone, hsp90. Importantly, this minimal ligand binding region could also mediate a dioxin-dependent release of hsp90 *in vitro*. Moreover, the ability of dioxin receptor fragments to associate with hsp90 was strictly correlated with their ability to bind ligand. In a similar fashion we have previously shown that there exists an inverse correlation between the DNA binding activity of the dioxin receptor and its association with hsp90 (Wilhelmsson *et al.*, 1990; Pongratz *et al.*, 1992). Taken together with our earlier findings that the dioxin receptor shows very low affinity for its ligand following artificially induced release of hsp90 (Pongratz *et al.*, 1992), these data strongly argue that hsp90 may be involved in at least two critical functions of the dioxin receptor: repression and the formation of a ligand binding conformation. The observation that the dioxin receptor loses its affinity for dioxin if the hsp90−receptor complex is disrupted (Pongratz *et al.*, 1992) can be interpreted to suggest that the receptor unfolds in the absence of hsp90. This model would lend substantial support to the notion that the role of hsp90 is to chaperone the correct folding of the ligand binding domain of the dioxin receptor. In addition, given the background that the bHLH factor, Arnt, can only dimerize with the ligand-stimulated form of dioxin receptor (Whitelaw *et al.*, 1993), it is possible that protein folding by hsp90 maintains the receptor in an inactive, repressed form that is incapable of, for instance, interaction with its partner factor. However, it is equally possible that repression of receptor function is simply mediated by steric hindrance, for instance by masking of the bHLH dimerization/DNA binding domain by hsp90. It will now be important to examine these two models experimentally.

The postulated function of hsp90 as a chaperone of the

folding of the ligand binding domain of the dioxin receptor is not without precedent. In an *in vitro* system, it has recently been demonstrated that purified bovine hsp90 greatly stimulates protein folding. Consistent with the model that hsp90 may be involved in maintaining a certain subset of proteins in an inactive form, it has also been shown that hsp90 is able to specifically recognize and bind non-native forms of proteins *in vitro* (Wiech *et al.*, 1992). Interestingly, hsp90 has also recently been reported to interact transiently with the bHLH factor MyoD and to be involved in the formation of a DNA binding competent form of MyoD (Shaknovich *et al.*, 1992). Thus, an additional role of hsp90 may be the conformational activation of the bHLH motif. We have hitherto not tested this possible function of hsp90 in the dioxin receptor system. Strikingly, hsp90 is thought to regulate negatively the activity and nuclear translocation of the glucocorticoid receptor by the formation of a large, heteromeric complex in the absence of ligand. Moreover, the hsp90 binding region is contained within the C-terminal ligand binding domain of the glucocorticoid receptor, and the hsp90-free form of glucocorticoid receptor does not express any high affinity hormone binding activity (for recent reviews see Pratt *et al.*, 1992; Smith and Toft, 1993). In contrast to the glucocorticoid receptor system, however, it may be interesting to note that the present study has indicated a region of the dioxin receptor (located between amino acids 230 and 337) that conferred on the τDBD receptor derivative constitutive negative regulation that was non-responsive to dioxin treatment. Interestingly, this fragment did not mediate binding to hsp90, indicating the intriguing but testable possibility that the dioxin receptor may harbor additional repression functions that may be distinct from hsp90-mediated mechanisms.

Regulation of dioxin receptor activity by dimerization with the bHLH Arnt auxiliary factor

The dioxin receptor requires interaction with the bHLH partner factor Arnt to be able to interact with the XRE DNA target sequence (Whitelaw *et al.*, 1993). In the absence of Arnt, for instance when expressed in Arnt-deficient Hepa-1-C4 mutant cells, the receptor exhibits no endogenous DNA binding activity and is non-functional (Cuthill *et al.*, 1991; Hoffman *et al.*, 1991 and references therein). It is therefore an interesting observation that the τDBD/83−805 chimeric receptor construct lacking the bHLH motif in the very N-terminus of the dioxin receptor was functional and showed ligand regulation when transiently transfected into the Hepa-1-C4 mutant cells. A similar ligand regulation of chimeric receptor τDBD/83−593 was seen in Hepa-1-C4 cells (data not shown). Thus, deletion of the bHLH motif of the dioxin receptor uncouples it from the requirement of the Arnt auxiliary factor for function, supporting the notion that, apart from being necessary for DNA binding activity of the bHLH motif of the dioxin receptor (Whitelaw *et al.*, 1993) and possibly nuclear translocation (Hoffman *et al.*, 1991), Arnt plays no role *per se* in the ligand responsiveness of the dioxin receptor. Although it is formally possible that Arnt may represent a novel class of bHLH orphan receptors that are capable of binding as yet unidentified categories of ligands, Arnt activity is not affected by dioxin treatment *in vitro* or *in vivo* (Whitelaw *et al.*, 1993). Our experiments indicate that one possible additional function of Arnt may be to contribute to the overall transcriptional potency of the ligand-activated dioxin receptor. It is striking that the activity of the ligand-activated τDBD/83−805 chimeric receptor construct was very similar if not identical to that of the parental τDBD glucocorticoid receptor fragment upon expression in CHO cells, suggesting the absence of a strong transactivating domain within the dioxin receptor itself. However, we observed a significant, albeit weak, potentiation of τDBD activity by dioxin receptor sequences following expression in COS and Hepa-1-C4 cells. It is therefore conceivable that transactivating functions of the dioxin receptor (possibly contained within the glutamine-rich sequence motif) may be cell type specific. In line with this model it is noteworthy that the activity of the dioxin-activated τDBD/83−805 chimeric receptor in Hepa-1-C4 was considerably weaker than that of the ligand-activated full-length glucocorticoid receptor used in control experiments (Figure 5), arguing that the major transactivating potential of the dioxin-activated heterodimeric, endogenous dioxin receptor may be contained within the bHLH Arnt partner protein. Clearly, further experiments, including identification and mutagenesis of transactivating domains within Arnt, are required to establish the role of Arnt in transcriptional regulation of target genes by the dioxin receptor.

Materials and methods

Plasmid constructions

cDNA fragments of the murine dioxin receptor encompassing codons 83−805, 83−593, 83−421 and 230−337 were primed from pSportAhR (containing the full-length mouse dioxin receptor; plasmid generously provided by Dr Christopher A.Bradfield, Northwestern University Medical School, Chicago, IL) by 20 cycles (1 min, 94°C; 1 min, 55°C; 3 min, 72°C) of PCR (Perkin-Elmer/Cetus). Primers were designed to provide *Xho*I restriction sites at their termini. The cDNA fragments were digested with *Xho*I and subcloned into the *Xho*I-digested pMT-GR/PPAR (Göttlicher *et al.*, 1992), replacing the ligand binding domain of the peroxisome proliferator-activated receptor and forming plasmids pMT-τDBD/230−337, pMT-τDBD/83−421, pMT-τDBD/83−593 and pMT-τDBD/83−805. These constructs contain the coding region for amino acids 1−500 of the human glucocorticoid receptor in-frame with the respective codons for amino acids 230−337, 83−421, 83−593 and 83−805 of the murine dioxin receptor, under the control of the metallothionein (MT) promoter. Codons for the additional amino acids Pro and Arg were incorporated at the fusion site between the glucocorticoid receptor and the dioxin receptor as a result of the *Xho*I linker, and similarly Pro, Arg and Val were introduced at the 3′ end of each chimera, with the exception of pMT-τDBD/230−337 which has Thr, Arg and Val at the 3′ end. Removal of the PPAR sequence from pMT-GR/PPAR by digestion with *Xho*I provided pMT-τDBD. pτDBD/GEM and pτDBD/CMV4 were generated by subcloning *Bam*HI−*Eco*RI and *Bam*HI−*Xba*I fragments of pMT-hGR (Alksnis *et al.*, 1991) or pMT-τDBD, respectively, coding for amino acids 1−500 of the human glucocorticoid receptor into pGEM-7Zf(+) (Promega) or pCMV4 (Andersson *et al.*, 1989). Excision of a *Cla*I−*Xba*I fragment from each pMT-τDBD/dioxin receptor plasmid and insertion into *Cla*I/*Xba*I digested pτDBD/GEM gave the plasmids pτDBD/230−337/GEM, pτDBD/83−421/GEM, pτDBD/83−593/GEM and pτDBD/83−805/GEM, which were used for *in vitro* translation. Plasmids pτDBD/230−421/GEM and pτDBD/230−593/GEM were constructed by excision of a *Cla*I−*Kpn*I fragment from pτDBD/230−337/GEM and insertion into *Cla*I/*Kpn*I digested pτDBD/83−421/GEM and pτDBD/83−593/GEM, respectively. Plasmid pτDBD/83−337/GEM was constructed by excising a *Cla*I−*Kpn*I fragment from pτDBD/83−421/GEM and inserting into *Cla*I/*Kpn*I digested pτDBD/230−337/GEM. Plasmids pMT-τDBD/83−337, pMT-τDBD/230−421 and pMT-τDBD/230−593 were obtained by excision of *Cla*−*Xba*I fragments from the relative GEM vector constructs and insertion into *Cla*I/*Xba*I digested pMT-τDBD. To generate chimeric receptor genes driven by the cytomegalovirus enhancer/promoter, *Cla*I−*Xba*I fragments of each corresponding GEM vector construct were subcloned into *Cla*I/*Xba*I digested pτDBD/CMV4. Sequences and direction of inserts were confirmed

by dideoxy sequencing and/or multiple restriction digestions and analysis of *in vitro* translation products. The pMMTV-AF (generously provided by Dr Stefan Nilsson, KaroBio Inc., Stockholm) and pXRE-MMTV-hGH2 reporter gene constructs have been previously described (Alksnis *et al.*, 1991; Whitelaw *et al.*, 1993).

Cell culture and transfections

CHO cells were cultured in Ham's F12 medium supplemented with 10% fetal calf serum, 100 U of penicillin and 100 μg of streptomycin per ml (Gibco/BRL). A mouse hepatoma cell line, Hepa 1c1c7, and the mutant C4 line derived from it were cultured in minimum essential medium as described previously (Wilhelmsson *et al.*, 1990). Cells were seeded at a density of 10^5 per 35 $\times$ 10 mm dish and grown for 24 h. Reporter constructs (1 μg) and metallothionein or CMV promoter-driven expression vectors (1 μg) were transfected with 10 μl of lipofectin (Bethesda Research Laboratories) or 7.5 μg of DOTAP (Boehringer) as described by the manufacturers. After 12 h of transfection the cells were induced with either 5 μM dexamethasone, the indicated amount of dioxin, or vehicle alone (0.1% DMSO) for 72 h. Secreted hGH levels produced by the XRE-MMTV-hGH reporter (Whitelaw *et al.*, 1993) were assayed in the cell medium by radioimmunoassay (Pharmacia). Secreted placental alkaline phosphatase from the pMMTV-AF reporter (Göttlicher *et al.*, 1992) was determined by a colorimetric assay. Briefly, cell supernatants were heated at 65°C for 30 min, then incubated at 37°C in a mixture containing 200 mM Tris (pH 8.8), 275 mM NaCl, 0.5 mM $MgCl_2$ and 5 mM *p*-nitrophenyl phosphate. Phosphatase levels were measured by the increase in absorbance at 405 nm. To obtain chimeric proteins for analysis by Western blot, CHO cells were transfected with 5 μg of each CMV-driven expression vector and 30 μg of DOTAP (Boehringer) in 60 $\times$ 10 mm dishes. After 48 h cells were collected, washed with PBS and dispersed in 50 μl whole cell extract buffer (20 mM HEPES pH 7.9, 1.5 mM $MgSO_4$, 0.2 mM EDTA, 0.42 M NaCl, 0.5% NP-40, 25% glycerol, 1 mM DTT, 1 mM PMSF). After 30 min shaking on ice extracts were centrifuged at 12 000 r.p.m. in an Eppendorf centrifuge (4°C) and the supernatants collected. Samples (60 μg protein) were subjected to 7.5% SDS–PAGE and transferred to nitrocellulose in a semi-dry blotter (Bio-Rad). Chimeric proteins were detected with monoclonal antibody 293 directed against the N-terminus of the glucocorticoid receptor (Okret *et al.*, 1984), using the ECL detection system (Amersham) according to the recommendations of the manufacturer.

Ligand binding assay

Chimeric proteins were *in vitro* translated from mRNAs [generated from pGEM (Promega) vector constructs] in reticulocyte lysates (Promega) in the presence of unlabeled methionine under conditions recommended by the manufacturer. Ligand binding was assayed by a hydroxylapatite (HAP) adsorption assay (Gasiewicz and Neal, 1982; Poellinger *et al.*, 1985). Aliquots of *in vitro* translation mixtures (10 μl) were incubated with 10 μl TEG buffer [20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol] containing 1 mM PMSF, 5 μg/ml aprotinin and $[^3H]$TCDD (40 Ci/mmol; Chemsyn, Lenexa, KS) at a final concentration of 2.5 nM. After incubation for 90 min at room temperature, a 50 μl suspension of HAP in TEG buffer (50:50, v/v) was added and the mixtures were shaken on ice for 30 min. After rapid centrifugation the supernatants were discarded and the pellets were washed four times with 500 μl TEG buffer containing 0.1% Tween 20. The pellets were then washed twice with 500 μl ethanol and the supernatants subjected to scintillation counting.

Immunoprecipitation with anti-hsp90 antibody

For immunoprecipitation experiments 5 μl of the monoclonal anti-hsp90 IgM antibody, 3G3 (Perdew and Whitelaw, 1991; purchased from Affinity Bioreagents), or an equal amount (2.5 μl) of the control mouse IgM antibody, TEPC 183 (Sigma), was incubated with 150 μl of a 1:4 suspension of Sepharose-coupled rabbit anti-mouse antibody (Dako) in MENG buffer (25 mM MOPS, 1 mM EDTA, 0.02% NaN_3, 10% glycerol, pH 7.5). After 90 min shaking on ice the suspension was rapidly pelleted and washed successively with 1 ml volumes of MENG–500 mM NaCl, MENG and finally buffer F (MENG–50 mM NaCl, 20 mM sodium molybdate, 2 mg/ml ovalbumin). The pellets were resuspended in 1 ml buffer F, then 5 μl of freshly *in vitro* translated chimeric receptor derivatives were added. The proteins were translated *in vitro* in reticulocyte lysates as described above, in the presence of $[^{35}S]$methionine. For the experiment of Figure 5C, translated τDBD/230–421 was pre-incubated with 10 nM TCDD, or vehicle alone (0.5% DMSO) at 25°C for 3 h. The reaction samples were then shaken on ice for a further 2 h, then pelleted by centrifugation. After four 1 ml washes with buffer F containing 0.05% Tween 20 the pellets were boiled in 2 $\times$ sample buffer and subjected to SDS–PAGE analysis. Gels were fixed in 20% methanol/10% acetic acid, soaked in Amplify (Amersham) for 30 min and subjected to autoradiography.

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