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Identification of Transactivation and Repression Functions of the Dioxin Receptor and Its Basic Helix-Loop-Helix/PAS Partner Factor Arnt: Inducible versus Constitutive Modes of Regulation

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Gene regulation by dioxins is mediated via the dioxin receptor, a ligand-dependent basic helix-loop-helix (bHLH)/PAS transcription factor. The latent dioxin receptor responds to dioxin signalling by forming an activated heterodimeric complex with a specific bHLH partner, Arnt, an essential process for target DNA recognition. We have analyzed the transactivating potential within this heterodimeric complex by dissecting it into individual subunits, replacing the dimerization and DNA-binding bHLH motifs with heterologous zinc finger DNA-binding domains. The uncoupled Arnt chimera, maintaining 84% of Arnt residues, forms a potent and constitutive transcription factor. Chimeric proteins show that the dioxin receptor also harbors a strong transactivation domain in the C terminus, although this activity was silenced by inclusion of 82 amino acids from the central ligand-binding portion of the dioxin receptor. This central repression region conferred binding of the molecular chaperone hsp90 upon otherwise constitutive chimeras in vitro, indicating that hsp90 has the ability to mediate a *cis*-repressive function on distant transactivation domains. Importantly, when the ligand-binding domain of the dioxin receptor remained intact, the ability of this hsp90-binding activity to confer repression became conditional rather than irreversible. Our data are consistent with a model in which crucial activities of the dioxin receptor, such as dimerization with Arnt and transactivation, are conditionally repressed by the central ligand- and-hsp90-binding region of the receptor. In contrast, the Arnt protein appears to be free from any repressive activity. Moreover, within the context of the dioxin response element (xenobiotic response element), the C terminus of Arnt conferred a potent, dominating transactivation function onto the native bHLH heterodimeric complex. Finally, the relative transactivation potencies of the individual dioxin receptor and Arnt chimeras varied with cell type and promoter architecture, indicating that the mechanisms for transcriptional activation may differ between these two subunits and that in the native complex the transactivation pathway may be dependent upon cell-specific and promoter contexts.

Potent toxicity and tumor promotion are responses to dioxins generally acknowledged to be mediated via the dioxin receptor, a ligand-activated transcription factor responsible for inducing genes encoding xenobiotic metabolizing enzymes. The dioxin receptor, also termed the aryl hydrocarbon receptor, belongs to the basic helix-loop-helix (bHLH) class of transcription factors (4, 9). The bHLH family includes protein complexes important in oncogenesis (Myc/Max), muscle differentiation (MyoD/E12), and *Drosophila* neurogenesis (achaete or scute/daughterless) (for recent reviews, see references 18 and 19). In analogy to these heterodimeric bHLH systems, the dioxin receptor recruits a specific bHLH partner protein, Arnt (15), to enable binding to a specific DNA target sequence, the XRE (xenobiotic response element) (8, 23, 39, 44). Juxtaposed to their bHLH domains, the dioxin receptor and Arnt share a second region of homology, the PAS domain, which is common to the *Drosophila* midline developmental protein Sim (28) and circadian oscillator Per (for a review, see reference 41). This domain distinguishes the dioxin receptor and Arnt from other mammalian bHLH proteins and may help specify formation of a functional dioxin receptor/Arnt complex. Consistent with this idea, the PAS domain of Per has been reported to form a dimerization interface (16).

In nonstimulated cells, the dioxin receptor is found as an inert, cytoplasmic (35) complex with the 90-kDa heat shock protein, hsp90 (7, 31, 46). Treatment with ligand initiates a transformation process whereby hsp90 is released, dimerization with Arnt is achieved, and the receptor is converted to a form tightly held in the nucleus. While our understanding of this fundamental transformation is rudimentary, sequential steps in the activation process are beginning to emerge. We and others have recently shown that dimerization between the dioxin receptor and Arnt is a ligand-dependent event (8, 23, 38, 44). We have also recently observed that Arnt, initially postulated to be essential for nuclear compartmentalization of the transformed receptor (15), may play an active role in releasing hsp90 from the ligand-bound receptor (25). In vitro dephosphorylation experiments have indicated that both heterodimerization between the receptor and Arnt and DNA binding of the dimeric complex are dependent upon phosphorylation (1). Consistent with this model, tetradecanoyl phorbol acetate-induced down-regulation of protein kinase C activity in vivo inhibits both DNA binding of the ligand-stimulated receptor (1, 5, 30) and activity of an XRE-driven reporter gene (1),

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suggesting a critical role for protein kinase C in receptor function.

Although no physiological ligand is currently known, the dioxin receptor appears to be unique in being the only bHLH protein to require a ligand for activation. We have recently mapped the core ligand-binding activity of the mouse receptor to a centrally located stretch of 190 amino acids (43), a region also encompassed by ligand cross-linking studies (8). Interestingly, this minimal region was found to form a stable complex with hsp90 in vitro (43), providing further evidence for the initial implications of a role for hsp90 in chaperoning the ligand-binding conformation of the receptor (36). Here we have created chimeric receptors in which the zinc finger DNA-binding domain of the glucocorticoid receptor has replaced the bHLH region of various murine dioxin receptor fragments, which are capable of inducing glucocorticoid response element-containing reporter genes. We have used this system in transient transfection assays to analyze transcription-activating potential within the dioxin receptor protein. Construction of a corresponding Arnt chimera in which the glucocorticoid receptor DNA-binding domain has replaced the Arnt bHLH motif has facilitated an independent dissection of the dioxin receptor system into its individual subunits.

In the case of the dioxin receptor, powerful, constitutive transcription factors were formed when C-terminal fragments were fused to the heterologous glucocorticoid receptor DNA-binding domain. Interestingly, this C-terminal transactivation function was repressed when chimeras were elongated to include the ligand-binding domain. In these constructs, repression was only partially reversed by treatment with dioxin. The central portions of the dioxin receptor conferring this repression were also shown to confer interaction with hsp90 in vitro, thus providing evidence for the involvement of hsp90 in negative regulation of receptor function. In contrast, a glucocorticoid receptor/Arnt chimera containing all but the N-terminal bHLH of Arnt formed a strong, constitutive transcription factor. These data are consistent with a model in which both subunits of the dioxin receptor complex can function as transactivating proteins, with this function being conditionally repressed in the dioxin receptor by the presence of ligand- and hsp90-binding activities. Within the native bHLH heterodimeric complex, Arnt contained a region with a strong, dominating transactivation function. Finally, the relative potencies of the individual Arnt and dioxin receptor chimeras showed cell- and promoter-specific differences, indicating that the two subunits may operate by different mechanisms to induce transcription.

MATERIALS AND METHODS

Plasmid constructions. Plasmids pMMTV-AF, pMT-GR, pMT-τDBD, pτDBD/GEM, pMT-τDBD/DR83-593, pMT-τDBD/DR83-805, pτDBD/CMV4, pCMV4Arnt, and pCMV 4DR have been previously described (22, 43). For construction of GR N-terminal regions containing a deletion of the τ1 domain, *Bgl*II fragments (containing τ1) were excised from pτDBD/GEM, pMT-τDBD, and pτDBD/CMV4 and then religated to give pGRDBD/GEM, pMT-GRDBD, and pGRDBD/CMV4, respectively. For construction of dioxin receptor chimeras, pSportAhR (containing complete mouse dioxin receptor cDNA; 4) was amplified by PCR with primers designed to provide segments of the dioxin receptor containing codons 340 to 805, 422 to 805, and 521 to 805. Inclusion of *Xho*I restriction sites flanking coding sequences of the primers allowed facile digestion and subcloning of fragments into *Xho*I-digested pGRDBD/GEM. PCR-derived C termini were replaced by original dioxin receptor cDNA from pDR/BS (25), using *Not*I and *Xba*I (for codons 340 to 805 and 422 to 805) and *Sph*I and *Apa*I (for codons 521 to 805) restriction sites, thus providing pGRDBD/DR340-805/GEM, pGRDBD/DR422-805/GEM, and pGRDBD/DR521-805/GEM. Fidelity of remaining PCR-derived sequences was verified by dideoxy sequencing. Plasmids pGRDBD/DR83-805/GEM, pMT-GR DBD/DR83-805, and pMT-GRDBD/DR83-593 were constructed by excising *Cla*I-*Xba*I fragments from pMT-τDBD/DR83-805 or pMT-τDBD/DR83-593 and subcloning into *Cla*I-*Xba*I-digested pGRDBD/GEM or pMT-GRDBD. In a similar fashion, pMT-GRDBD/DR340-805 and pMT-GRDBD/DR422-805 were constructed by subcloning *Cla*I-*Xba*I fragments from the relative GEM vectors into *Cla*I-*Xba*I-digested pMT-GRDBD. To create pMT-GRDBD/DR521-805 and pGRDBD/DR521-805/CMV4, a *Cla*I fragment was excised from pGRDBD/DR521-805/GEM and subcloned into *Cla*I-digested pMT-GRDBD and pGRDBD/CMV4, respectively. Other CMV4 expression vectors were obtained by subcloning *Cla*I-*Xba*I fragments of GR/DR chimeras into *Cla*I-*Xba*I-digested pGRDBD/CMV4.

For construction of chimeric Arnt plasmids, a section of pBM5NeoM1-1 (containing the human Arnt cDNA [15]) was amplified by PCR with primers designed to provide codons 128 to 407 of the Arnt gene. Inclusion of *Xho*I restriction sites flanking the primers allowed subcloning into pτDBD/GEM as detailed above, thus producing pτDBD/Arnt128-407/GEM. Fidelity of the PCR was verified by dideoxy sequencing. A *Cel*II-*Xba*I fragment of pArnt/GEM (44) was subcloned into *Cel*II-*Xba*I-digested pτDBD/Arnt128-407/GEM to provide the full C-terminus-coding region of Arnt in pτDBD/Arnt128-774/GEM. Excision of a *Cla*I-*Xba*I fragment from this GEM plasmid and subsequent ligation into *Cla*I-*Xba*I-digested pMT-τDBD, pMT-GRDBD, and pGRDBD/CMV4 gave pMT-τDBD/Arnt128-774, pMT-GRDBD/Arnt128-774, and pGRDBD/Arnt128-774/CMV4, respectively. These GR/Arnt chimeras code for extra amino acids Pro, Arg, and Val at the juncture between the glucocorticoid receptor and Arnt by way of the *Xho*I linker, while all dioxin receptor chimeras code for Pro, Arg, and Gly at this fusion site. DR593/CMV4 was constructed by subcloning a *Not*I-*Xba*I fragment from pMT-GRDBD/DR83-593 into *Not*I-*Xba*I-digested pCMV4DR. Arnt603/CMV4 (24) contains codons 1 to 603 of the human Arnt cDNA from pBM5NeoM1-1. A *Bam*HI-*Kpn*I fragment from pMT-GRDBD/Arnt128-774 was subcloned into *Bgl*II-*Kpn*I-digested Arnt603/CMV4 to provide pGRDBD/Arnt128-603/CMV4.

The promoter region of reporter plasmid pGREII-Oct-AF contains two copies of the consensus palindromic glucocorticoid response element (AGAACACAGTGTTCT) separated by a 6-base sequence (GCTAGC), located upstream of an octamer-binding site (ATGTAAAT) and a TATA box (TATAAA) (21). A distance of 42 bases separates the GRE repeat from the octamer sequence, with 15 bases between the octamer site and TATA box. Arrangement of the promoter in pXRE-Oct-AF is identical except for replacement of the GRE repeat with a 38-bp sequence spanning the XRE1 motif from the upstream (−1 kb) region of the rat cytochrome P4501A1 gene (11). Reporter gene pXRE-MMTV-AF was formed by subcloning a *Hin*dIII-*Xba*I fragment from pXRE-MMTV-hGH (44) into *Hin*dIII-*Nhe*I-digested pMMTV-AF.

Cell culture and transient transfections. CHO cells were grown in Ham's F12 medium, Hepa 1c1c7 and mutant hepatoma C4 and C12 cells were grown in minimal essential medium supplemented with 2 mM L-glutamine, COS7 cells were grown in Dulbecco's modified Eagle medium, and HepG2 cells were grown in RPMI 1640 supplemented with 2

mM L-glutamine. All media were supplemented with 10% fetal calf serum, 100 U of penicillin, and 100 μg of streptomycin (Gibco/BRL) per ml. Cells were seeded at a density of 10^5 per dish (35 by 10 mm) and left 24 h for recuperation. Reporter plasmids and either metallothionein (MT) or cytomegalovirus (CMV) promoter-driven expression plasmids (1 μg each of reporter and expression vector unless otherwise indicated) were transfected with 7.5 μl of *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammoniummethylsulfate (DOTAP) according to the manufacturer's instructions (Boehringer). After a 12-h transfection period, cells were induced with ligand (5 μM dexamethasone or 10 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD]) or vehicle alone (0.1% dimethyl sulfoxide) for periods of up to 72 h as indicated. Secreted alkaline phosphatase was assayed by colorimetry as previously described (43). All transfections were performed in duplicate, and the average values were recorded. Duplicate transfections were repeated at least three times, and the values are representative data from these independent transfection experiments.

In vitro translation and immunoprecipitation experiments. Chimeric proteins were translated from GRDBD/DR/GEM plasmids in rabbit reticulocyte lysates (Promega), in the presence of [^{35}S]methionine, as recommended by the manufacturer. Aliquots (2 μl) of each translation mixture were separated by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by fluorography. Radiolabelled bands were analyzed on a PhosphorImager (Fuji), which allowed the calculation of relative protein quantities because of incorporated [^{35}S]methionine. For immunoprecipitations, translation mixtures containing equivalent quantities of chimeric receptors (between 8 and 12 μl) were made up to a total volume of 15 μl with blank translation mixture and then added to 2 ml of immunoprecipitation buffer (25 mM MOPS [morpholinepropanesulfonic acid], 1 mM EDTA, 0.02% NaN_3, 10% glycerol, 2 mg of ovalbumin per ml, 50 mM NaCl, 0.05% Tween 20; pH 7.5). This mixture was incubated on ice for 2 h before 1-ml aliquots were immunoprecipitated with resins containing preadsorbed anti-hsp90 antibody 3G3 (Affinity Bioreagents [32], or control immunoglobulin M antibodies (Sigma), as previously described (43). After four 1-ml washes with the above immunoprecipitation buffer, immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography.

Immunoblotting. CHO cells (in dishes [60 by 10 mm]) were transiently transfected with CMV-driven expression vectors (6.5 μg) and DOTAP (25 μl) according to the manufacturer's instructions. After a transfection period of 36 h, whole-cell extracts were obtained as previously described (43), and samples (100 μg of protein) were subjected to SDS–7.5% PAGE. Proteins were transferred to nitrocellulose in a semidry blotter (Bio-Rad). GR/dioxin receptor and GR/Arnt fusion proteins were detected with a rabbit polyclonal antiserum raised against an N-terminal peptide of the human GR (PA1-511; Affinity Bioreagents). Previously described rabbit polyclonal antisera directed against N-terminal peptides of the murine dioxin receptor (44) or human Arnt (25) were used to detect either transiently expressed native dioxin receptor and deletion mutant DR593 or native Arnt and deletion mutant Arnt603, respectively.

RESULTS

Identification of a ligand-activated transactivation domain in the dioxin receptor. The activated wild-type dioxin receptor is a potent inducer of transcription for various genes involved in xenobiotic metabolism, of which the most characterized is cytochrome P4501A1 (for a recent review, see reference 10). In studies designed to map the ligand-binding domain of the dioxin receptor, we previously constructed chimeric receptors consisting of the N-terminal τ1 transactivation and zinc finger DNA-binding domains (here termed τDBD) of the glucocorticoid receptor, fused to various C-terminal fragments of the dioxin receptor (43). Two of these chimeras, containing segments of the dioxin receptor between amino acids 83 to 805 and 83 to 593 (Fig. 1A and B), showed distinct dioxin inducibility when cotransfected into CHO cells with a glucocorticoid response element-containing reporter gene, pMMTV-AF. In the absence of ligand, however, these segments of the dioxin receptor mediate repression of the τ1 glucocorticoid receptor transactivation region contained in these chimeras (Fig. 2A).

Since the ligand-induced activities of these chimeras were similar to that of the constitutive τDBD fragment (Fig. 2A), it was difficult to ascertain whether ligand stimulation invoked primarily derepression of τ1 or activated some bona fide transactivation function within the dioxin receptor. In an attempt to resolve this issue, we constructed analagous chimeras, termed GRDBD/DR, in which the τ1 domain of the GR has been deleted (Fig. 1B). Chimeras τDBD/DR83-805 and GRDBD/DR83-805 both exhibited ligand responsiveness when cotransfected with reporter gene pMMTV-AF into CHO cells (Fig. 2A). However, upon deletion of the τ1 domain, the ligand-stimulated activity of the resulting GRDBD/DR83-805 chimera was reduced to approximately 30% of the corresponding activity of τDBD/DR83-805. This result would indicate that the major response seen upon ligand treatment of τDBD/DR83-805 results from a derepression of τ1 activity, with a smaller contribution coming from transactivation function(s) within the dioxin receptor. Support for such a mechanism is given by comparing the activities of chimeras τDBD/DR83-593 and GRDBD/DR83-593. While ligand induction is seen for τDBD/DR83-593, restoring activity to almost the level of the parental τDBD construct, little or no ligand responsiveness is seen for GRDBD/DR83-593. These results indicate the existence of a region in the dioxin receptor between amino acids 83 and 593 acting as a regulable inactivation domain and strongly support a model in which activity of τDBD/DR83-593 reflects a ligand-induced derepression of τ1 activity. A comparison of the activities of GRDBD/DR83-805 and GRDBD/DR83-593 suggest that a transactivating function of the dioxin receptor might lie in the C-terminal 212 amino acids. Since a portion of this region is glutamine rich, an amino acid pattern observed for other common transcription factors (for a review, see reference 26), the hypothesis that a glutamine-rich domain contributes to dioxin receptor transactivation is an attractive one. Indeed, homopolymeric stretches of glutamine have recently been shown to activate transcription in vitro and in vivo when fused to a heterologous DNA-binding domain (12).

A glucocorticoid receptor/Arnt chimera forms a strong, constitutive transcription factor. Several lines of evidence indicate that Arnt is an essential component of the transcriptionally active, native dioxin receptor complex. Complementation of a dioxin-resistant hepatoma cell line with Arnt restores a dioxin-responsive phenotype (15), while in vitro experiments have shown that the ligand-bound receptor forms a heterodimer with Arnt, a step required for XRE-binding activity (8, 23, 39, 44). It is therefore critical for understanding the dioxin receptor as a transcription factor to elucidate whether one or both protein subunits harbor transactivation functions. In a fashion similar to construction of the above dioxin receptor chimeras, we replaced the N-terminal bHLH region of Arnt with the glucocorticoid receptor zinc finger DNA-binding domain, providing the ability to bind glucocorticoid

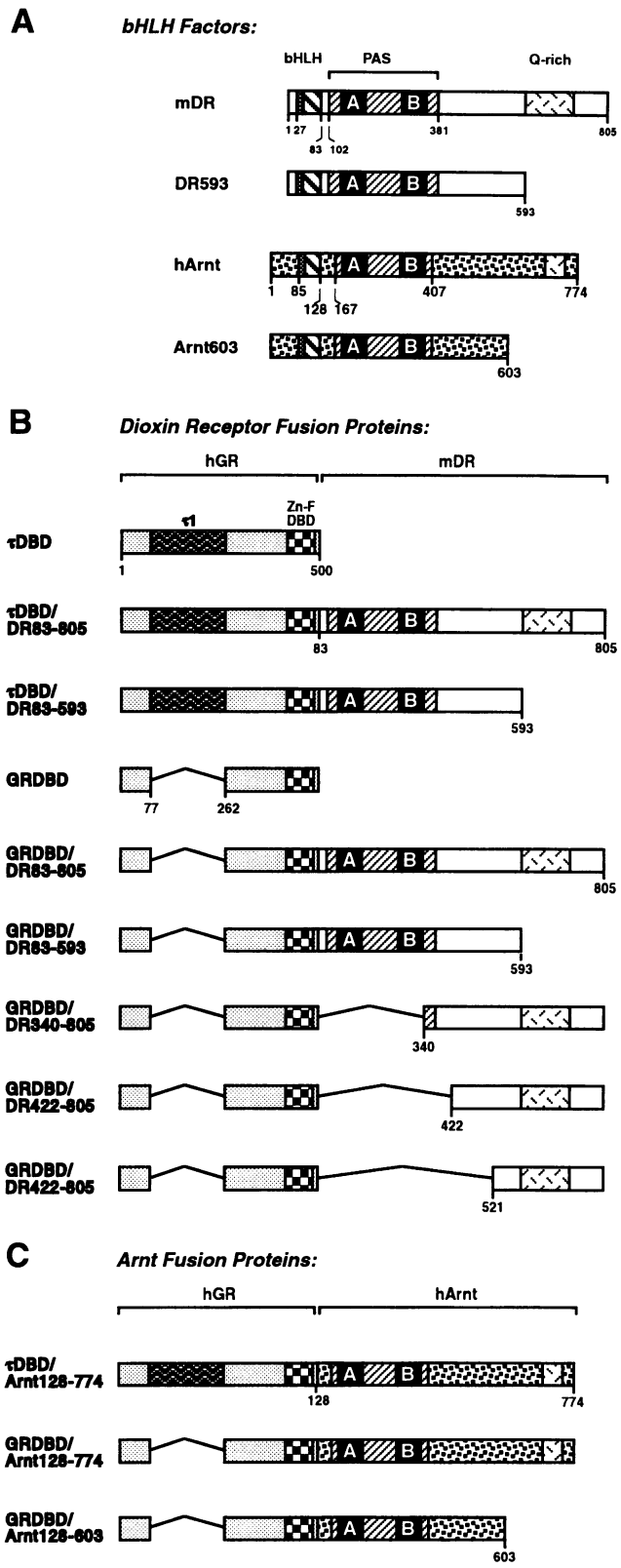


FIG. 1. Structural organization within the dioxin receptor, Arnt, and chimeric constructs of the glucocorticoid receptor/dioxin receptor and glucocorticoid receptor/Arnt. (A) Schematic of dioxin receptor and Arnt proteins, and C-terminally truncated mutants, showing locations of bHLH motifs, regions with PAS (Per-Arnt-Sim) homology, and glutamine (Q)-rich regions. (B) Glucocorticoid receptor/dioxin receptor chimeras. The N-terminal 500 amino acids of the human glucocorticoid receptor, containing a transactivation (τ1) domain and zinc finger (Zn-F) DNA-binding domain, termed τDBD, or a deletion mutant lacking τ1, termed GRDBD, were fused to various C-terminal fragments of the murine dioxin receptor. (C) Glucocorticoid receptor/Arnt chimeras, lacking the N-terminal bHLH region of Arnt, analogous to those of the dioxin receptor in panel B.

response elements (Fig. 1C). The GRDBD/Arnt128-774 chimera contains 84% of the Arnt protein, making it a close counterpart to the dioxin receptor chimera GRDBD/DR83-805, which contains 90% of the dioxin receptor. When transiently transfected into CHO cells, these two fusion proteins were expressed at similar levels as assessed by immunoblot analysis (Fig. 2C), thus permitting a direct comparison of the transactivation potentials of the two subunits of the dioxin receptor complex. Transfection of GRDBD/Arnt128-774 into CHO cells produced a strong response on reporter gene pMMTV-AF (Fig. 2B). Interestingly, this activity was unaffected by ligand, demonstrating that the Arnt protein harbors a strong, constitutive transactivation function.

The strong transactivating potency of the Arnt chimera is illustrated by comparing its activity to that seen with full-length, ligand-activated glucocorticoid receptor on the same reporter gene (Fig. 2B). More importantly, the activity of GRDBD/Arnt128-774 was five- to eightfold greater than that of the corresponding GRDBD/DR83-805 dioxin receptor chimera. This difference was observed when chimeras were compared in either MT or CMV expression vectors (data not shown). The difference in transactivation potency was also observed in a number of different cell lines including CHO cells (Fig. 2B), COS7 cells, Hepa 1c1c7 hepatoma cells (data not shown), and human keratinocytes (13). In contrast to GRDBD/DR83-805 (Fig. 2A), however, the activity of GRDBD/Arnt128-774 could not be elevated by inclusion of the glucocorticoid receptor τ1 transactivation domain, again emphasizing the potency of the transactivation function inherent in the Arnt protein (Fig. 2B). This constitutive potency of the Arnt chimera on the mouse mammary tumor virus (MMTV)-driven reporter gene promoter also establishes that Arnt can be functionally uncoupled from its natural bHLH/PAS partner factor, forming a very active transcription factor when attached to a heterologous DNA-binding domain.

Fusion of the dioxin receptor C terminus onto GRDBD produces a strong, constitutive transcription factor. To further analyze the region with the implied transactivation function in the C terminus of the dioxin receptor, we constructed chimera GRDBD/DR521-805, containing the very C-terminal 284 amino acids of the dioxin receptor fused to the zinc finger motif of the glucocorticoid receptor (Fig. 1B). Surprisingly, when this chimera was cotransfected into CHO cells with reporter gene pMMTV-AF, an activity equal to that given by the potent Arnt chimera GRDBD/Arnt128-774 was seen (Fig. 3A). This activity was unaffected by ligand, consistent with deletion of the minimal ligand-binding domain centered around amino acids 230 to 421 (43). Since the strong transactivation function of the dioxin receptor observed with GRDBD/DR521-805 was not observed within the fusion protein GRDBD/DR83-805, we reasoned that the longer portion of the dioxin receptor might harbor an inhibitory activity which cannot be fully derepressed by ligand treatment alone. To investigate whether this repression function was mediated strictly by the ligand-binding domain of the dioxin receptor, we constructed a chimera containing amino acids immediately C

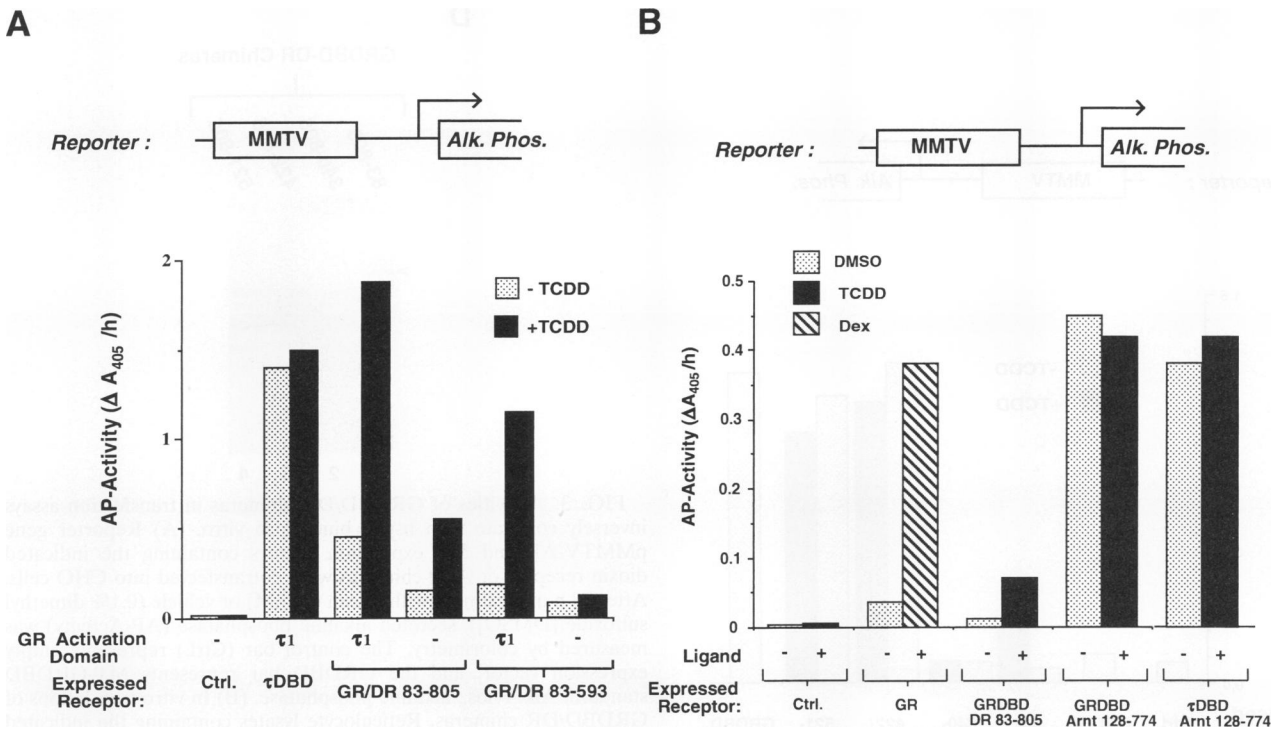
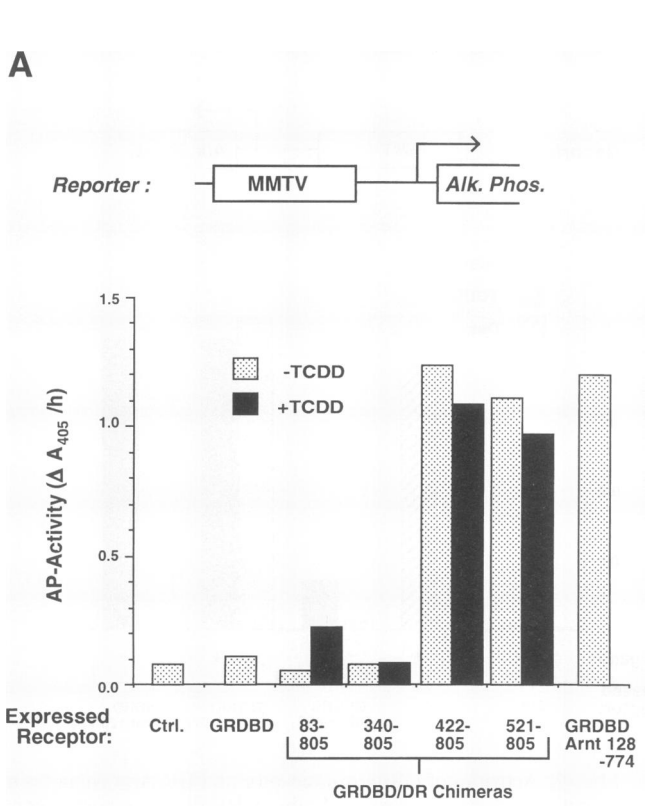


FIG. 2. Activities of GR/dioxin receptor and GR/Arnt chimeras in transient transfection assays with the GRE-driven reporter gene pMMTV-AF. (A) CHO cells were cotransfected with reporter gene and chimeric MT-GR/dioxin receptor expression vectors either containing the GR transactivation domain (τ1 [termed τDBD/DR in Fig. 1B]) or omitting the τ1 domain (− [termed GRDBD/DR in Fig. 1B]) as indicated. After 72 h of treatment with 10 nM dioxin or 0.1% dimethyl sulfoxide (DMSO) vehicle, secreted alkaline phosphatase (AP-Activity) was measured by colorimetric assay. The control bar (Ctrl.) represents empty expression vector. Alk. Phos., alkaline phosphatase. (B) CHO cells were cotransfected with reporter gene and GRDBD/DR83-805, GRDBD/Arnt128-774, τDBD/Arnt128-774, or wild-type GR MT expression vectors as indicated. After 48 h of treatment with dexamethasone (Dex) (5 μM), dioxin (10 nM), or vehicle (0.1% DMSO), secreted alkaline phosphatase (AP-Activity) was measured by colorimetry. The control bar (Ctrl.) represents empty expression vector. Alk. Phos., alkaline phosphatase. (C) Detection of fusion proteins by Western blotting. Whole-cell extracts were prepared 36 h after CHO cells had been transfected with GRDBD, GRDBD/DR83-805, or GRDBD/Arnt128-774 expression vector. Fusion proteins were detected with polyclonal antisera raised against a GR N-terminal epitope, as indicated by the arrowheads.

terminally to the ligand-binding core (GRDBD/DR422-805) and a chimera, GRDBD/DR340-805, containing amino acids C terminally to PAS B, thus encompassing the C-terminal half of the ligand binding core (Fig. 1). As shown in Fig. 3A, chimera GRDBD/DR422-805 exhibited a strong, constitutive activity equal to that produced by GRDBD/DR521-805, indicating that regions immediately C terminally to the ligand-binding domain are uncoupled from repression. In direct contrast, however, the chimera containing amino acids 340 to 805 was repressed in both the absence or presence of ligand, resulting in very low levels of transactivation. The relative abilities of these chimeras to activate the reporter gene were conserved when chimeras were expressed from either MT or CMV (data not shown) expression vectors. Moreover, Western blotting of extracts from transfected cells revealed the chimeras to be expressed at similar levels (Fig. 3D and 2C), ruling out the possibility that differences in reporter activity were caused by variable expression levels. Absence of activity seen with chimera GRDBD/DR340-805 is consistent with a *cis*-repression function localized within the ligand-binding domain. Because only the C-terminal half of the ligand-binding domain is contained within GRDBD/DR340-805, this chimera remained unresponsive to ligand. In excellent agreement with this observation, a dioxin receptor fragment spanning residues 340 to 421 is devoid of ligand-binding activity in vitro (24), whereas a fragment spanning amino acids 230 to 421 shows strong ligand-binding activity (43).

hsp90 confers repression on GRDBD/dioxin receptor chimeras. As outlined above, the reduced activities seen for dioxin receptor chimeras containing sequences N terminally to amino



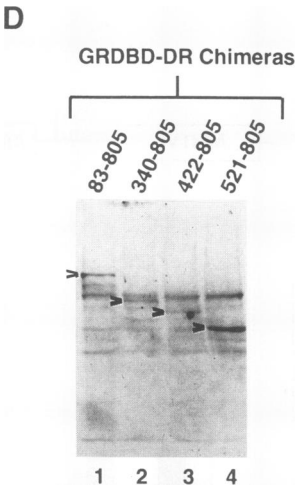


FIG. 3. Activities of GRDBD/DR chimeras in transfection assays inversely correlate with hsp90 binding in vitro. (A) Reporter gene pMMTV-AF and MT expression vectors containing the indicated dioxin receptor or Arnt chimeras were cotransfected into CHO cells. After 72 h of treatment with dioxin (10 nM) or vehicle (0.1% dimethyl sulfoxide [DMSO]), secreted alkaline phosphatase (AP-Activity) was measured by colorimetry. The control bar (Ctrl.) represents empty expression vector, and the GRDBD bar represents MT-GRDBD standard. Alk. Phos., alkaline phosphatase. (B) In vitro translations of GRDBD/DR chimeras. Reticulocyte lysates containing the indicated [^{35}S]methionine-labelled proteins (2 μl) were analyzed by SDS-PAGE and fluorography. (C) Coimmunoprecipitation of in vitro-translated, [^{35}S]methionine-labelled GRDBD/DR fusion proteins with antibodies directed against hsp90. Reticulocyte lysates containing equivalent concentrations of the indicated fusion proteins were subjected to immunoprecipitation with resins containing preadsorbed anti-hsp90 antibody 3G3 or control immunoglobulin M antibodies. After the proteins were washed extensively, immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography. C lanes contain control antibody immunoprecipitates, S lanes contain specific anti-hsp90 immunoprecipitates, and M lanes contain molecular mass standards. (D) Detection of GR/dioxin receptor fusion proteins by Western blotting. Whole-cell extracts were prepared 36 h after CHO cells had been transfected with expression plasmids containing the indicated chimeras. Fusion proteins were detected with polyclonal antisera raised against a GR N-terminal epitope, as indicated by the arrowheads.

acid 422 suggest that a region involved in repression lies within the ligand-binding domain. We have previously recognized that the core dioxin receptor ligand-binding activity, located between amino acids 230 and 421, also mediates binding of the molecular chaperone hsp90 (43). It is therefore possible that stable interaction with hsp90 may provide a mechanism by which this region could mediate repression. To test this hypothesis, dioxin receptor chimeras were translated in reticulocyte lysate in the presence of [^{35}S]methionine and coimmunoprecipitated with an antibody directed against hsp90. SDS-PAGE analysis and subsequent autoradiography showed that the chimeric proteins were expressed at very similar levels (Fig. 3B). The radiolabelled bands were quantitated with a PhosphorImager, and then equivalent amounts of chimeric proteins were subjected to coimmunoprecipitation experiments performed with the monoclonal anti-hsp90 antibody 3G3, which is known to bind hsp90 complexed with other proteins (32). As shown in Fig. 3C, chimeric proteins GRDBD/DR83-805 and GRDBD/DR340-805 showed specific coimmunoprecipitation with the anti-hsp90 antibody (lanes 2 and 4), since a control antibody absorbed only minor background levels of the la-

belled proteins (lanes 1 and 3). In strong contrast, chimeras GRDBD/DR422-805 and GRDBD/DR521-805 showed no significant coimmunoprecipitation with the specific hsp90 antibody, giving levels similar to or only slightly above those seen with control antibody (compare lanes 5 and 6 and lanes 7 and 8). Thus, for dioxin receptor chimeras, a strong inverse correlation exists between the interaction with hsp90 in vitro and the ability of the chimera to activate a reporter gene in transfection experiments. Stable interaction with hsp90 may therefore provide the inhibitory function which silences the transactivation domain within the C terminus of the dioxin receptor.

In the case of GRDBD/DR340-805, we observed repression to be irreversible, since this chimera contains only part of the minimal ligand-binding domain between amino acids 230 and 421. In contrast, the complete ligand-binding domain is present within GRDBD/DR83-805, and this chimera showed distinct ligand responsiveness, although derepression appeared to be incomplete (Fig. 3A). We have previously noted that the strong interaction between hsp90 and chimera τDBD/DR83-805 is very difficult to disrupt by in vitro treatment with ligand (25). The incomplete derepression of GRDBD/DR83-805 upon ligand treatment in transfection assays is most probably a reflection of this difficulty in releasing hsp90 by ligand treatment alone. In support of this model, we have recently shown that a cellular factor, possibly the Arnt partner factor, is necessary to enhance release of hsp90 when the native dioxin receptor is exposed to ligand in vitro. In fact, native Arnt can promote hsp90 release from the native dioxin receptor, whereas a mutant Arnt lacking the bHLH region cannot. However, we failed to detect any Arnt-promoted release of hsp90 from the bHLH-deficient chimera τDBD/DR83-805 (25). Taken together, these data support a model in which chimera GRDBD/DR83-805, which lacks the bHLH motif of the dioxin receptor, is unable to be fully derepressed upon ligand treatment because of impaired interaction with Arnt or an Arnt-like factor via the bHLH dimerization interface.

The functional activities of dioxin receptor and Arnt chimeras are dependent upon cell type and promoter context. To examine the influence of promoter architecture on the potencies of the dioxin receptor and Arnt chimeras, we tested their abilities to activate an artificial promoter that was less complex than the wild-type MMTV promoter. Reporter GREII-Oct-AF contains two copies of a glucocorticoid response element upstream of a binding site for octamer transcription factors and a TATA box, linked to the placental alkaline phosphatase gene (21). Figure 4 shows that chimeras GRDBD/DR422-805 and GRDBD/DR521-805 were also strong constitutive activators of this reporter construct, while GRDBD/DR340-805 was irreversibly repressed and GRDBD/DR83-805 showed remarkably weak, ligand-dependent activity. Thus, for dioxin receptor chimeras, the pattern of activity on this synthetic promoter was similar to that on the wild-type MMTV promoter. However, activation by GRDBD/Arnt128-774 relative to the levels produced by the constitutive dioxin receptor derivative GRDBD/521-805 was about 50% lower on this promoter than on the MMTV promoter (Fig. 4). This decreased potency of GRDBD/Arnt128-774 on the synthetic promoter GREII-Oct appeared to be a general phenomenon, also occurring in COS7 and mouse hepatoma cell lines (data not shown).

To show that the chimeras activated the GREII-Oct target promoter via the glucocorticoid response elements, transfections were also performed with a reporter gene XRE-Oct-AF (21) in which the GREs have been replaced by a response element recognized by the native dioxin receptor complex, the XRE. The strong activators GRDBD/Arnt128-774 and GRDBD/DR521-805 failed to induce this XRE-driven promoter, proving specificity for the GRE reporter gene and demonstrating that these dioxin receptor and Arnt fusion proteins had completely switched their DNA target element from an XRE to a GRE.

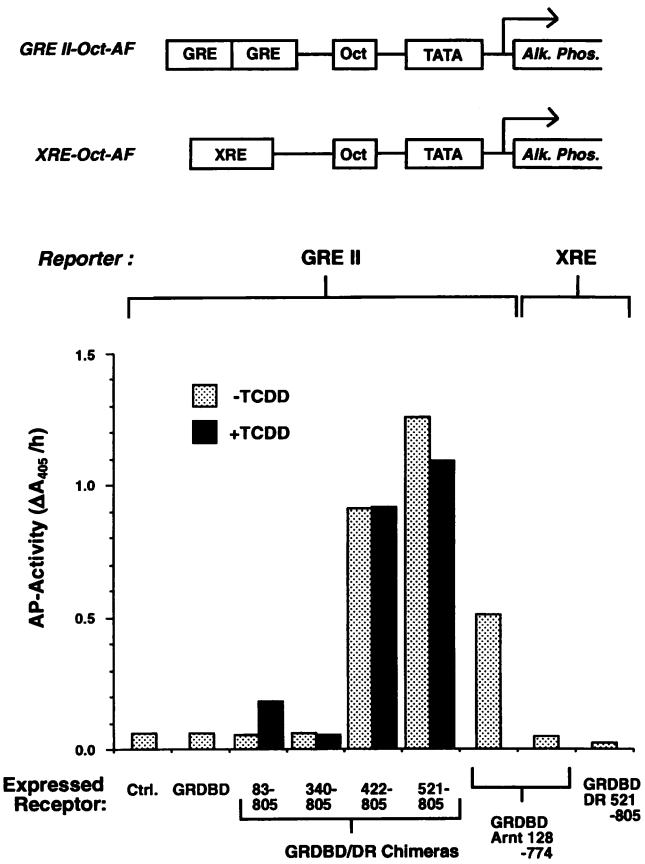


FIG. 4. Activities of GRDBD/DR and GRDBD/Arnt chimeras on simplified GRE and XRE-driven reporter genes. CHO cells were cotransfected with the indicated reporter gene and MT expression vectors containing either GRDBD, the indicated GRDBD/DR chimeras, or GRDBD/Arnt128-774. After 72 h of treatment with dioxin (10 nM) or vehicle (0.1% dimethyl sulfoxide [DMSO]), secreted alkaline phosphatase was measured by colorimetry. The control bar (Ctrl.) represents empty expression vector. Alk. Phos., alkaline phosphatase; AP-Activity, alkaline phosphatase activity.

The observation that relative potencies of dioxin receptor and Arnt chimeras could vary with promoter context stimulated us to investigate if their relative potencies might also vary with cell type. We therefore tested the abilities of the constitutive dioxin receptor chimeras, GRDBD/DR422-805 and GRDBD/DR521-805, and the Arnt chimera GRDBD/Arnt128-774 to activate reporter gene pMMTV-AF in a variety of cell lines. For this experiment, activities of the chimeras were normalized against the activity of the nonchimeric parental GRDBD fragment. Reported values are therefore the fold increase each chimera displays over the value for the parental GRDBD, in order to correct for transfection efficiencies between different cell lines. As illustrated in Fig. 5, CHO (hamster ovary), COS7 (monkey kidney), and Hepa 1c1c7 (murine hepatoma) cells revealed that the constitutive dioxin receptor and Arnt chimeras activated the MMTV promoter with similar potencies. Unexpectedly, a marked contrast was

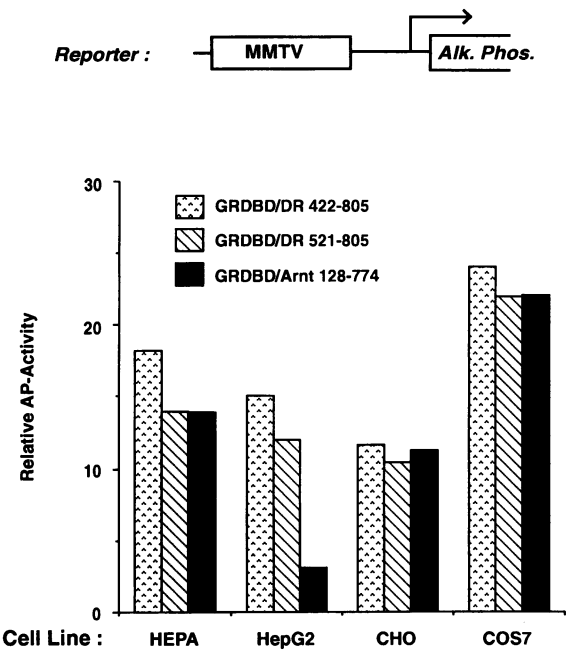


FIG. 5. Activities of constitutive GRDBD/dioxin receptor and GRDBD/Arnt chimeras in a variety of cell lines. Each indicated cell line was cotransfected with reporter gene pMMTV-AF and MT expression vectors containing either GRDBD, GRDBD/DR422-805, GRDBD/DR521-805, or GRDBD/Arnt128-774. After 48 h, secreted alkaline phosphatase (AP-activity) was measured by colorimetry. Values for each chimera were normalized against the GRDBD standard in each cell line to correct for transfection efficiencies between cell lines. Alk. Phos., alkaline phosphatase.

seen in HepG2 cells (human hepatoma), where GRDBD/Arnt128-774 had a four- to fivefold-lower effect on the MMTV promoter than the dioxin receptor chimeras. It is of particular interest that such a difference in relative potency exists between mouse and human hepatoma cell lines. This observation argues against a general tissue-specific phenomenon, while strong activity of the Arnt chimera in human keratinocytes (13) argues against a general species-specific phenomenon. While the reason for altered relative potencies in HepG2 cells remains unclear, it indicates that signalling of chimeric dioxin receptor and Arnt molecules differs between HepG2 and other cell lines. It is plausible that receptor and Arnt proteins communicate differently with the basic transcription apparatus, possibly contacting separate members of the core assembly complex or binding different intermediary factors. An alteration in Arnt signalling in HepG2 cells could be easily explained, for instance, if these cells contained reduced levels of specific intermediary TBP (TATA-binding protein)-associated factors. It should be possible to provide an answer to this question with the chimeric proteins in reconstituted, cell-free transcription models.

C-terminal transactivation functions of the native dioxin receptor/Arnt complex. The abrogation of transcriptional activity seen when dioxin receptor chimera GRDBD/DR83-805 was C terminally truncated to GRDBD/DR83-593 (Fig. 2A), together with the strong activity shown by GRDBD/DR521-805 (Fig. 3A and 4), indicated that a strong transactivation domain resides in the dioxin receptor C terminus. To explore the effect of this region in a more natural context, we truncated the C-terminal 213 amino acids of the native dioxin receptor (DR593 [Fig. 1A]) and analyzed its activity on an XRE-driven promoter in the presence of native Arnt. Cotransfection of full-length dioxin receptor and Arnt expression vectors into CHO cells led to a strong dioxin-activated response on reporter gene XRE-MMTV-AF, high above the level obtained from the endogenous receptor and Arnt proteins (Fig. 6A). In contrast, a 50% decrease in reporter activity was observed when, in the presence of dioxin, Arnt was coexpressed with DR593 in lieu of wild-type dioxin receptor (Fig. 6A), consistent with a loss of transactivation potency from the dioxin receptor. In order to test for a corresponding transactivation function within the C terminus of Arnt, we created an Arnt deletion mutant, Arnt603, which was C terminally truncated by 171 amino acids (Fig. 1A). Surprisingly, when the full-length dioxin receptor was coexpressed with Arnt603 and stimulated by ligand treatment, only very low levels of reporter activity were observed, corresponding to approximately 10% of the activity obtained in cotransfection experiments with full-length Arnt (Fig. 6A). When Arnt603 was paired with DR593, a large decrease in activity was noted in comparison with cotransfection with DR593/Arnt. In fact, reporter activity was repressed to levels below the background response produced by the endogenous dioxin receptor/Arnt complex. Following coexpression of DR593 and Arnt603, the truncated proteins would be expected to dimerize with their endogenous partner factors, as well as each other, thus titrating out activity of the endogenous dioxin receptor/Arnt complex and explaining the decrease to below background levels. Immunoblot analysis from extracts of transfected cells revealed that the truncated proteins were expressed at levels similar to those produced by the full-length proteins (Fig. 6B and C), ruling out the possibility that poor expression levels of these deletion mutants led to decreased transcriptional activities. Moreover, the dramatic loss of activity seen with Arnt603 cannot be explained by this truncation producing a nonfunctional protein, since gel mobility shift analysis revealed it to be fully active in dimerization with the dioxin receptor and binding the XRE sequence (data not shown), consistent with dimerization and DNA recognition motifs for Arnt being localized in the N terminus.

The Arnt C terminus provides a dominant transactivation function in the native dioxin receptor/Arnt complex. The low transactivation potency of the dioxin receptor-Arnt603 complex (Fig. 6A) indicates that a powerful transactivation domain might lie within the C terminus of Arnt. To test the activity of the Arnt C-terminal deletion mutant in a situation independent of the dioxin receptor, we created chimera GRDBD/Arnt128-603 (Fig. 1C). In transient transfection experiments, this fusion protein was expressed at the same level as the other GRDBD chimeras (data not shown), although it produced a very weak response when tested on the MMTV-AF reporter gene, giving a 10-fold-lower activity in CHO cells than the full-length chimera GRDBD/Arnt128-774 (Fig. 7A). The strong transactivation function of the Arnt subunit is therefore almost totally abrogated by this C-terminal deletion. To compare the activities of native Arnt and Arnt603 in a situation devoid of endogenous Arnt protein, we performed cotransfection experiments with the XRE-MMTV-AF reporter gene in the Arnt-deficient, mutant hepatoma C4 (15) cell line. Consistent with C4 cells containing wild-type levels of dioxin receptor (44), transfection of an Arnt expression vector rescued dioxin responsiveness, as measured by activity on the XRE reporter gene (Fig. 7A). A large increase in activity was also observed in the absence of dioxin, a phenomenon noted previously by us (22) and others (23) when either Arnt or the dioxin receptor (Fig. 7C) is transiently expressed in cells. In a situation of high transient expression, it is possible that heterodimeric dioxin

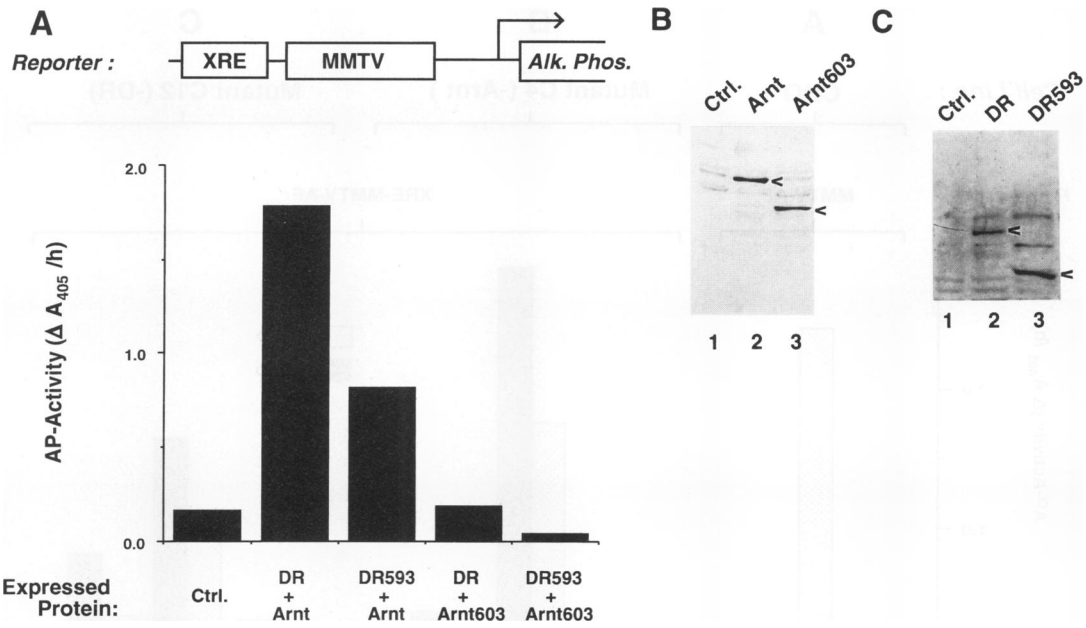


FIG. 6. Analysis of dioxin receptor and Arnt C-terminal deletion mutants. (A) CHO cells were cotransfected with reporter gene pXRE-MMTV-AF (100 ng) and CMV expression vectors containing the indicated combination of native dioxin receptor or deletion mutant DR593, paired with either native Arnt or deletion mutant Arnt603 (1 μg of each expression vector). After 72 h of treatment with dioxin (10 nM), secreted alkaline phosphatase (AP-Activity) was measured by colorimetry. The control bar (Ctrl.) represents empty expression vector (2 μg). Alk. Phos., alkaline phosphatase. (B) Detection of Arnt proteins by Western blotting. Whole-cell extracts were prepared 36 h after CHO cells had been transfected with expression vectors containing native Arnt or deletion mutant Arnt603. Transiently expressed proteins were detected with polyclonal antisera raised against an N-terminal epitope of Arnt, as indicated by the arrowheads. The control (Ctrl.) lane contains an equal amount of protein from a whole-cell extract of nontransfected cells. (C) Detection of dioxin receptor proteins by Western blotting. Transiently expressed native dioxin receptor and deletion mutant DR593 were visualized with polyclonal antisera raised against an N-terminal epitope of the dioxin receptor, following the protocol outlined above for panel B.

receptor/Arnt complexes may form in the absence of ligand, leading to the observed increase in nonstimulated reporter activity. The large increase in activity seen when Arnt was expressed in C4 cells was abrogated when the Arnt603 deletion mutant substituted for native Arnt (Fig. 7B). Residual levels of activity in this experiment probably reflect transactivation from the dioxin receptor subunit. Taken together, these results imply that Arnt is the major transactivating species within the native dioxin receptor complex. To further explore this idea, we performed transfections in the mutant hepatoma C12 cell line, which expresses wild-type Arnt activity but very low levels of dioxin receptor (4, 22). Consistent with low but detectable levels of endogenous dioxin receptor, only minor dioxin induction was seen on the XRE reporter gene (Fig. 7C). Activity on the reporter in the presence of dioxin was increased fivefold by cotransfecting a dioxin receptor expression vector, while this increase was lowered only twofold when DR593 replaced the full-length dioxin receptor (Fig. 7C). Expression of truncated Arnt and dioxin receptor proteins in these mutant hepatoma cell lines therefore confirms their observed activities in CHO cells (Fig. 6A) and emphasizes that, in the context of the MMTV promoter, the Arnt partner factor of the dioxin receptor plays a dominant role in the transactivation process.

DISCUSSION

Dissection of the dioxin receptor transcription factor complex into individually functioning subunits. The wild-type dioxin receptor and its bHLH/PAS partner factor Arnt fail to recognize the XRE target sequence individually and show, when exposed to one another in the presence of ligand, highly cooperative dimerization and XRE-binding activities in vitro (8, 22, 23, 44), as well as cooperative activation of XRE-driven target genes in vivo (22, 23, 44). Moreover, consistent with its role in mediating dimerization processes of other bHLH factors, the bHLH motif is necessary for dioxin receptor-Arnt interaction (22, 44). Deletion of the bHLH motif of Arnt thus impairs the biological activity of the dioxin receptor complex (44). To study the transactivating potentials of the dioxin receptor and Arnt individually, we therefore substituted the bHLH motifs in the N termini of both proteins with the DNA-binding domain of the glucocorticoid receptor, thus permitting by transient expression in different cell lines examination of transcription initiated from glucocorticoid response element-controlled reporter genes. Using this strategy, we have previously established that a glucocorticoid-dioxin receptor fusion protein, τDBD/DR83-805, containing the τ1 transactivation function and the DNA-binding domain of the glucocorticoid receptor, is functionally uncoupled from Arnt, since it is conditionally active when expressed in Arnt-deficient, mutant hepatoma cells (43). In the present study, we show that both the dioxin receptor and Arnt fusion protein constructs could activate glucocorticoid response element-driven promoters independently of one another. Whereas the dioxin receptor chimera was conditionally regulated by dioxin, the corresponding Arnt fusion protein was constitutively active.

To investigate transactivation function(s) within the dioxin receptor and Arnt, we deleted the endogenous τ1 transactivation domain of the glucocorticoid receptor in our chimeric receptor constructs. As expected, the resulting parental glu-

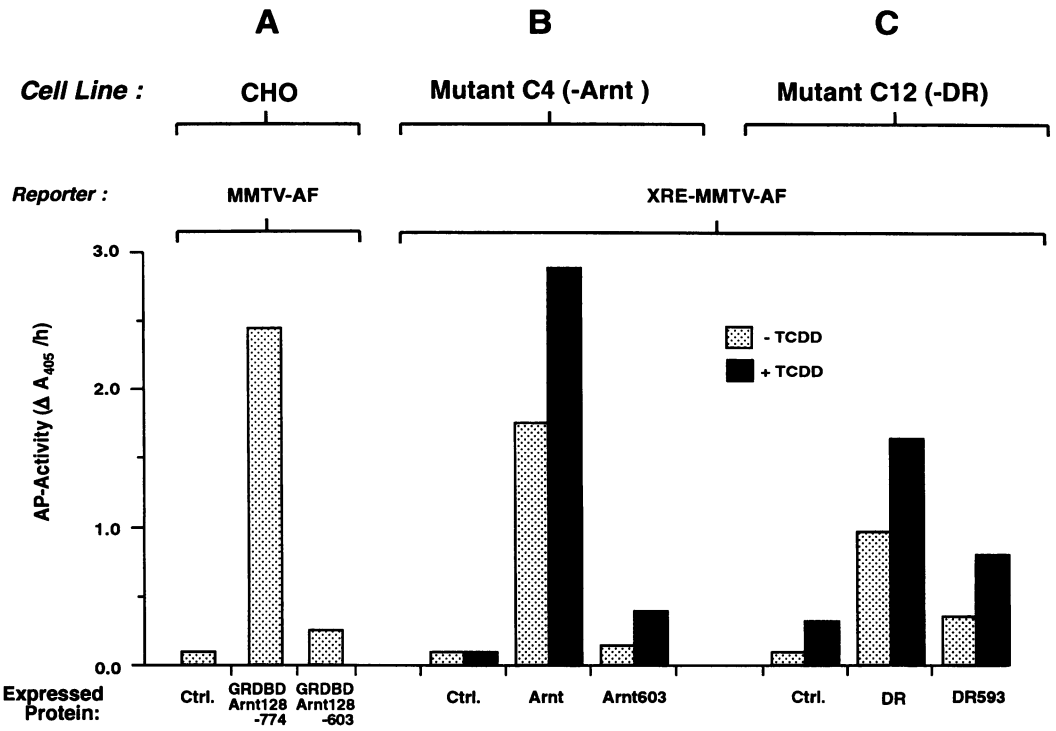


FIG. 7. A potent transactivation function in the Arnt C terminus provides dominant activity on the XRE-MMTV-AF reporter gene. (A) Arnt harbors a potent C-terminal transactivation function. CHO cells were cotransfected with reporter gene pMMTV-AF and a CMV expression vector containing either GRDBD/Arnt128-774 or the C-terminally truncated GRDBD/Arnt128-603. After 48 h, secreted alkaline phosphatase was measured by colorimetry. (B) Mutant hepatoma C4 cells (Arnt deficient) were cotransfected with reporter gene pXRE-MMTV-AF (1 μg) and a CMV expression vector (0.5 μg) containing either native Arnt or deletion mutant Arnt603. After 72 h of treatment with dioxin (10 nM) or vehicle (0.1% dimethyl sulfoxide [DMSO]), secreted alkaline phosphatase was measured by colorimetry. (C) Mutant hepatoma C12 cells (dioxin receptor depleted) were cotransfected with pXRE-MMTV-AF (0.5 μg) and a CMV expression vector (1.0 μg) containing either native dioxin receptor or deletion mutant DR593. After 72 h of treatment with dioxin (10 nM) or vehicle (0.1% DMSO), secreted alkaline phosphatase was measured by colorimetry. For panels A to C, control bars (Ctrl.) represent values obtained from transfecting empty expression vector. Reported values represent the fold induction over the value obtained for the control transfection in the absence of ligand, which for this experiment has been arbitrarily set to 0.1 in each cell line. AP-Activity, alkaline phosphatase.

cocorticoid receptor fragment harboring only the DNA-binding domain GRDBD showed very low background levels of activity on glucocorticoid response element-containing reporter genes. When all residues of receptor or Arnt located C terminally to the bHLH domains were fused to GRDBD, transactivating chimeras were formed. Thus, both the dioxin receptor and Arnt contain endogenous transactivating functions. Since the major portions of receptor and Arnt proteins are contained in these chimeras (90 and 84%, respectively), it is very plausible that these constructs might reflect the transactivation potential for each partner of the native ligand-induced dioxin receptor complex. In a variety of cell lines (CHO, COS7, hepatoma cells, and keratinocytes), the Arnt fusion protein GRDBD/Arnt128-774 was at least fivefold more potent than the dioxin receptor chimera GRDBD/DR83-805. The subtle, dioxin-induced activation of the receptor chimera containing amino acids 83 to 805 was abrogated in a receptor chimera containing residues 83 to 593, indicating the presence of a transactivation domain in the C terminus. Surprisingly, when C-terminal fragments of the dioxin receptor spanning this putative transactivating function, DR521-805 and DR422-805, were fused to the GRDBD, potent, constitutive transactivation was obtained. The functional activities of these constructs were comparable to that seen for the Arnt chimera on the MMTV promoter, thus demonstrating that both Arnt and the dioxin receptor have strong independent transactivation domains that provide regulation at cognate DNA target motifs.

Functional architecture of the dioxin receptor versus Arnt. Our results indicate that a strong activation domain is located in the C terminus of the dioxin receptor. This region of the receptor contains a glutamine-rich stretch of amino acids that has been implicated in transcriptional regulation by a number of transcription factors which act from remote or proximal promoter positions (for reviews, see references 12 and 26). As schematically represented in Fig. 8, a glutamine-rich region is also found in the very C terminus of Arnt, indicating that both proteins may have similar functional architecture. In agreement with this model, we have localized the transactivation domain of Arnt to lie within the C terminus. In contrast to Arnt, however, our data strongly argue that the activation domain of the dioxin receptor is normally repressed within the context of structures of the receptor that overlap at least in part with the PAS homology region. Thus, although both the receptor and Arnt share this region of similarity, it appears to confer distinct modes of regulation onto the activation domains of the receptor and Arnt, respectively. A rather simple model for the dramatic increase in the activity of the dioxin receptor when deletions are introduced into centrally located structures of the molecule is that derepression of the masked activation domain requires a conformational change.

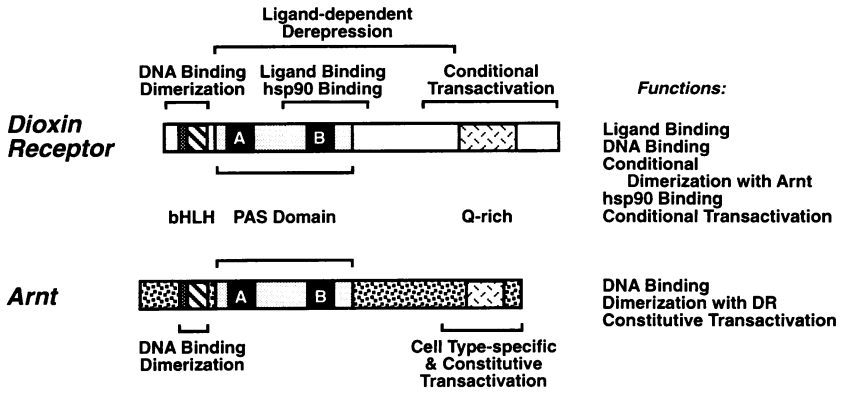


FIG. 8. Functional architecture of dioxin receptor and Arnt proteins. N-terminal bHLH domains mediate dimerization and DNA binding, while regions with transactivation activities are located in the C termini. In the case of the dioxin receptor, a central portion encompassing PAS B forms the core ligand- and hsp90-binding activities. This domain confers conditional repression upon dimerization with Arnt and transactivation capability of the C terminus. In contrast, dimerization and transactivation functions of Arnt appear to act constitutively, with transactivation capability of Arnt showing cell type specificity. See text for details.

hsp90: a mediator of conditional repression of the dioxin receptor? hsp90 is a highly abundant intracellular protein, which associates with a diverse array of other proteins including steroid hormone receptors, tyrosine kinases, and structural proteins. While the full relevance of hsp90 association with these proteins is not clear, hsp90 appears to have at least two important functions. First, it has been postulated to act as a molecular chaperone (for a recent review, see reference 17), since hsp90 has been shown to bind denatured proteins and enhance refolding in vitro (27, 45). Consistent with this hypothesis, both the glucocorticoid and dioxin receptors lose ligand-binding capacity when stripped of hsp90 in vitro (3, 29, 36). Moreover, yeast systems designed to study the function of glucocorticoid receptor/hsp90 interaction have shown decreased glucocorticoid sensitivity in *Saccharomyces cerevisiae* strains expressing low levels of hsp90 (33) or mutant hsp90 proteins (2). Similar experiments investigating $pp60^{v\text{-}src}$/hsp90 function have shown decreased tyrosine kinase activity in a low hsp90 environment (47). A second function of hsp90 appears to be of an inhibitory nature, since both the glucocorticoid and dioxin receptors are maintained in their latent, repressed, non-DNA-binding forms by hsp90 interaction (for recent reviews, see references 37 and 40). Our results show that the constitutive, strong transcription factor formed when amino acids 422 to 805 of the dioxin receptor are fused to GRDBD can be completely repressed when the dioxin receptor portion of the chimera is extended to include a further N-terminal 82 amino acids (i.e., GRDBD/DR340-805). Strikingly, this extension also confers a strong interaction in vitro with hsp90, not seen with the constitutive chimera GRDBD/DR422-805, arguing that hsp90 is the agent of repression. It will now be important to validate this model with in vivo experiments, possibly by using *S. cerevisiae* strains in which the level of hsp90 can be manipulated.

The mechanism by which hsp90 conveys repression is presently unclear. In the case of the native dioxin receptor, in vitro studies have shown that the hsp90-bound receptor is incapable of binding DNA, while artificial stripping of hsp90 allows formation of a DNA-binding species (36, 46). Since DNA binding of the receptor is normally contingent upon ligand-induced heterodimerization with Arnt (22, 23, 38, 44), a repressive function of hsp90 appears to lay in blocking receptor interaction with Arnt. Whether this blockage occurs merely by steric interference or by invoking a receptor conformation unable to interact with Arnt in the absence of ligand remains to be elucidated. In the case of the dioxin receptor chimeras, the site of interaction with hsp90 (amino acids located N terminally to residue 422) is distinct from the transactivation domain which is located C terminally to residue 521 (24, 43). It is therefore unlikely that repression of transactivation seen for GRDBD/DR chimeras is via steric blocking of the C terminus, although an hsp90-chaperoned conformation unable to interact with components of the basal transcription apparatus is a possible and attractive model. All the tested dioxin receptor and Arnt chimeras contain a nuclear localization signal, inherent in the DNA-binding domain of the glucocorticoid receptor (34), although this signal may be masked in chimeras which bind hsp90. The repressive mechanism of hsp90 may therefore be inhibition of nuclear translocation, a hypothesis we are currently investigating. Consistent with this possibility, a role for hsp90 in cytoplasmic/nuclear shuttling of the glucocorticoid receptor has recently been suggested (20).

Separation of ligand-binding and transactivation domains in the dioxin receptor is analogous to separation of steroid binding and activation domains in steroid hormone and retinoic acid receptors. This class of transcription factors contain an AF-1 transactivation domain, which can act constitutively on heterologous DNA-binding domains, and a second, ligand-dependent transactivation domain, AF-2, which overlaps the ligand-binding domain (for reviews, see references 6, 14, and 42). In the dioxin receptor, no AF-2 type transactivation domain was detected, since chimera DBD/DR83-593, which contains residues beyond the minimal ligand-binding core from amino acids 230 to 421, showed no ligand-induced activity. The C terminus of the dioxin receptor, being constitutive on a heterologous DNA-binding domain, appears therefore to be equivalent to an AF-1-type activity, which is blocked in the natural dioxin receptor by distant residues interacting with a separate factor, hsp90. Finally, while the dioxin receptor chimera GRDBD/DR83-805 is repressed in the absence of ligand, the counterpart Arnt chimera, GRDBD/Arnt128-774 shows strong, unconditional activity. In strong support of the model that repression of the dioxin receptor chimera may be mediated by hsp90, native Arnt clearly shows no interaction with hsp90 (25, 38).

Comparing the potent activities of chimeras containing

dioxin receptor segments between residues 422 to 805 and 521 to 805, respectively, with the repressed activity of the chimera containing amino acids 340 to 805 has indicated that an inhibitory activity, lying within an 82-amino-acid stretch between amino acids 340 to 421, has the ability to confer repression upon a distant transactivation domain (located C terminally of amino acid 521). Against the background outlined above, we propose that binding of hsp90 mediates this repression, and therefore, hsp90 may have two important modes of repression in the native dioxin receptor. The first can be referred to as a "*trans* repression," or blocking of an intermolecular event, represented by dimerization with the cofactor Arnt (44). This mode of repression is inferred by earlier observations that artificial stripping of hsp90 from the receptor in the absence of ligand results in formation of a constitutively DNA-binding complex (36). The second mode can be termed a "*cis* repression" or blocking of some intramolecular event within the receptor protein, inferred by the repression of dioxin receptor chimeras which are functionally uncoupled from Arnt as a cofactor. It will now be important to examine whether this intramolecular event may be the chaperoning of a conformation which is inert in the absence of ligand or perhaps simply the blocking of a nuclear localization signal.

Implications for gene regulation by dioxin signalling. The abrogation of transactivation potency seen when dioxin receptor and Arnt chimeras are C terminally truncated strongly suggests that transactivation functions reside in the C termini of both proteins. To investigate the influence of these transactivating regions in a more natural context and to explore the important question of whether one or both proteins contribute to transactivation in the ligand-activated dioxin receptor/Arnt heterodimeric complex, we analyzed dioxin receptor and Arnt proteins spanning the N-terminal bHLH dimerization motif but containing C-terminal deletions. The loss of activity seen when DR593 and Arnt603 are coexpressed confirms the localization of transactivation activity to the C termini of both proteins. Analyses of each truncated mutant with its full-length bHLH partner factor in both CHO and mutant C4 (Arnt-deficient) or C12 (receptor-depleted) hepatoma cell lines have indicated that Arnt provides the dominant transactivation function of the native dioxin receptor/Arnt complex. Surprisingly, our assay system shows the strong transactivation domain of the dioxin receptor C terminus to be largely silent in the native dioxin receptor/Arnt complex. However, while we have shown dioxin receptor and Arnt chimeras to enhance transcription on both natural (MMTV) and artificial (GREII-Oct) promoters, we have found that the relative strengths of these chimeras vary between the two promoters. It is therefore possible that in the natural dioxin receptor/Arnt complex, one subunit or the other may play a dominant role, depending on promoter architecture. To pursue this question, we are currently analyzing activities of our dioxin receptor and Arnt transactivation-deficient mutants on a variety of reporter genes to investigate the mechanisms by which transcription is enhanced in natural promoter contexts. Furthermore, our data indicate that cell type specificity may determine whether the dioxin receptor or Arnt is the major transactivating protein. In contrast to CHO, Hepa 1c1c7, and COS7 cells, in which dioxin receptor and Arnt chimeras were of similar potencies, HepG2 cells showed receptor chimeras to be four- to fivefold more active. Taken together, these results suggest that the two factors may have different mechanisms for transcription activation, possibly by making different contacts with components of the basal transcription machinery or cell-specific intermediary cofactors. Our results therefore provide a direction for studies aimed at elucidating how these subunits of the native dioxin receptor complex communicate with other factors to mediate dioxin-induced transcription.

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