MOLECULAR AND CELLULAR BIOLOGY, Apr. 1993, p. 2504–2514
0270-7306/93/042504-11$02.00/0

Vol. 13, No. 4

Ligand-Dependent Recruitment of the Arnt Coregulator Determines DNA Recognition by the Dioxin Receptor

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Received 23 October 1992/Returned for modification 18 December 1992/Accepted 6 January 1993

The intracellular basic region/helix-loop-helix (bHLH) dioxin receptor mediates signal transduction by dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) and functions as a ligand-activated DNA binding protein directly interacting with target genes by binding to dioxin response elements. Here we show that the partially purified, ligand-bound receptor alone could not bind target DNA. In contrast, DNA binding by the receptor could be induced by addition of a cytosolic auxiliary activity which functionally and biochemically corresponded to the bHLH factor Arnt. While Arnt exhibited no detectable affinity for the dioxin response element in the absence of the dioxin receptor, it strongly promoted the DNA binding function of the ligand-activated but not the ligand-free receptor forms. Arnt also functionally reconstituted in vitro the DNA binding activity of a mutant, nuclear translocation-deficient dioxin receptor phenotype in cytosolic extracts from a dioxin-resistant hepatoma cell line. Importantly, coimmunoprecipitation experiments showed that Arnt physically interacted in solution with the ligand-activated dioxin receptor but failed to heterodimerize with the ligand-free, hsp90-associated receptor form. Mutational analysis suggested that the functional interaction between these two factors occurred via the bHLH motif of Arnt. These data suggest that dioxin receptor activity is governed by a complex pattern of combinatorial regulation involving repression by hsp90 and then by ligand-dependent recruitment of the positive coregulator Arnt. The dioxin receptor system also provides the first example of signal-controlled dimerization of bHLH factors.

Signal transduction by dioxins (most notably 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD]) is mediated by the intracellular dioxin (or aryl hydrocarbon) receptor. The receptor binds dioxin and its planar aromatic congeners in a saturable manner with high affinity (for reviews, see references 22 and 49). The potent toxicity of dioxins is well established in animal models but is a matter of debate in humans (for a recent review, see reference 19). In animals the toxic effects are typified by thymic wasting and immune suppression, severe epithelial disorders, and tumor promotion (for a review, see reference 51). At the molecular level, dioxins are very potent inducers of transcription of a battery of target genes encoding xenobiotic metabolizing enzymes such as cytochrome P-450IA1, glutathione *S*-transferase Ya, aldehyde dehydrogenase, and quinone oxidoreductase (for a review, see reference 34). In addition, dioxin appears to transcriptionally regulate the expression of the growth modulatory genes for interleukin-1β and plasminogen activator inhibitor-2 (59). The dioxin induction response is mediated by single or multiple copies of dioxin-inducible transcriptional control elements (xenobiotic response elements [XREs]) in target promoters (16, 18, 47). In analogy to the current model of action of steroid hormone receptors (for a review, see reference 3), the ligand-activated dioxin receptor appears to transmit the gene induction signal from the cytoplasm to the nucleus, where it interacts with its cognate DNA response element to activate transcription (for reviews, see references 34 and 49). Finally, the endogenous ligand, if any, of the dioxin receptor has not been identified (for a review, see reference 49).

The detailed mechanism of signal transduction through the dioxin receptor is currently unclear. The ligand-binding form of receptor is a ubiquitous ~100-kDa protein which was recently shown to contain a putative basic region/helix-loop-helix (bHLH) motif (8, 15). In contrast, the zinc finger motif (27, 38 [and references therein]) is highly conserved among members of the steroid receptor superfamily. Thus, the dioxin receptor belongs to a distinct class of ligand-activated nuclear receptors. Moreover, the receptor is structurally related to the bHLH factor Arnt which functionally complements C4 mutant hepatoma cells which are nonresponsive to dioxin and express a nuclear translocation-deficient (nt^-) dioxin receptor phenotype (8, 15, 30). Arnt has therefore been postulated to govern nuclear translocation of the ligand-activated dioxin receptor form (30).

Interestingly, in analogy to a distinct subgroup of the zinc finger receptors including the glucocorticoid receptor (for a review, see reference 3), the latent (i.e., non-DNA binding) form of dioxin receptor is found complexed with hsp90 (11, 48, 61). Exposure to dioxin in vivo (13, 18, 26, 45, 61) or in vitro treatment with dioxin under high-ionic-strength conditions (10, 52) leads to release of hsp90 and a transformed dioxin receptor form with high affinity for XRE sequences. This DNA binding form of the dioxin receptor has a native molecular mass of about 200 kDa (26, 55), indicating that it either represents a homodimer or a heterodimeric complex with a factor of similar molecular mass. The latter possibility is favored by DNA cross-linking studies which have identified two distinct proteins (~100 and ~110 kDa) in contact with the XRE target sequence (14). A good candidate for a receptor partner is the ~85-kDa structurally related bHLH factor Arnt. In line with this model, Arnt has recently been reported to be a component of the nuclear dioxin receptor complex after treatment of target cells with dioxin in vivo (57).

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We have previously shown that the isolated, hsp90-free form of dioxin receptor shows constitutive DNA binding activity and does not form a stable complex with dioxin (52). Therefore, hsp90 may serve as a cellular chaperone molecule, which, in addition to repressing the DNA binding activity of the receptor, stabilizes and maintains a ligand-binding configuration of the protein. To further explore the mechanism of regulation of dioxin receptor activity, we have investigated the DNA binding properties of the partially purified, hsp90-associated form of dioxin receptor. We show here that it was not possible to in vitro activate DNA binding of this receptor form, even though it can bind ligand (52). In complementation assays, we identified a distinct 4S-5S activity which strongly enhanced the binding of the receptor to its responsive element. This activity corresponded by functional and biochemical criteria to the Arnt coregulator. Coimmunoprecipitation experiments demonstrated that Arnt formed a strong complex with the ligand-activated dioxin receptor in solution. Moreover, neither Arnt nor the dioxin receptor showed any detectable affinity for the XRE target sequence unless the heteromeric complex was formed. The bHLH region of Arnt was required for functional interaction with the dioxin receptor. Importantly, however, this interaction also required the presence of ligand and depended on the dose and receptor affinity of the employed ligand. In agreement with these observations, Arnt failed to physically associate with the ligand-free, hsp90-containing dioxin receptor form. Thus, hsp90 appears to prevent Arnt from interacting with the receptor. Taken together, activation of the dioxin receptor appears to be a multistep process involving ligand-induced release of hsp90 and subsequent interaction with the Arnt coregulator prior to binding to DNA target sequences.

MATERIALS AND METHODS

Recombinant plasmids. The Arnt expression vector pBM5-NEO-M1-1 has previously been described (30). The in vitro transcription vector pArntΔHLH was constructed by subcloning of a *Hin*dIII fragment containing the full-length Arnt coding region from pBM5-NEO-M1-1 into pGem7Z (Promega), excision of a 390-bp *Bcl*I fragment from the Arnt coding region, and subsequent religation of the plasmid. The ArntΔHLH coding region was then excised as a *Cla*I-*Xba*I fragment and inserted into the corresponding sites in pCMV4 (2) to create the expression vector pCMVArntΔHLH. The XRE-driven reporter gene was created by substituting the glucocorticoid response element in pMMTV-hGH2 (1) with a single, synthetic wild-type or point-mutated XRE element to create the reporter constructs pXRE-MMTV-hGH or pXM1-MMTV-hGH, respectively.

Cells, transient transfection, and extract preparation. The wild-type, dioxin-responsive Hepa 1c1c7 cell line and the mutant, dioxin-resistant nt^- C4 cell line derived from it (25) were grown in minimum essential medium as described previously (61). Cells were grown to near confluence in an atmosphere of 6% CO_2. Cells were transiently transfected with 1 μg of the wild-type or mutant Arnt expression vector and 1 μg of the wild-type or point-mutated XRE reporter gene, respectively, with lipofectin (Bethesda Research Laboratories). Secreted hGH levels were assayed in the cell medium by a radioimmunoassay (Pharmacia). Cytosolic extracts were prepared by homogenization of untreated cells in 1 volume of TEG buffer (20 mM Tris-HCl [pH 7.4], 1 mM EDTA, 10% [wt/vol] glycerol, 1 mM dithiothreitol) and centrifugation at 120,000 × *g* for 45 min. The resulting supernatant was taken as the cytosolic fraction and either used immediately or frozen in small aliquots at −70°C.

Fractionation of the dioxin receptor and the auxiliary factor on sucrose density gradients. Wild-type or mutant cell cytosol (500 μl; about 4 mg of protein per ml of cytosol) was layered on 10 to 40% (wt/vol) linear sucrose gradients prepared in TEG buffer containing 50 mM NaCl. The gradients were centrifuged at 300,000 × *g* to a cumulative centrifugal effect of 1.7×10^{12} rad^2/s in a Beckman L8-60 ultracentrifuge. Fractions were collected by gravity flow, starting from the bottom of the gradients. ^{14}C-labeled immunoglobulin G (IgG) (6.6S) and bovine serum albumin (4.4S) were used as external sedimentation marker proteins.

DNA binding assay. The DNA binding activity of the dioxin receptor was monitored by a gel mobility shift assay performed essentially as described previously (26, 44). DNA binding reactions were assembled with the indicated protein fractions in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 5% (vol/vol) glycerol, 0.5 mM dithiothreitol, 2.5 mM $MgCl_2$, 1 mM EDTA, 0.08% (wt/vol) Ficoll, and 4 mM spermidine at a final concentration of 60 mM NaCl in a final volume ranging between 20 and 50 μl. A 36-bp ^{32}P-3'-end-labeled, double-stranded oligonucleotide XRE (10) spanning the dioxin-responsive XRE1 element of the rat cytochrome P-450IA1 upstream-promoter region (18) was added to the reactions as a specific probe in the presence of 1 μg of poly(dI-dC) (Pharmacia) nonspecific competitor DNA and incubated for 30 min at 25°C. Bound and free DNAs were electrophoretically separated under conditions described previously (26). The double-stranded oligonucleotide XM1 (10) containing a single-point mutation of the XRE target sequence was used in DNA binding competition experiments.

Expression of Arnt by in vitro translation. Wild-type or mutated Arnt mRNAs were generated from pBM5-NEO-M1-1 or pArntΔHLH using T7 polymerase and used for in vitro synthesis of labeled or unlabeled Arnt proteins in rabbit reticulocyte lysates (Promega) in the presence of either [^{35}S]methionine (New England Nuclear) or 20 to 30 μM unlabeled methionine under conditions suggested by the manufacturers. For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, prestained and ^{14}C-labeled M_r marker proteins were purchased from Bio-Rad and Amersham, respectively.

Noncovalent and covalent labeling of the dioxin receptor. The crude or ~9S dioxin receptor from wild-type or mutant hepatoma cells was noncovalently labeled by incubation for 2 to 3 h at 25°C with the indicated concentration of nonradioactive dioxin or [^3H]dioxin (Chemsyn, Lenexa, Kans.; specific activity, 40 Ci/mmol). The affinity ligand 2-azido-3-[^{125}I]2-iodo-7,8-dibromo-dibenzo-*p*-dioxin ([^{125}I]dioxin, specific activity, ~2,000 Ci/mmol) was synthesized and purified essentially as previously described (50, 52). The crude cytosolic dioxin receptor was covalently labeled by incubation for 1 h at 0 to 4°C with [^{125}I]dioxin and subsequent UV irradiation at 330 nm for 15 min as previously described (48, 50). Prior to UV irradiation, the sample was treated with dextran-coated charcoal to remove nonbound ligand.

Dioxin receptor antiserum, immunoblot, and immunoprecipitation experiments. Peptide corresponding to amino acids 12 to 31 of the murine dioxin receptor (8, 15) was synthesized and coupled to ovalbumin (62), and antisera were prepared from rabbits by standard techniques (28). In the immunoblot experiments, proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were subsequently incubated with preim-

mune or anti-receptor antisera at 1:10 dilutions and stained by peroxidase-conjugated swine anti-rabbit immunoglobulins (Dako). For the immunoprecipitation experiments, cytosolic extracts from nt$^-$ mutant C4 cells were treated with 10 nM TCDD at 25°C for 3 h, in the presence of the protease inhibitors aprotonin (5 μg/ml) and 1 mM phenylmethylsulfonyl fluoride. In vitro-translated [^{35}S]methionine-labeled Arnt (5 μl) was added to 35 μl of untreated or ligand-treated C4 cytosol (6 mg of protein per ml) and incubated at 25°C for 20 min. Immune or preimmune serum (20 μl) was then added and gently shaken for 1 h. Immunoprecipitation was carried out by the addition of 100 μl of a 50% slurry of protein A-Sepharose in PEG buffer (20 mM sodium phosphate [pH 7.2], 1 mM EDTA, 10% [wt/vol] glycerol) containing 150 mM NaCl, 1% Triton X-100, and 1 mM dithiothreitol. After being shaken for 30 min, the resin was washed four times with the same buffer and the immunoprecipitated proteins were separated through an SDS–7.5% polyacrylamide gel. For fluorography, gels were fixed in 20% methanol–10% acetic acid, immersed in Amplify (Amersham) for 30 min, dried, and exposed to film.

Safety precautions. In experiments involving the use of dioxin, special handling procedures were employed (61 and references therein), and contaminated materials were disposed of by high-temperature incineration.

RESULTS

Failure to activate the isolated, hsp90-associated dioxin receptor form to a DNA binding species by ligand treatment in vitro. In an effort to in vitro reconstitute the process of activation of the latent dioxin receptor to a DNA binding form, we partially purified the ~9S (63), ligand-free form of receptor by fractionation on sucrose density gradients of a cytosolic extract from untreated Hepa 1c1c7 hepatoma cells. This form of receptor is tightly associated with hsp90 (11, 48, 61) and shows dioxin-binding activity in vitro (52). The ~9S receptor form was identified by immunoblot analysis of the individual sucrose gradient fractions. To this end, we used an anti-receptor antiserum, the specificity of which is shown in Fig. 1A. The ~95-kDa receptor was specifically recognized by the antiserum but not by preimmune serum following fractionation of a crude cytosolic extract from Hepa 1c1c7 cells by SDS-PAGE (Fig. 1A, compare lanes 1 and 3). A similar immunoblot analysis of Hepa 1c1c7 cell cytosol following fractionation on a sucrose gradient demonstrated that the ligand-free dioxin receptor was recovered as a distinct symmetric peak in the 9S region of the gradient (Fig. 1B).

In cytosolic extracts from a number of cells, it is possible to activate the DNA binding function of the dioxin receptor by in vitro treatment with ligand and salt at moderate temperatures (10, 18, 44, 52). Thus, in gel mobility shift assays the ligand-free dioxin receptor in crude cytosol from untreated Hepa 1c1c7 cells is inactive and shows no detectable affinity for the XRE target sequence, whereas it is activated to maximal levels of DNA binding activity by exposure to 5 to 10 nM dioxin in vitro (10) (Fig. 1C, compare lanes 1 and 2). Remarkably, however, it was not possible to convert the isolated 9S dioxin receptor to a DNA binding species by ligand treatment in vitro (Fig. 1C, compare lanes 2 to 7) by the protocol which readily activated the receptor in crude cytosol. Even in the presence of very high concentrations (100 nM) of dioxin, no DNA binding activity was detected in the 9S fraction (Fig. 1C, lane 7). Although this

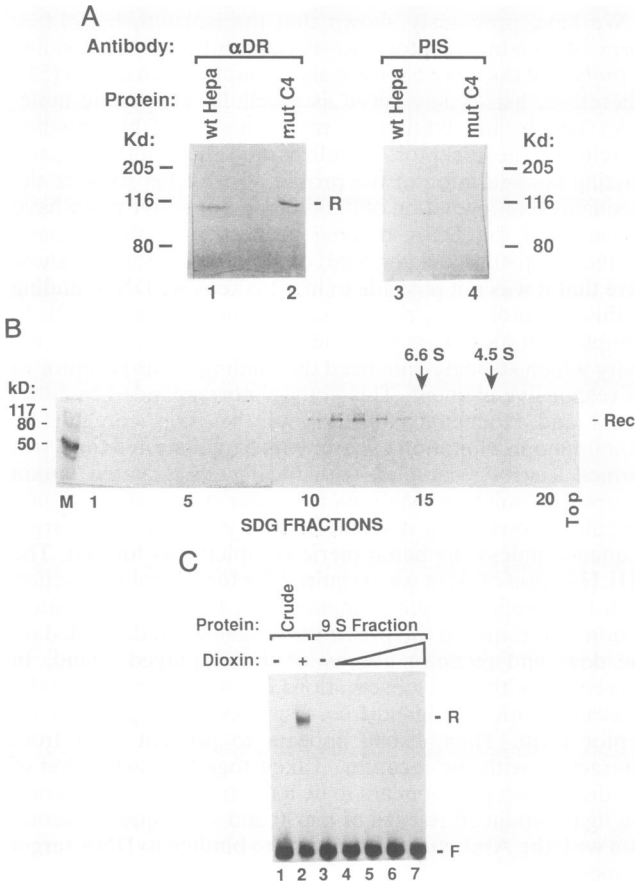

FIG. 1. Failure to activate the ~9S dioxin receptor form by ligand treatment in vitro. (A) Immunoblot analysis of dioxin receptor. Crude cytosolic extracts (~100 μg of protein) from wild-type (wt Hepa; lanes 1 and 3) or nt$^-$ mutant C4 (mut C4; lanes 2 and 4) hepatoma cells were fractionated on an SDS–7.5% polyacrylamide gel and analyzed on immunoblots. Note that the anti-receptor antiserum (αDR; lanes 1 and 2) recognized a single band (indicated by R) in both wild-type and mutant cell extracts, whereas no staining of the filters was obtained with preimmune serum (PIS; lanes 3 and 4). (B) Immunoblot analysis of ligand-free dioxin receptor following fractionation on a sucrose density gradient (SDG). A cytosolic extract (500 μl; ~4 mg of protein per ml) from untreated wild-type hepatoma cells was fractionated on a 10 to 40% (wt/vol) sucrose gradient, and the individual fractions were analyzed by immunoblot analysis as described above with the anti-receptor antiserum. The position of the receptor (Rec) is indicated. On top of the blots are indicated the position of sedimentation marker proteins (albumin, 4.5S; immunoglobulin G, 6.6S) run on separate gradients. (C) DNA binding analysis. Ligand-free cytosol from wild-type hepatoma cells was fractionated on sucrose gradients as described above. The receptor sedimenting in the 9S fraction was incubated in the absence (lane 3) or presence of increasing concentrations of dioxin (0.1 to 100 nM; lanes 4 to 7). As a control, the crude input cytosolic extract was treated in the absence (lane 1) or presence (lane 2) of 5 nM dioxin. DNA binding activities were analyzed by gel mobility shift analysis with a ^{32}P-labeled XRE oligonucleotide probe. The relative mobilities of the receptor-dependent XRE complex (R) and the free probe (F) are indicated.

form of receptor avidly binds ligand (52) (data not shown), ligand binding alone is not sufficient for activation.

We conclude from these experiments that the partially purified dioxin receptor showed very low, if any, intrinsic affinity for DNA. To bind efficiently to DNA, the receptor

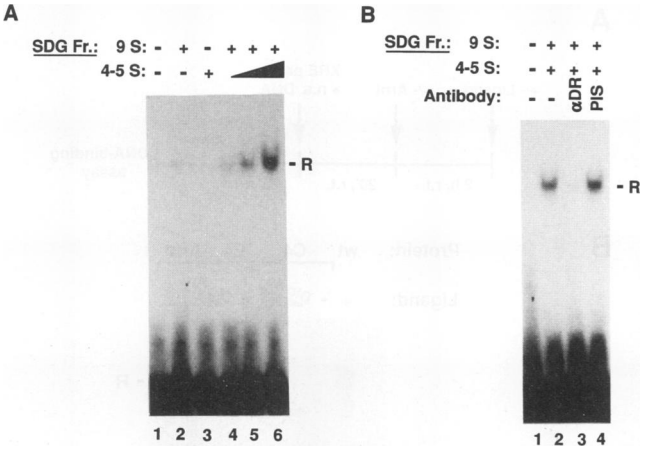

FIG. 2. An auxiliary, cytosolic activity is required to convert the dioxin receptor into a DNA binding form. Wild-type Hepa 1c1c7 cytosol was incubated with 10 nM [^{3}H]dioxin and fractionated on a 10 to 40% (wt/vol) sucrose density gradient (SDG) under low-salt conditions (50 mM NaCl). Under these conditions the receptor-hsp90 complex is not disrupted by the ligand during sucrose gradient centrifugation (44 [and references therein]). (A) The ~9S dioxin receptor form was identified by scintillation counting of the individual gradient fractions and incubated at 25°C for 30 min in the absence (lane 2) or presence (lanes 4 to 6) (1 to 10 μl) of increasing concentrations of cytosolic material sedimenting in the 4-5S region of the gradient. The XRE binding activity of the isolated 4-5S fraction is shown in lane 3. (B) The specificity of the regenerated complex was assessed in antibody-mediated DNA binding inhibition experiments. The mixture of the 9S and 4-5S fractions was preincubated in the absence or presence of receptor-specific antibody (αDR) or preimmune serum (PIS) for 20 min at 25°C prior to assembly of the XRE binding reactions. The position of the receptor-dependent XRE complex (R) is shown.

appears rather to require an auxiliary factor that is present in crude cytosolic extracts but not in the 9S fraction of sucrose gradients.

Presence in hepatoma cells of a cytosolic activity which strongly enhances binding of the dioxin receptor to target DNA. To identify and characterize such a possible auxiliary factor(s), we used the inert 9S fraction containing hsp90-bound dioxin receptor in complementation experiments, in which we screened the ability of other fractions from the sucrose gradient to reconstitute DNA binding activity of the receptor. Importantly, the dioxin-labeled receptor-hsp90 complex is very stable and is not disrupted by sucrose gradient centrifugation under low-salt conditions (44 [and references therein]). Thus, following fractionation of dioxin-labeled crude Hepa 1c1c7 cytosol on sucrose gradient, little, if any, specific XRE binding activity was detected in gel mobility shift assays with the recovered 9S fraction (Fig. 2A, lane 2) or other fractions of the sucrose gradient (52) (data not shown). Interestingly, however, addition of increasing concentrations of an activity in the 4-5S region strongly promoted in a concentration-dependent manner XRE binding in the presence of the ligand-treated 9S fraction (Fig. 2A, compare lanes 4 to 6). Addition of other sucrose gradient fractions or nonspecific proteins (e.g., reticulocyte lysate) to the receptor-containing 9S fraction did not have any effect on the XRE binding activity (data not shown). Moreover, the 4-5S fraction itself did not generate any detectable complex with the XRE probe in the absence of the receptor-containing material (Fig. 2A, lane 3). Finally, the XRE complex that was generated by coincubating the 9S and 4-5S fractions contained the dioxin receptor as shown by the inhibition of XRE DNA-protein complex formation following exposure to the receptor-specific antibody (Fig. 2B, compare lanes 2 and 3). Control antibodies did not have any effect on this DNA binding activity (Fig. 2B, lane 4). From these data we conclude that the 4-5S fraction of sucrose gradients contains an activity facilitating binding of the ligand-activated dioxin receptor to target DNA. This activity appears to be distinct from the receptor, since no receptor-dependent immunoreactivity or any receptor-dependent ligand or DNA binding activities were found in this region of the gradient. Thus, this activity could represent the postulated auxiliary factor.

The Arnt factor substitutes for the 4-5S activity in promoting the DNA binding function of the dioxin receptor. Given the background that the Arnt protein is structurally related to the dioxin receptor (8, 15, 30) and that it apparently regulates nuclear translocation of the ligand-activated receptor, it is a very plausible candidate for a functional partner of the receptor. Moreover, Arnt has recently been reported to be associated with the nuclear receptor form (57). We therefore tested the hypothesis that the function of Arnt is to promote DNA binding of the ligand-activated receptor.

In vitro translation of Arnt mRNA in the presence of [^{35}S]methionine produced a single labeled protein of ~85 kDa as assessed by SDS-PAGE analysis (see, for instance, Fig. 5B and 6B, lane 1). Centrifugation of the labeled Arnt protein through a sucrose gradient reveals Arnt sedimenting in the 4-5S region of the gradient. More importantly, a strong protein-XRE complex was generated by coincubating the ligand-occupied 9S receptor fraction with increasing concentrations of Arnt, indicating that Arnt promotes DNA binding cooperatively with the receptor (data not shown; see Fig. 6C, lane 6), consistent with the hypothesis that Arnt corresponds to the putative auxiliary factor.

Arnt reconstitutes in vitro the DNA binding activity of the mutant nt$^-$ dioxin receptor. To further examine the functional properties of the Arnt protein, we used a cytosolic extract from Arnt-deficient, dioxin-resistant C4 mutant hepatoma cells (25, 30 [and references therein]). These cells express an nt$^-$ dioxin receptor phenotype which has the same molecular mass as the wild-type receptor and is present at levels similar to those expressed by the parental Hepa 1c1c7 cells (Fig. 1A, compare lanes 1 and 2). We therefore wanted to examine the effect of in vitro-expressed Arnt on the XRE binding activity of the nt$^-$ receptor. Dioxin treatment in vitro fails to activate the DNA binding activity of the cytosolic nt$^-$ receptor (10) (Fig. 3A, lane 1). Furthermore, we observed no receptor-dependent XRE binding activity endogenous to the unprogrammed rabbit reticulocyte lysate (lane 2), nor in the lysate containing in vitro-translated Arnt (lane 3) or in a coincubated mixture of unprogrammed lysate and the dioxin-occupied nt$^-$ receptor (lane 4). In contrast, incubation of the ligand-occupied nt$^-$ receptor with in vitro-translated Arnt generated an XRE complex with mobility equivalent to that produced by the activated wild-type receptor (lane 5). This protein-DNA complex is further characterized in Fig. 3C. In DNA competition experiments, an excess of the unlabeled XRE target sequence abrogated formation of the complex by the mixture of Arnt and the nt$^-$ receptor (compare lanes 1 and 2), whereas the levels of this complex were not altered (compare lanes 1 and 3) in the presence of an identical excess of a single point mutant XM1 (as detailed in Fig. 3B) that is unable to bind the ligand-activated wild-type receptor (10). Finally, in the presence of the receptor-specific antiserum but not in the presence of preimmune serum, formation of

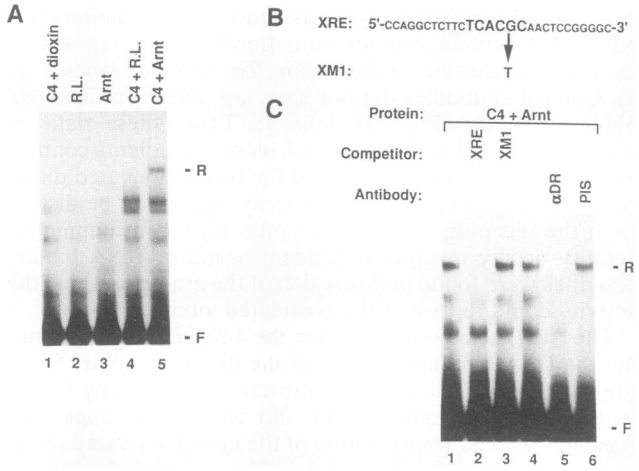

FIG. 3. In vitro reconstitution of the DNA binding activity of the nt⁻ dioxin receptor phenotype by Arnt. (A) The nt⁻ dioxin receptor in C4 mutant hepatoma cell cytosol (~4 mg of protein per ml) was treated with 5 nM dioxin. Gel mobility shift assays were performed with the ^{32}P-labeled XRE probe in the presence of 5 μl of the ligand-treated nt⁻ receptor alone (lane 1), 5 μl of unprogrammed reticulocyte lysate (R.L.; lane 2), 5 μl of reticulocyte lysate containing in vitro-translated Arnt (Arnt; lane 3), or reaction mixtures containing the ligand-treated nt⁻ receptor that had been coincubated for 20 min at 25°C with either 5 μl of unprogrammed reticulocyte lysate (lane 4) or 5 μl in vitro-translated Arnt (lane 5). (B) Sequences of the wild-type XRE motif and a single-point mutant (XM1) deficient in binding the ligand-activated wild-type receptor. (C) The specificity of the reconstituted XRE complex was assessed by DNA competition and antibody experiments. The ligand-treated nt⁻ receptor was coincubated with in vitro-translated Arnt as described above in the absence (lanes 1 and 4) or in the presence of an excess of either the unlabeled wild-type XRE (lane 2) or XM1 mutant (lane 3) oligonucleotides or receptor-specific (αDR; lane 5) or preimmune (PIS; lane 6) antibodies. XRE binding activities were monitored by gel mobility shift analysis. The positions of the reconstituted, receptor-dependent complex (R) and the free probe (F) are indicated. As shown in the competition experiments, the protein-XRE complexes of faster mobility than the receptor complex are the result of nonspecific protein-DNA interactions.

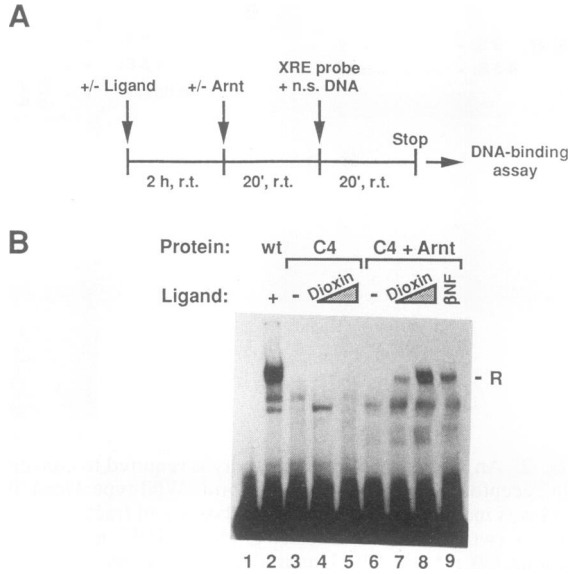

FIG. 4. Requirement of ligand for conversion of the dioxin receptor into a DNA binding form by the Arnt coregulator. (A) Schematic diagram of the experimental protocol for ligand-dependent in vitro reconstitution of the DNA binding activity of the nt⁻ mutant dioxin receptor. (B) Cytosol from mutant C4 cells (~6 mg of protein per ml) was treated with no dioxin (lanes 3 and 6) or with 1 (lanes 4 and 7) or 10 (lanes 5 and 8) nM dioxin or with 500 nM β-naphthoflavone (lane 9) and further incubated in the absence (lanes 3 to 5) or presence (lanes 6 to 9) of 5 μl of reticulocyte lysate containing in vitro-translated Arnt. As a control, wild-type (wt) hepatoma cell cytosol (~6 mg of protein per ml) was treated with 10 nM dioxin (lane 2). XRE binding activities were monitored by the gel mobility shift assay. The position of the receptor-dependent complex is indicated.

this complex was disrupted (Fig. 3C, compare lanes 4 to 6), indicating that the nt⁻ receptor was a component of the XRE complex following addition of Arnt. Taken together, these results show that Arnt recruited the receptor into the XRE binding complex. Moreover, these experiments demonstrate that, individually, the receptor and Arnt proteins bind very poorly, if at all, on their own to the DNA target sequence.

The ligand regulates the functional interaction between the dioxin receptor and Arnt. We next explored the cell-free reconstitution assay to investigate whether the receptor ligand influenced the effect of Arnt on the DNA binding activity of the nt⁻ dioxin receptor. The scheme for activation of the receptor is detailed in Fig. 4A: incubation of the C4 cytosolic extract with ligand proceeded for 2 h at room temperature; Arnt was added and further incubated before standard gel mobility shift analysis. As expected, treatment of the cytosolic nt⁻ receptor with increasing concentrations (1 to 10 nM) of dioxin failed to induce an XRE complex in the absence of Arnt (Fig. 4B, compare lanes 3 to 5), while addition of Arnt to the ligand-occupied nt⁻ receptor produced the receptor-dependent XRE complex which, in turn, increased in intensity at the higher concentration of dioxin (compare lanes 7 and 8). Moreover, the receptor-XRE complex was also generated in the presence of a high concentration (500 nM) of the weaker dioxin receptor agonist β-naphthoflavone. This complex was less intense than the corresponding dioxin-induced complex (compare lanes 8 and 9), consistent with the about 50- to 100-fold-lower affinity of β-naphthoflavone for the dioxin receptor in vitro (20). For reference, the cytosolic, wild-type dioxin receptor was in vitro activated by exposure to 10 nM dioxin. Interestingly, the resulting XRE complex did not only comigrate with the corresponding complex produced by Arnt and the nt⁻ receptor (compare lane 2 with lanes 7 to 9) but showed an intensity that was only slightly stronger than that generated in the reconstituted system in the presence of an identical concentration (10 nM) of dioxin (compare lanes 2 and 8). Against the background that both the wild-type and C4 cytosolic extracts were matched with regard to protein concentration and that the receptor is expressed at similar levels in both the wild-type and nt⁻ mutant C4 cells (Fig. 1A), these data clearly indicate that Arnt is a potent regulator which, in the presence of maximal ligand stimulation, appears to be both necessary and sufficient to restore the DNA binding activity of the nt⁻ receptor to almost wild-type levels.

It is important that the receptor-dependent XRE complex was not detected if Arnt was incubated with the ligand-free nt⁻ receptor (Fig. 4B, compare lanes 6 to 8). Thus, functional interaction between Arnt and the dioxin receptor was a strictly ligand-dependent event. In the case of the wild-type receptor, we have previously shown that the intensity

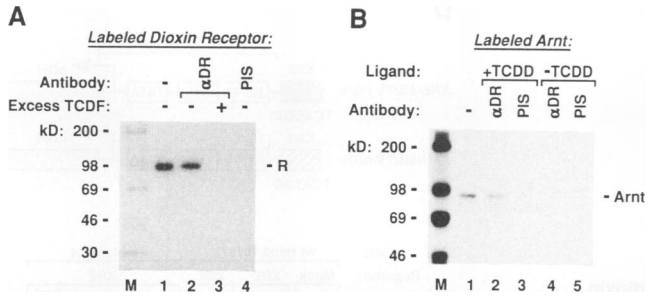

FIG. 5. The ligand-activated dioxin receptor and the Arnt coregulator are tightly associated in solution. (A) Immunoprecipitation of the dioxin receptor. The nt$^-$ dioxin receptor in cytosol (~2 mg of protein per ml) from C4 mutant hepatoma cells was covalently labeled with [^{125}I]dioxin in the absence or presence of the unlabeled competitor ligand prior to immunoprecipitation with receptor-specific (αDR) antibodies or preimmune serum (PIS), as indicated. Input material (lane 1) or the immunoprecipitated complexes were separated on a 7.5% SDS–polyacrylamide gel and analyzed by autoradiography. The position of the receptor (R) is indicated. (B) Coimmunoprecipitation experiments. Arnt was labeled by in vitro translation in the presence of [^{35}S]methionine and incubated with 10 μl of a cytosolic extract (~6 mg of protein per ml) from C4 mutant hepatoma cells that had been pretreated in the absence (lanes 4 and 5) or presence (lanes 2 and 3) of 20 nM dioxin. These reaction mixtures were immunoprecipitated with the receptor-specific antibodies (αDR) or preimmune serum (PIS) and analyzed by SDS-PAGE and subsequent fluorography. As a reference, the labeled, input Arnt material was also analyzed on the gel (lane 1). The molecular mass marker lanes (M) are indicated.

of the receptor-XRE complex is directly proportional to the dose and receptor affinity of the ligand used (10). Employing in vitro-translated Arnt in artificial conversion of the nt$^-$ receptor to a DNA binding species therefore seems to successfully and faithfully mimic the dioxin activation process. Finally, it is noteworthy that Arnt fails to promote the XRE binding activity of the ligand-free nt$^-$ dioxin receptor. Since the ligand-free dioxin receptor is characterized by a stable heteromeric complex with hsp90 (52 [and references therein]), it is interesting to consider that hsp90 may prevent Arnt from functionally interacting with the receptor by steric interference or by simply masking a possible dimerization interface.

Arnt forms a stable physical complex with the ligand-activated dioxin receptor in solution. The results of the reconstitution experiments suggest that the positive effect of Arnt on the XRE binding activity of ligand-occupied receptor may be due to direct interactions between these two proteins. To investigate this possibility, we employed the receptor-specific polyclonal antibodies in immunoprecipitation assays. In control experiments the nt$^-$ dioxin receptor present in crude cytosol from C4 mutant hepatoma cells was covalently labeled with the photoaffinity ligand [^{125}I]dioxin. By this procedure the receptor was visualized as an ~95-kDa protein by SDS-PAGE and subsequent autoradiography (Fig. 5A, lane 1), in perfect agreement with the molecular mass determination of the nt$^-$ receptor by immunoblot analysis (Fig. 1A). Thus, these two independent assays confirmed that the nt$^-$ receptor shows wild-type properties with regard to ligand-binding activity and molecular mass. Immunoprecipitation of the covalently labeled material with the receptor-specific antibodies and protein A-Sepharose produced the labeled ~95-kDa ligand-receptor complex (Fig. 5A, lane 2). This complex was not recovered by the receptor-specific antibodies, however, if the labeling reaction of the receptor was performed in the presence of an excess of the nonradioactive high-affinity ligand 2,3,7,8-tetrachlorodibenzofuran (lane 3), nor was it precipitated by the preimmune antibodies (lane 4). Subjecting either the crude input C4 cytosol or the immunoprecipitate produced by the receptor-specific antibodies to SDS-PAGE, followed by transfer to a filter and subsequent immunodetection, provided a single ~95-kDa band. No receptor band was seen by this technique when preimmune serum was used to immunoprecipitate crude C4 cytosol (data not shown).

To examine whether Arnt can physically interact with the ligand-activated dioxin receptor in the absence of DNA, ^{35}S-radiolabeled Arnt was synthesized by in vitro translation and incubated with the ligand-activated, nonradioactive nt$^-$ dioxin receptor. Immunoprecipitation of the receptor by the receptor antibodies revealed that the labeled ~85-kDa Arnt protein coprecipitated with the receptor (Fig. 5B, lane 2). Moreover, the labeled Arnt protein was not detected following immunoprecipitation with control antibodies (lane 3), indicating that Arnt formed a strong complex with the ligand-activated receptor independent of the XRE sequence. To investigate the role of the receptor ligand in this process, we performed similar coimmunoprecipitation experiments employing the ligand-free nt$^-$ receptor and the radiolabeled Arnt protein. Little or no coimmunoprecipitation of Arnt was observed under these conditions (Fig. 5B, lanes 4 and 5), strongly arguing that physical association between Arnt and the receptor is a ligand-dependent event and that the dimerization interface may be masked in the latent receptor form. A strict requirement of ligand for Arnt-receptor interaction was also observed in a precipitation assay employing a biotinylated XRE probe and streptavidin-agarose (data not shown). Upon addition of the ligand-occupied nt$^-$ receptor, it was also possible to specifically shift the sedimentation position of the labeled Arnt protein to the bottom fractions of a sucrose gradient with the receptor-specific receptor antibodies (data not shown). In conclusion, the results obtained by these different experimental approaches clearly indicate that the Arnt and dioxin receptor proteins physically associate with each other in solution, resulting in conversion of the receptor to its DNA binding form. The data further strongly suggest that this interaction is ligand dependent.

The bHLH region of Arnt is required for functional interaction with the dioxin receptor. To study the role of the bHLH motif in Arnt-dioxin receptor interaction we constructed the Arnt deletion mutant ArntΔHLH. As shown schematically in Fig. 6A, the deletion encompasses the entire 56-amino-acid bHLH consensus motif (43) of Arnt. Upon in vitro translation of the deletion mutant mRNA, a [^{35}S]methionine-labeled protein of ~75 kDa versus the ~85-kDa wild-type protein was detected (Fig. 6B, compare lanes 1 and 2), in accordance with the calculated molecular mass of the deletion mutant. DNA binding reconstitution experiments using either the ligand-occupied nt$^-$ receptor or the partially purified, ligand-occupied ~9S form of wild-type receptor demonstrated that deletion of the bHLH domain-containing region of Arnt led to a complete loss of XRE binding (Fig. 6C), indicating that this region of Arnt is required for binding of the receptor to an isolated XRE element. Thus, these results support the notion that the bHLH motif constitutes the dimerization interface between these two regulatory factors.

In transient transfection experiments with an XRE-containing pXRE-MMTV-hGH reporter gene, we observed strong activation in wild-type Hepa 1c1c7 cells in the pres-

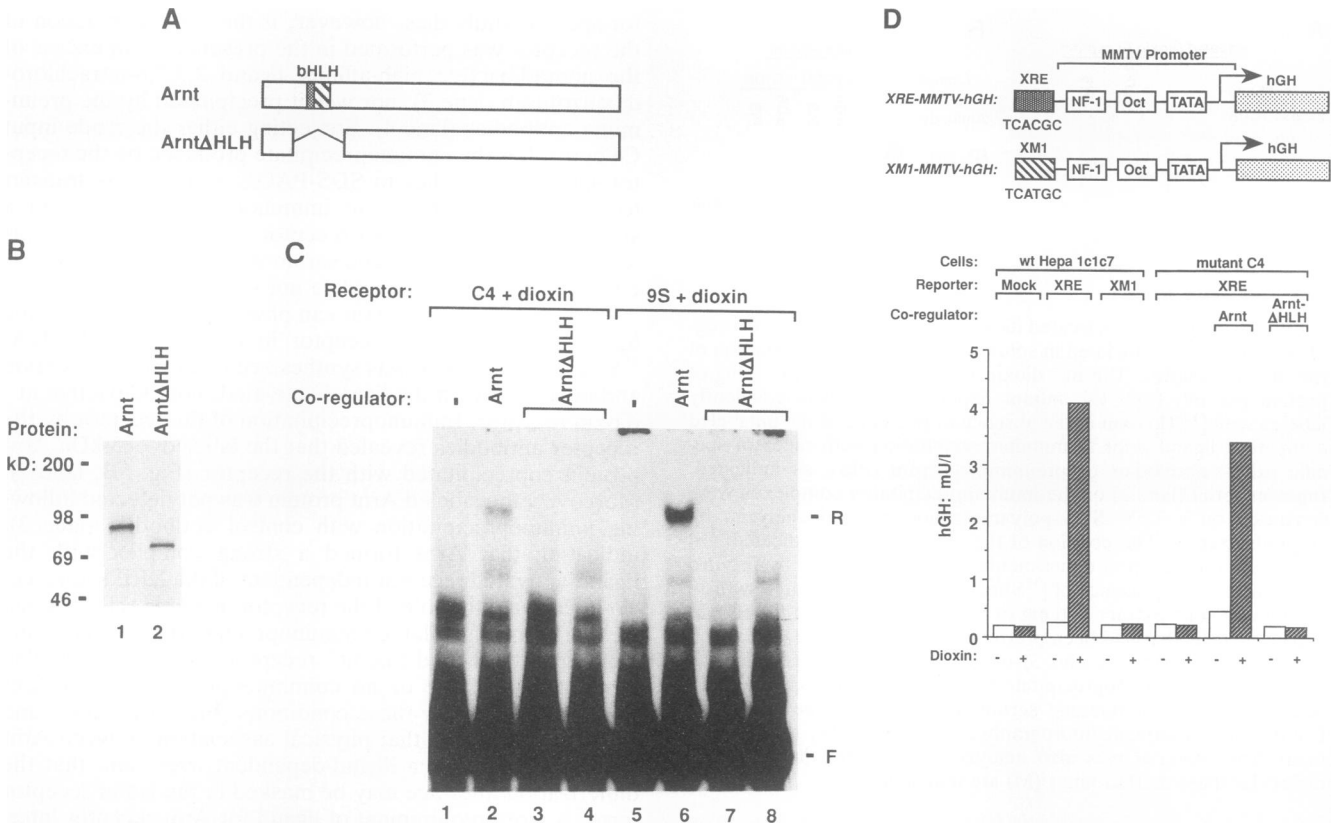

FIG. 6. Arnt requires a region spanning the bHLH domain for functional interaction with the ligand-activated dioxin receptor. Wild-type Arnt or the Arnt deletion mutant ArntΔHLH schematically drawn in panel A was expressed by in vitro translation in reticulocyte lysate. ArntΔHLH lacks amino acids 65 to 195 spanning the entire bHLH region. (B) [^{35}S]methionine-labeled, in vitro-translated products were analyzed by SDS-PAGE and fluorography as indicated. (C) In vitro-translated Arnt (10 μl) or ArntΔHLH (lanes 3 and 7, 1 μl; lanes 4 and 8, 10 μl) was mixed with either 5 μl of dioxin-labeled receptor in cytosolic extracts from Arnt-deficient, mutant C4 cells (lanes 1 to 4) or with 5 μl of the dioxin-occupied, partially purified ~9S form of wild-type receptor (lanes 5 to 8). The reaction mixtures were then analyzed for DNA binding activity by the gel mobility shift assay with a labeled XRE probe. The positions of the receptor complex (R) and the free probe (F) are indicated. (D) The induction of an XRE-driven reporter construct by dioxin is dependent on the Arnt bHLH region. The mouse mammary tumor virus (MMTV)-hGH reporter constructs contain single copies of wild-type or point-mutated (XM1) XRE elements in front of the mouse mammary tumor virus promoter. Wild-type Hepa 1c1c7 or mutant C4 cells were transiently transfected with the reporter constructs in the absence or presence of Arnt or ArntΔHLH expression vectors, as indicated. Transfected cells were treated with no receptor ligand (open bars) or with 10 nM dioxin (hatched bars). Results of a representative experiment are shown. Secreted growth hormone levels were assayed by a radioimmunoassay.

ence of dioxin, whereas the reporter construct showed very low basal activity in the absence of receptor ligand (Fig. 6D). A single point mutation of the core XRE hexamer motif (shown schematically in Fig. 3B) did not only abrogate binding of the receptor in vitro (10) (Fig. 3C) but also completely abolished dioxin induction of the reporter gene (Fig. 6D). Furthermore, these data demonstrate that the induction response was mediated by the XRE sequence. Importantly, the wild-type reporter gene was not activated in the presence of dioxin following transient transfection of the Arnt-deficient, mutant C4 cells (Fig. 6D). Cotransfection experiments using the wild-type Arnt expression vector, however, produced a significant dioxin induction response. In the absence of dioxin, transfection of C4 cells with the Arnt expression vector slightly enhanced the basal activity of the reporter. In contrast, neither the increase in basal activity nor that in dioxin inducibility of the reporter was observed upon cotransfection with the ArntΔHLH deletion mutant expression vector. Taken together, these data strongly suggest that the region spanning the bHLH region of Arnt is critical for functional interaction with the dioxin receptor. Finally, these results demonstrated that the functional interaction between Arnt and the receptor required the presence of the receptor ligand.

DISCUSSION

We have shown that the dioxin receptor, a regulatory protein carrying the bHLH motif, requires an auxiliary factor indistinguishable from the bHLH factor Arnt to recognize dioxin response elements in vitro. Reconstitution experiments using extracts from Arnt-deficient, mutant hepatoma cells as a receptor source demonstrated that Arnt exerted its function as a coregulator of receptor activity via the bHLH dimerization motif only in the presence of the ligand-activated receptor and not the in the presence of the ligand-free receptor. Furthermore, we have shown that the receptor and Arnt physically associated with each other in solution and that the receptor ligand provided the signal triggering this interaction. Thus, the Arnt coregulator failed to both functionally and physically interact with the ligand-free receptor form which is characterized by a stable asso-

ciation with hsp90. Therefore, this model provides the first example of signal-regulated dimerization of bHLH factors.

Regulation of dioxin receptor function by Arnt. Although Arnt has been implicated in the regulation of nuclear translocation of the dioxin receptor (30), the mechanism by which it modulates dioxin receptor function has hitherto been unclear. The present experiments demonstrate that both the ligand-activated dioxin receptor and Arnt individually exhibited poor, if any, intrinsic XRE binding activity. The formation of a heteromeric protein complex in vitro between these two factors, however, dramatically increased the binding affinity of the complex for the XRE target sequence. On the basis of these criteria, Arnt is not only a physical partner of the receptor but also functions as a critical coregulator protein serving to target the receptor to its DNA response element.

Given the background that the nuclear dioxin receptor has a native molecular mass of approximately 200 kDa (26, 55), the Arnt-receptor complex most probably represents a heterodimer. It is formally possible, however, that this complex may harbor additional, low-molecular-weight components. Once the XRE binding heteromeric complex has been formed, it appears that both the receptor and Arnt are able to interact directly with the XRE target sequence, since UV DNA cross-linking experiments have indicated the specific binding of both an ~110 and ~100-kDa protein, respectively, to an XRE probe following dioxin activation (14). Interestingly, however, the ~100-kDa component (presumably Arnt) is only detected at late times and at very high energies of UV irradiation (14, 61, 62). These data suggest that, within the ligand-activated, heteromeric complex, Arnt may exhibit a significantly lower affinity than the receptor protein for the XRE target sequence.

Deletion experiments indicated that the region containing the bHLH region of Arnt was critical for XRE binding and/or functional interaction with the dioxin receptor. Among proteins in the growing family of factors bearing the bHLH motif, bHLH-mediated heterodimerization processes appear to constitute a critical mechanism of regulation of the DNA binding activity of these factors. For instance, c-Myc (which contains a bHLH region contiguous with a leucine zipper motif) has to heterodimerize with the structurally related Max factor to efficiently bind E box (CACGTG)-related DNA sequences (5, 6, 31, 54). In similar fashion, the bHLH protein E12 appears to be a functional and physical partner protein of the bHLH myogenic factor MyoD in the activation of muscle-specific gene expression. While the E12 factor itself shows low affinity for E box sequences, the binding activity of the heterodimeric complex is greatly enhanced (35, 42, 56). Finally, the bHLH factor TFEB binds to a related sequence motif as a heterodimeric complex with the bHLH factor TFE3 (17). Thus, a strikingly distinct and restricted pattern of heterodimerization has been observed among these factors, even though they are capable of binding to very similar, if not identical, target DNA sequences in vitro. It may be noteworthy that the core of the conserved motif (GCGTGA [see reference 49 for a review]) in several dioxin response elements bears some similarity to the E box motif (see the underlined sequence). However, the Arnt-receptor complex does not bind the E box, nor does the in vitro-expressed bHLH factor USF (which strongly binds to the E box motif and lacks a known dimerization partner protein [23]) affect the XRE binding activity of the receptor (data not shown).

Mechanism of activation of the dioxin receptor. Dioxin receptor activity appears to be highly controlled by a complex pattern of protein-protein interactions resulting in either negative or positive regulation. Although the mechanism of activation of the dioxin receptor remains obscure, it is clear that the decision to convert from a latent into a functional receptor form is strictly governed by the presence of receptor ligand (10). A striking feature of the latent dioxin receptor is that it is stably associated with hsp90 in a large, ~300-kDa complex (11, 48, 61). In analogy to the glucocorticoid receptor (12 [and references therein]), ligand-induced activation of the DNA binding activity of the dioxin receptor involves the release of hsp90 (52, 61). These observations can be interpreted to suggest that hsp90 passively inhibits Arnt from interacting with the receptor simply by steric hindrance, resulting in inhibition of the XRE binding function. As detailed in the model in Fig. 7, this mode of repression is readily reversed upon binding of ligand. Alternatively, hsp90 maintains the ligand-free receptor polypeptide in a specific conformation, in which the dimerization interface with Arnt may be masked. In support of the latter scheme, we have recently demonstrated that hsp90 maintains and stabilizes the dioxin receptor in a ligand binding conformation, whereas the hsp90-free form shows no detectable dioxin binding activity in vitro (52). Conversely, artificially induced release of hsp90 results in derepression of the receptor, manifested in constitutive XRE binding activity (52). In support of this model, it has recently been shown that purified hsp90 chaperones protein folding in vitro (60). Given the background that Arnt-deficient hepatoma cells are characterized by a nuclear translocation-deficient dioxin receptor phenotype in vivo (30 [and references therein]), these data raise the interesting possibility, that, in the absence of ligand, hsp90 maintains the receptor in a specific, repressed conformation that precludes DNA binding, nuclear localization, or both receptor activities, by preventing Arnt from interacting with the receptor. We are currently attempting to examine this hypothesis in closer detail.

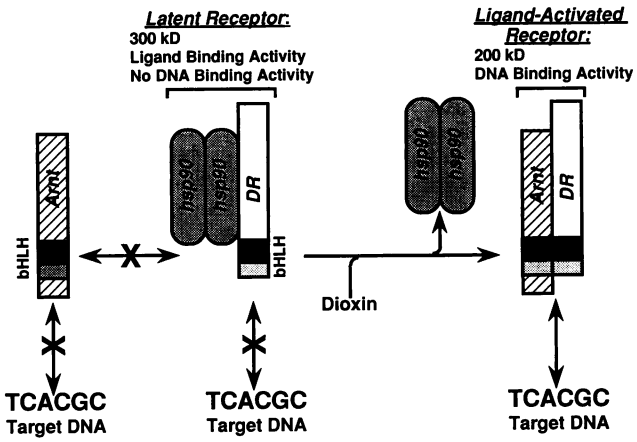

FIG. 7. Hypothetical model for ligand-dependent activation of the dioxin receptor into a DNA binding form. The ligand binding, but non-DNA binding ~300-kDa form of dioxin receptor (DR) is stably associated with hsp90 (represented as a putative dimer) and is defined as the latent (inactive) receptor form. Ligand-induced activation of the receptor into the ~200-kDa DNA binding, nuclear form appears to involve the following critical steps: (i) binding of ligand (dioxin), (ii) release of hsp90, and (iii) subsequent interaction with the Arnt coregulator. The bHLH motifs of the receptor and Arnt are indicated. In our model, the receptor is derepressed simply by dissociation of hsp90, resulting in interaction with Arnt. It is also conceivable that the ligand induces a specific receptor conformation that is critical for dimerization with Arnt. See text for details.

Common functional properties of coregulators of ligand-activated nuclear receptor proteins. Although both the dioxin receptor and Arnt are bHLH factors, there are striking functional similarities to the mechanism of gene regulation by members of the steroid receptor (here referred to as zinc finger receptors) superfamily. Most importantly, the retinoid X receptor (RXR) appears to function as a critical coregulator of a broad number of zinc finger receptors including the retinoic acid, thyroid hormone, vitamin D, and peroxisome proliferator-activated receptors. In a fashion similar to that of Arnt, RXR seems to enhance or modulate the DNA binding activity in vitro of partner zinc finger receptors by the formation of a heterodimeric complex (7, 21, 24, 32, 33, 36, 39, 64, 65). Since many of the partner receptors of RXR (such as the thyroid hormone receptor) are constitutive nuclear proteins (see reference 3 for a review), it is presently unclear whether RXR plays any role in the nuclear translocation process of its partner receptors. In the case of the dioxin receptor system it will be of interest to examine whether Arnt associates with the receptor in the cytoplasmic or nuclear compartment of target cells. Clearly, there existed a 4-5S cytosolic activity that functionally corresponded to Arnt with regard to promoting the DNA binding activity of the dioxin receptor in vitro. While there is only indirect evidence that the Arnt coregulator may be important for nuclear import of the ligand-activated dioxin receptor (30 [and references therein]), it has been established that distinct cytosolic factors are required for the two critical steps during nuclear translocation processes: targeting of the nuclear envelope and import through the nuclear pore (41 [and references therein]).

Role of ligand in the interaction of nuclear receptors with partner factors. One important feature of the dioxin receptor system appears to be that the receptor strictly required ligand for functional interaction with the Arnt coregulator. Moreover, the level of receptor-dependent XRE binding activity that was reconstituted by addition of Arnt to the receptor closely mirrored the dose and receptor affinity of the employed ligand. This mechanism of regulation of receptor activity has so far not been observed with regard to the effect of the RXR coregulator on zinc finger receptor function. However, the recent observation that the 9-*cis* retinoic acid (a ligand of RXR [29, 37]) induces homodimer formation of RXR (66) opens the interesting possibility that the ligand plays a more central role than previously anticipated in regulating the formation of homo- or heteromeric zinc finger receptor complexes.

Finally, by analogy to the regulation of RXR function by 9-*cis* retinoic acid, one may consider the possibility that the Arnt protein could bind a natural ligand and thus represent a novel class of bHLH orphan receptors. This therefore leads to the intriguing question of whether the interaction of the receptor and Arnt in fact reflects the physical convergence of two distinct signal transduction pathways. Clearly, however, Arnt does not bind dioxin (30), nor did dioxin treatment induce any Arnt-dependent XRE binding activity (data not shown).

Implications for the dioxin signal transduction process. Combinatorial regulation of dioxin receptor activity by protein-protein interactions with hsp90 and the Arnt coregulator may create the basis for a very complex and cell-type-specific pattern of modulation of receptor function. For instance, given the complex regulatory networks of other bHLH factors, it is an interesting possibility that the receptor, Arnt, or both proteins interact with additional, as-yet-unidentified dimerization partners in target cells. Such putative partners may or may not be structurally related to Arnt and the receptor. For instance, the *Drosophila* neural regulator *sim* is structurally related to the dioxin receptor and Arnt (8, 15, 30, 43), implying that putative partner proteins may be found in developmental regulatory pathways. Under conditions in which Arnt or the receptor is limiting, such patterns of protein-protein interactions may result in differential regulation of the receptor and possibly novel phenomena of cross-coupling of distinct regulatory pathways with the dioxin signalling process. In the case of the zinc finger receptor family, a number of its members have been reported to be negatively regulated by interaction with the c-Fos/c-Jun complex belonging to the leucine zipper family of DNA binding proteins (reviewed in references 40 and 58). Moreover, the bHLH factor MyoD is similarly repressed by direct interaction with c-Jun but not c-Fos (4). Finally, the DNA-binding activity of the dioxin receptor appears to be mediated by protein kinase C-dependent phosphorylation processes (4a, 9, 46, 53).

The efficiency of the putative Arnt-receptor heterodimer formation may constitute a crucial step in regulation of receptor activity. Interestingly, the amount of added Arnt protein was clearly critical for efficient in vitro promotion of the DNA binding activity of the receptor (data not shown), indicating that a threshold level had to be surpassed for the functional effect to occur. These preliminary observations raise the interesting possibility that the level of expression of Arnt (or the pool of Arnt available to dimerize with the receptor) may be limiting for the receptor following ligand activation and may thus provide an important mechanism for determining cellular sensitivity and responsiveness to dioxins.

ACKNOWLEDGMENTS

M.W. and I.P. are equal first authors. We thank Oliver Hankinson (UCLA) for providing the full-length Arnt cDNA clone and wild-type and C4 mutant Hepa 1c1c7 cells and Åsa Andersson and Kristina Sjöblom for excellent technical assistance. We also appreciate the comments from Anna Berghard, Anders Berkenstam, and Martin Göttlicher on the manuscript.

M.W. was supported by a fellowship from the Swedish Cancer Society. This work was supported by grants from the Swedish Cancer Society.

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