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Distinct Roles of the Molecular Chaperone hsp90 in Modulating Dioxin Receptor Function via the Basic Helix-Loop-Helix and PAS Domains

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The intracellular dioxin receptor mediates signal transduction by dioxin and functions as a ligand-activated transcription factor. It contains a basic helix-loop-helix (bHLH) motif contiguous with a Per-Arnt-Sim (PAS) homology region. In extracts from nonstimulated cells the receptor is recovered in an inducible cytoplasmic form associated with the 90-kDa heat shock protein (hsp90), a molecular chaperone. We have reconstituted ligand-dependent activation of the receptor to a DNA-binding form by using the dioxin receptor and its bHLH-PAS partner factor Arnt expressed by in vitro translation in reticulocyte lysate. Deletion of the PAS domain of the receptor resulted in constitutive dimerization with Arnt. In contrast, this receptor mutant showed low levels of xenobiotic response element-binding activity, indicating that the PAS domain may be important for DNA-binding affinity and/or specificity of the receptor. It was not possible to reconstitute dioxin receptor function with proteins expressed in wheat germ lysate. In line with these observations, reticulocyte lysate but not wheat germ lysate promoted the association of de novo synthesized dioxin receptor with hsp90. At least two distinct domains of the receptor mediated interaction with hsp90: the ligand-binding domain located within the PAS region and, surprisingly, the bHLH domain. Whereas ligand-binding activity correlated with association with hsp90, bHLH-hsp90 interaction appeared to be important for DNA-binding activity but not for dimerization of the receptor. Several distinct roles for hsp90 in modulating dioxin receptor function are therefore likely: correct folding of the ligand-binding domain, interference with Arnt heterodimerization, and folding of a DNA-binding conformation of the bHLH domain. Thus, the dioxin receptor system provides a complex and interesting model of the regulation of transcription factors by hsp90.

The intracellular dioxin receptor (also termed the aryl hydrocarbon receptor) is a ubiquitous basic helix-loop-helix (bHLH) factor (4, 10) that mediates signal transduction by the toxic environmental contaminant dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) (for recent reviews, see references 33, 42, and 47). In the absence of ligand the receptor exists in an inducible cytoplasmic form. Dioxin induces nuclear translocation of the receptor (reference 34 and references therein) and regulates dimerization with the bHLH partner factor Arnt, enabling both proteins to specifically recognize cognate response elements (xenobiotic response elements [XREs]) within regulated genes (9, 24, 38, 46). Individually, neither the receptor nor Arnt shows any detectable affinity for this target sequence (23, 46). Moreover, they do not appear to bind the twofold symmetric CACGTG or CAGCTG E box motifs (23, 46) that are recognized by the great majority of bHLH and bHLH-leucine zipper (bHLH-Zip) proteins, including lymphoid transcription factors, the oncoproteins Myc and Max, and factors involved in vertebrate myogenesis and *Drosophila* neurogenesis (for recent reviews, see references 20 and 21). Structural analysis of the protein-DNA complexes generated by a homodimer of the bHLH-Zip factors Max and USF has demonstrated E box recognition by the basic region and the formation of a parallel four-helix bundle by the two helices in the HLH domain (11, 12).

In the absence of ligand, the dioxin receptor is stably associated with the 90-kDa heat shock protein hsp90 (29, 50), which in certain model systems has been demonstrated to act as a molecular chaperone (48, 26). Although the role of hsp90 in dioxin receptor function remains unclear, it appears that hsp90 both blocks receptor-Arnt dimerization (46), resulting in repression of receptor function, and chaperones a high-affinity ligand-binding conformation of the receptor (35). Ligand-induced receptor activation involves release of hsp90 and concomitant unmasking of the dimerization and DNA-binding activities of the receptor (35, 46, 50). Ligand-binding (9, 45) and hsp90-binding (45) activities of the receptor are colocalized within the C-terminal half of the PAS domain, a region with homology to Arnt and the *Drosophila* factors Per and Sim that is contiguous with the bHLH motif of the receptor (reviewed in reference 43). Interestingly, the PAS domain has recently been reported to mediate homodimerization processes of the *Drosophila* factor Per (19), which, in contrast to the dioxin receptor, Arnt, and Sim, does not contain a bHLH motif.

In our efforts to understand the mechanism of activation of the dioxin receptor and to further examine the functional architecture of the receptor, we have reconstituted ligand-dependent DNA and dimerization activities of the receptor by using receptor and Arnt proteins expressed in vitro in reticulocyte lysate. Deletion of the PAS domain of the receptor resulted in constitutive dimerization with the bHLH partner factor Arnt.

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However, this receptor mutant showed low levels of XRE-binding activity, suggesting that the PAS domain may be important for the DNA-binding specificity of the receptor. Interestingly, it was not possible to reconstitute dioxin receptor function with proteins translated in wheat germ lysate. Although Arnt was functional in reconstitution experiments upon expression in wheat germ lysate, receptor expressed in this translation system failed to bind ligand or dimerize with Arnt and bind target DNA. In correlation with these observations, the dioxin receptor was associated with hsp90 upon expression in rabbit reticulocyte lysate but not when it was expressed in wheat germ lysate, indicating a role for hsp90 in the folding of a functional receptor form. We have previously observed association of hsp90 with the ligand-binding domain that is located within the C-terminal half of the PAS region of the dioxin receptor (45). In the present studies we have found that hsp90 also physically interacts with the bHLH motif of the dioxin receptor, an event that appears to be important for conversion of the bHLH domain into a DNA-binding conformation but not for dimerization processes.

MATERIALS AND METHODS

Plasmid constructions. Plasmids used to translate full-length dioxin receptor (pDR/ATG/BS) and Arnt (pGEM/Arnt) have previously been described (25, 46). For construction of plasmid DRΔPAS/GEM, fragments of plasmid pSportAhR (4) were amplified by PCR with primers designed to provide segments containing codons 1 to 82 (bHLH) and 340 to 805 (C terminus) of the murine dioxin receptor gene. Inclusion of *Cla*I and *Xho*I restriction sites in the primers used to amplify the bHLH coding sequence allowed facile digestion and subcloning into *Cla*I-*Xho*I-digested pGEM7Zf(+) (Promega), thus yielding pDRbHLH/GEM. Inclusion of *Xho*I restriction sites flanking primers used to amplify the C-terminal coding sequence allowed subcloning of this fragment into *Xho*I-digested pDRbHLH/GEM, yielding pDRΔPAS/GEM. A *Not*I-*Xba*I fragment from pDR/ATG/BS was subcloned into *Not*I-*Xba*I-digested pDRΔPAS/GEM, thus replacing most of the PCR-derived C terminus with original cDNA sequence. pDRΔPAS codes for the extra amino acids A, R, and G at the *Xho*I linker. For construction of pDRbHLH/Arnt, plasmid pBM5Neo M1-1 (17) was amplified by PCR with primers designed to provide codons 128 to 774 of the human Arnt cDNA. Inclusion of *Xho*I sites flanking the coding sequence allowed facile digestion and subcloning into a pGEM7 shuttle vector, replacement of much of the PCR-derived sequence with a *Cel*II-*Xba*I fragment from pBM5Neo M1-1, and then subsequent *Xho*I digestion and subcloning of the Arnt C-terminal fragment into *Xho*I-digested pDRbHLH/GEM, thus yielding pDRbHLH/Arnt. This plasmid encodes the extra amino acids A, R, and V at the *Xho*I linker. The fidelity of all remaining PCR-derived segments of these plasmids was verified by dideoxy sequencing.

Cells and extract preparation. Mutant, dioxin-resistant Hepa-1 c4 or Hepa-1 c12 cells (15) were grown in minimal essential medium supplemented with 10% fetal calf serum, 100 U of penicillin, and 100 mg of streptomycin (GIBCO-BRL) per ml as previously described (50). When near confluency, the cells were harvested and homogenized in TEG buffer (20 mM Tris-HCl [pH 7.4], 1 mM EDTA, 10% [wt/vol] glycerol, 1 mM dithiothreitol) and centrifuged at 120,000 × *g* for 45 min. The resulting supernatant was either used as a cytosolic extract (typical protein concentration, ~5 mg/ml) immediately or stored at −70°C until required.

In vitro expression of proteins. Wild-type or mutant dioxin receptor forms or Arnt were expressed by in vitro transcription and translation of the corresponding plasmids in rabbit reticulocyte lysate or wheat germ lysate (Promega) under conditions recommended by the manufacturers. The bHLH factors E12 and E47 were expressed by in vitro translation in reticulocyte lysate following transcription of the plasmids E12R and E47S, respectively (27). For immunoprecipitation, labeled proteins were generated by in vitro translation in the presence of [^{35}S]methionine (New England Nuclear).

DNA and ligand-binding assays. In vitro-translated dioxin receptor or dioxin receptor in crude cytosolic cell extracts was treated in the presence or absence of 10 nM dioxin for 2 to 3 h at 25°C. Specific ligand-binding activity of in vitro-translated proteins was determined as previously described (25), by using [^{3}H]dioxin (40 Ci/mmol; Chemsyn) as a ligand, with the exception that the washing steps were carried out in the presence of 0.5% Tween 20. The DNA-binding activity of the dioxin receptor was monitored by a gel mobility shift assay performed essentially as described previously (16, 28). Briefly, DNA-binding reaction mixtures were assembled with the indicated in vitro-translated proteins or cellular extracts in 10 mM HEPES, (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9)–5% (vol/vol) glycerol–0.5 mM dithiothreitol–2.5 mM $MgCl_2$–1 mM ethylene diaminetetraacetic acid–0.08 (wt/vol) Ficoll at a final concentration of 50 mM NaCl and in a final volume ranging between 30 and 50 μl. A 36-bp ^{32}P-3′-end-labeled, double-stranded oligonucleotide XRE (5), spanning the dioxin-responsive XRE1 element of the rat cytochrome P-4501A1 promoter region (13), was added to the reaction mixtures as a specific probe in the presence of 1 μg of poly(dI-dC) (Pharmacia) nonspecific competitor DNA. After addition of the specific probe, the reaction mixtures were incubated for 20 min at 25°C. Protein-DNA complexes were then immediately analyzed on a 4% (acrylamide/bisacrylamide ratio of 29:1) low-ionic-strength native polyacrylamide gel at 30 mA, by using a Tris-glycine-EDTA buffer (16).

Antibodies and immunoprecipitation experiments. For coimmunoprecipitation experiments, wild-type or mutant dioxin receptor or Arnt was labeled by in vitro translation in the presence of [^{35}S]methionine prior to addition to the unlabeled, in vitro-translated partner factor. Protein mixtures were incubated in a total volume of 20 μl with or without 10 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) at 25°C for 2 h, in the presence of 1 mg each of the protease inhibitors aprotinin and leupeptin per ml. The samples were then precleared with preimmune serum (10 μl) for 15 min; this step was followed by the addition of 50 μl of a 50% slurry of protein A-Sepharose (Pharmacia) in TEG-150 buffer (TEG buffer containing 150 mM NaCl and 0.1% Triton X-100), incubation for 15 min under gentle shaking, and centrifugation. Supernatants were then incubated with either dioxin receptor antiserum (46) or Arnt antiserum (23) for 30 min at room temperature to coimmunoprecipitate the heterologous dimerization partner with protein A-Sepharose as described above. The resin was washed three times with TEG buffer. To reduce nonspecific protein adsorption, protein A-Sepharose had been equilibrated in TEG buffer containing 1 mg of bovine serum albumin per ml prior to use. Immunoprecipitated proteins were separated through a sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel. For fluorography, gels were soaked in 1 M salicylic acid (Sigma) for 20 min, dried and exposed to film, and/or analyzed by phosphoimaging (Fuji). Association of in vitro-translated wild-type or mutant dioxin receptor with hsp90 was analyzed in an hsp90 coimmunoprecipitation assay that was performed as recently described (25, 45) by using either the monoclonal anti-hsp90 immunoglobulin M (IgM) antibody 3G3 (30) (Affinity Bioreagents) or an equal concentration of the control mouse IgM antibody TEPC 183 (Sigma). In the experiment presented in Fig. 4C, the precipitated material was further analyzed by immunoblotting by using the monoclonal anti-hsp90 antibody 29A (49).

RESULTS

In vitro reconstitution of dioxin-dependent activation of the dioxin receptor-Arnt complex. In initial reconstitution experiments, we analyzed the ability of the dioxin receptor and Arnt to bind DNA by using proteins expressed in vitro in rabbit reticulocyte lysate. As expected (9, 46), the expressed proteins did not recognize the XRE target sequence individually, as assessed by gel mobility shift analysis (Fig. 1A, lanes 2 to 4). Moreover, in the absence of one another, in vitro-expressed factors did not bind to DNA, even following dioxin treatment in vitro (compare lanes 2 to 4 in Fig. 1A). In contrast to previously reported findings (9), in vitro-translated dioxin receptor was stably repressed in the absence of ligand treatment, resulting in no detectable levels of XRE-binding activity upon the addition of in vitro-translated Arnt (Fig. 1A, lane 5). In the presence of dioxin, however, a protein-XRE complex was generated by the in vitro-translated dioxin receptor and Arnt (Fig. 1A, lane 6). Specific polyclonal antibodies against the dioxin receptor (2, 46) or Arnt (23) were used in DNA-binding inhibition or protein complex supershift experiments to confirm that the dioxin-induced XRE complex harbored both the dioxin receptor and Arnt (Fig. 1B). Thus, the DNA-binding function of the in vitro-translated dioxin receptor was strictly ligand dependent. In agreement with this observation, the dioxin receptor shows specific high-affinity dioxin-binding activity in vitro following expression in reticulocyte lysate (9, 25) (see Fig. 4D).

Differences in functional activities between dioxin receptor and Arnt following expression in reticulocyte versus wheat germ lysates. We next compared in vitro translation of both the dioxin receptor and Arnt in rabbit reticulocyte lysate and wheat germ lysate. As assessed by [^{35}S]methionine labeling and SDS-polyacrylamide gel electrophoresis (PAGE) analysis, both factors were routinely expressed as intact proteins in wheat germ lysate at levels (approximately 50 to 80%) that were roughly comparable to those generated in reticulocyte

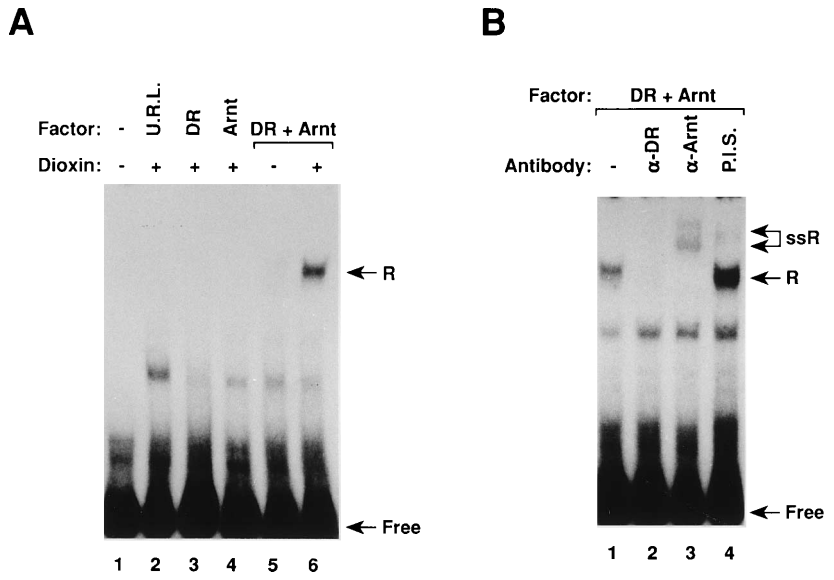

FIG. 1. In vitro reconstitution of ligand-inducible DNA-binding activity by the dioxin receptor and Arnt expressed in reticulocyte lysate. (A) Aliquots of unprogrammed reticulocyte lysate (10 μl; lane 2) or translation mixtures containing dioxin receptor (10 μl; lane 3), Arnt (15 μl; lane 4), or dioxin receptor (10 μl) plus Arnt (15 μl) (lanes 5 and 6) were incubated with 10 nM dioxin or vehicle alone (0.1% dimethyl sulfoxide) for 2 h at 25°C; this step was followed by gel mobility shift assay with a ^{32}P-labeled XRE oligonucleotide probe. Lane 1 shows the mobility of the free XRE probe. (B) The ligand-induced, reconstituted XRE-binding activity was analyzed by gel mobility shift assay in the absence or presence of antireceptor (α-DR; lane 2), anti-Arnt (α-Arnt; lane 3), or preimmune (P.I.S.; lane 4) serum. The positions of the free probe (Free), the receptor-containing complex (R), and a supershifted receptor complex (ssR) are indicated. Fast-migrating XRE complexes are the result of nonspecific protein-DNA interaction.

lysate (data not shown). However, it was not possible to reconstitute either constitutive or dioxin-induced XRE-binding activity by the dioxin receptor and Arnt with wheat germ lysate as a protein source (Fig. 2A, lanes 6 to 9). In control reactions, similar levels of dioxin receptor and Arnt expressed in reticulocyte lysate showed strong XRE-binding activity in the presence of dioxin (Fig. 2A, compare lanes 5 and 9).

To examine which protein was functionally deficient upon translation in wheat germ lysate, we attempted to use these proteins to reconstitute the DNA-binding activity of the dioxin receptor by in vitro complementation of extracts from either Arnt- or receptor-deficient hepatoma cells. The ligand-occupied cytosolic dioxin receptor expressed in Arnt-deficient Hepa-1 c4 cells does not exhibit any detectable levels of specific XRE-binding activity in vitro (5) (Fig. 2B, lane 2). Following the addition of increasing concentrations of Arnt expressed in wheat germ lysate, however, the XRE-binding activity of the ligand-stimulated mutant Hepa-1 c4 receptor was reconstituted in a dose-dependent manner (Fig. 2B, compare lanes 5 to 8). As demonstrated with specific antibodies, the reconstituted XRE complex harbored both the dioxin receptor and Arnt (Fig. 2C). Conversely, dioxin receptor expressed in wheat germ lysate was used to functionally complement a cytosolic extract from dioxin receptor-deficient Hepa-1 c12 cells. Even in the presence of high concentrations of in vitro-translated dioxin receptor, no receptor-dependent XRE-binding activity was detected (Fig. 2D). In control experiments, the dioxin receptor was translated in vitro in reticulocyte lysate prior to its addition to the crude receptor-deficient Hepa-1 c12 extract. Whereas no receptor-dependent XRE-binding activity was observed when using the dioxin-treated extract or reticulocyte lysate-expressed receptor alone, this form of receptor strongly promoted XRE-binding activity following its addition to the extract (Fig. 2E, compare lanes 2 to 6), which is consistent with the strong cooperativity in DNA binding between the dioxin receptor and Arnt (23).

The wheat germ lysate-expressed dioxin receptor form fails to dimerize with the Arnt partner factor. In excellent agreement with the failure of the ligand-free dioxin receptor expressed in reticulocyte lysate to show XRE-binding activity following incubation with Arnt (Fig. 1A), this form of receptor did not dimerize with Arnt in coimmunoprecipitation experiments using specific antibodies against Arnt (Fig. 3A, lane 1). In the presence of dioxin, however, the receptor formed a stable physical complex with Arnt, resulting in coimmunoprecipitation with Arnt antibodies of the [^{35}S]methionine-labeled, in vitro-translated dioxin receptor (Fig. 3A, compare lanes 1 and 2). In contrast, similar levels of dioxin receptor produced in wheat germ lysate did not physically interact with Arnt expressed in either wheat germ lysate (Fig. 3B) or reticulocyte lysate (data not shown).

The dioxin receptor expressed in wheat germ lysate is not associated with the molecular chaperone hsp90. To monitor physical interaction in solution between de novo synthesized dioxin receptor and the molecular chaperone hsp90, we employed in coimmunoprecipitation experiments a monoclonal IgM antibody, 3G3, that is capable of specifically recognizing either free hsp90 or hsp90 complexed with other proteins (30). These antibodies specifically coimmunoprecipitate the dioxin receptor expressed in reticulocyte lysate (25) (Fig. 4A, compare lanes 1 and 2), demonstrating stable association of the receptor with hsp90, but fail to precipitate the dioxin receptor form expressed by in vitro translation in wheat germ lysate. In the case of the dioxin receptor form expressed in wheat germ lysate, only low levels of nonspecific interaction were observed with the anti-hsp90 antibody compared with that observed with an irrelevant IgM monoclonal antibody (Fig. 4B, compare lanes 1 and 2). Thus, the dioxin receptor failed to become associated with the wheat germ hsp90 homolog. In control experiments, material precipitated by the 3G3 antibodies from either reticulocyte or wheat germ lysate was analyzed in immunoblot experiments by using a distinct anti-hsp90 antibody,

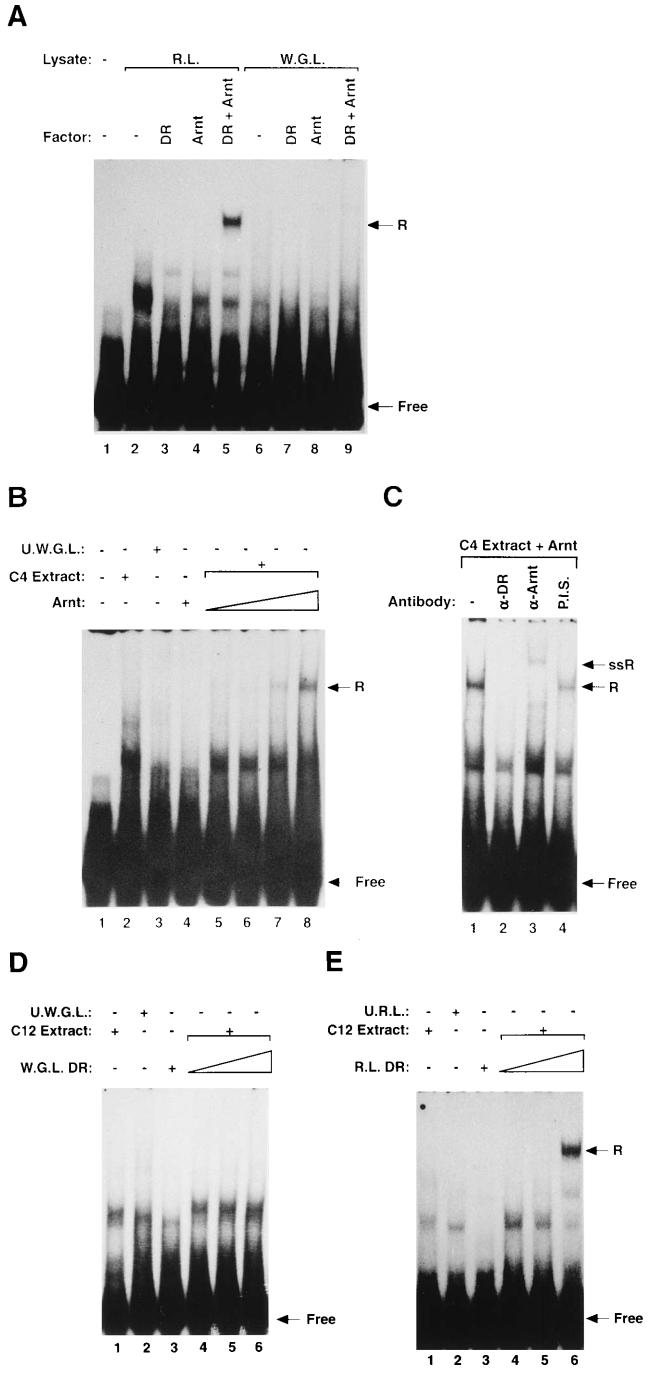

FIG. 2. Differences in XRE-binding activity between dioxin receptor (DR) and Arnt following expression in reticulocyte lysate versus wheat germ lysate. (A) Aliquots of dioxin receptor (10 μl) and Arnt (15 μl) expressed in reticulocyte lysate (lanes 3 to 5) or wheat germ lysate (lanes 7 to 9) were incubated with 10 nM dioxin for 2 h at 25°C; this step was followed by gel mobility shift analysis with a ^{32}P-labeled XRE probe. The positions of the receptor complex (R) and free probe (Free) are indicated. (B) The mutant Arnt-deficient Hepa-1 c4 cell extract (10 μl; 4 mg of protein per ml) was incubated in the absence (lane 2) or the presence of increasing concentrations (7.5 to 20 μl) of Arnt expressed in wheat germ lysate (lanes 5 to 8). The mixtures were incubated with 10 nM dioxin for 2 h at 25°C before XRE-binding activity was monitored by gel mobility shift analysis. The XRE-binding activity of 15 μl of unprogrammed wheat germ lysate (U.W.G.L.) and the highest concentration of Arnt alone are shown in lanes 3 and 4, respectively. Lane 1 shows the mobility of the free XRE probe. (C) Specificity of the reconstituted XRE complex was assessed by antibody experiments. The c4 extract was coincubated with Arnt expressed in wheat germ lysate (15 μl) in the presence of 10 nM dioxin for 2 h at 25°C, in the absence (lane 1) or presence of antireceptor (α-DR; lane 2), anti-Arnt (α-Arnt; lane 3) or preimmune (P.I.S.; lane 4) serum. The positions of the free probe, the receptor-containing complex (R), and a supershifted receptor complex (ssR) are indicated. (D) The mutant receptor-deficient Hepa-1 c12 cell extract (10 μl; 4 mg of protein per ml) was incubated in the absence (lane 1) or presence of increasing concentrations (5 to 15 μl) of the dioxin receptor expressed in wheat germ lysate (W.G.L. DR; lanes 4 to 6). After treatment with 10 nM dioxin (for 2 h at 25°C), XRE-binding activity was analyzed by gel mobility shift analysis. XRE-binding activities by unprogrammed wheat germ lysate (U.W.G.L.) (15 μL) and the highest concentration of dioxin receptor are shown in lanes 2 and 3, respectively. The position of the free probe is indicated. (E) The gel mobility shift protocol mentioned in the legend to panel D was repeated with dioxin receptor expressed in reticulocyte lysate (R.L. DR). The positions of the free probe and the receptor-containing complex (R) are indicated. U.R.L., unprogrammed reticulocyte lysate.

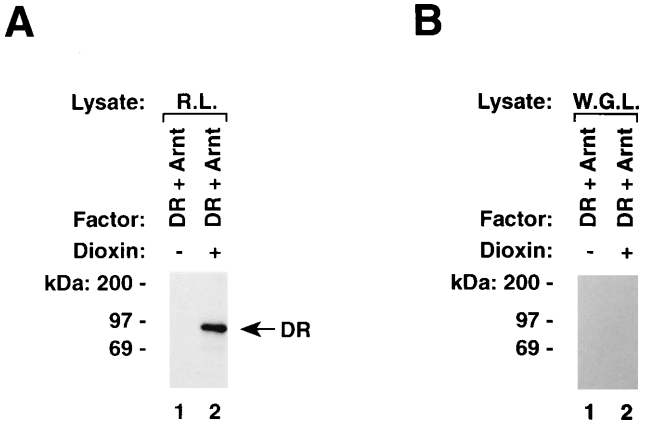

FIG. 3. Dimerization between the dioxin receptor (DR) and Arnt can be reconstituted in reticulocyte lysate (R.L.) but not wheat germ lysate (W.G.L.). (A) Aliquots of Arnt (10 μl) and ^{35}S-methionine-labeled dioxin receptor (10 μl) translated in reticulocyte lysate were incubated in the absence (lane 1) or presence (lane 2) of 10 nM dioxin for 2 h at 25°C; this step was followed by immunoprecipitation with anti-Arnt polyclonal antiserum. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by fluorography. (B) The immunoprecipitation protocol described in the legend to panel A was repeated with ^{35}S-methionine-labeled dioxin receptor (10 μl) expressed in wheat germ lysate. The positions of coimmunoprecipitated dioxin receptor and of molecular mass standard proteins are indicated.

29A (49). This analysis demonstrated that similar levels of rabbit or wheat hsp90 were precipitated by the 3G3 antibodies (Fig. 4C). Given the background that the dioxin receptor present in hepatoma cells shows no dioxin-binding activity in vitro following salt- and temperature-induced disruption of the receptor-hsp90 complex (35), we were curious to examine the ligand-binding activity of the wheat germ lysate-expressed dioxin receptor form. In contrast to the dioxin receptor expressed in reticulocyte lysate (25) but consistent with its inability to generate a stable complex with hsp90, dioxin receptor expressed in wheat germ lysate did not show any detectable levels of [^{3}H]dioxin-binding activity in vitro (Fig. 4D). In conclusion, the dioxin receptor was defective in three critical functional properties upon in vitro translation in wheat germ lysate compared with that in reticulocyte lysate: (i) binding of ligand, (ii) association with hsp90, and (iii) dimerization with its bHLH partner factor Arnt and, consequently, DNA binding.

Role of the bHLH and PAS domains in DNA recognition by the dioxin receptor. The carboxy-terminal half of the PAS domain of the receptor harbors both ligand-binding (9, 45) and hsp90-binding (45) activities. Moreover, the PAS domain motif has recently been reported to represent a dimerization motif within the *Drosophila* non-bHLH factor Per (19). To study PAS function in our reconstituted system with reticulocyte

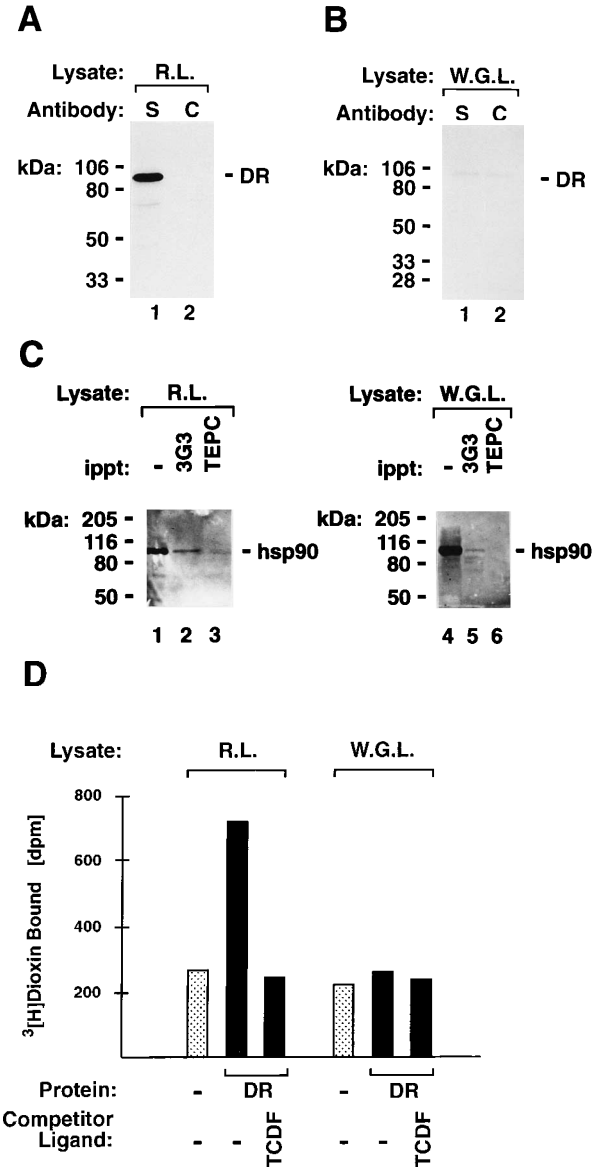

FIG. 4. Dioxin receptor (DR) expressed in reticulocyte lysate but not wheat germ lysate is associated with hsp90. The dioxin receptor was translated in reticulocyte lysate (R.L.) (A) or wheat germ lysate (W.G.L.) (B) containing ^{35}S-methionine. Aliquots of the labeled receptor (10 μl) were incubated with anti-hsp90 antibody 3G3 (5 μl) or control IgM ascites fluid which had been preadsorbed to a resin of goat anti-mouse IgM–Sepharose. After extensive washing, immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography. Lanes 1 and 2 show the dioxin receptor coimmunoprecipitated by the hsp90-specific antibody (S) and the control IgM antibody (C), respectively. The positions of precipitated dioxin receptor and of the molecular mass standard proteins are indicated. 3G3 or irrelevant TEPC monoclonal antibodies were used to precipitate material from unprogrammed reticulocyte lysate (R.L.) or wheat germ lysate (W.G.L.), as indicated (C). The precipitates was separated by SDS-PAGE and analyzed by immunoblotting with the monoclonal antibody 29A. Lanes 1 and 4 show nonprecipitated, crude lysates. (D) Ligand-binding experiments. Unprogrammed reticulocyte lysate (stippled bars) or reticulocyte lysate containing in vitro-translated dioxin receptor (solid bars) was incubated with 2.5 nM [^{3}H]dioxin for 90 min at 25°C; this step was followed by adsorption to hydroxylapatite at 4°C for 30 min. After extensive washing, bound [^{3}H]dioxin was determined by scintillation counting. In control experiments, the specificity in the binding reaction was assessed by performing the reaction in the presence of a 150-fold molar excess of tetrachlorodibenzofuran (TCDF). Results of a representative experiment are shown. R.L., reticulocyte lysate; W.G.L., wheat germ lysate.

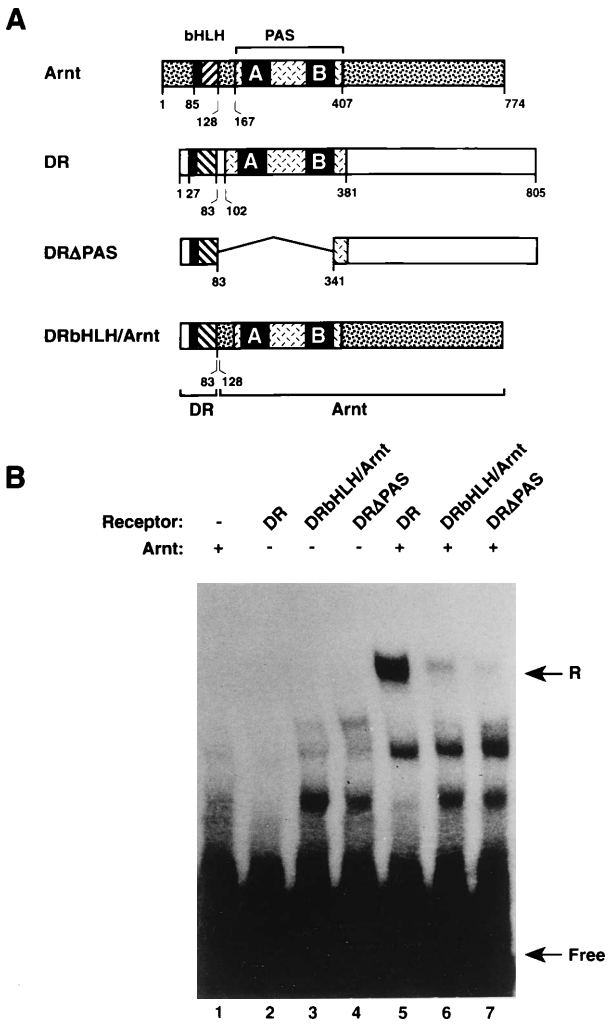

FIG. 5. Both the bHLH and PAS domains are required for strong binding of the dioxin receptor to the XRE. (A) Schematic structures of Arnt, wild-type dioxin receptor (DR), a dioxin receptor deletion mutant lacking almost the entire PAS motif (DRΔPAS), and a swap mutant in which the bHLH domain of Arnt has been substituted with that of the dioxin receptor (DRbHLH/Arnt). Codon numbers are indicated. (B) Gel mobility shift assays were performed according to the protocol outlined in the legend to Fig. 1 with dioxin-treated, reticulocyte lysate-expressed Arnt (10 μl; lane 1), dioxin receptor (DR) (10 μl; lane 2), swap mutant DRbHLH/Arnt (10 μl; lane 3), deletion mutant DRΔPAS (10 μl; lane 4), or mixtures of Arnt and the dioxin receptor (10 μl each; lane 5), Arnt and DRbHLH/Arnt (10 μl each; lane 6), and Arnt and DRΔPAS (10 μl each; lane 7). The positions of the free probe and the receptor-containing complex (R) are indicated. XRE complexes of faster mobility represent nonspecific protein-DNA interactions.

lysate-expressed proteins, we compared the XRE-binding activity of the wild-type dioxin receptor to that of the dioxin receptor deletion mutant DRΔPAS (shown schematically in Fig. 5A) lacking almost the entire PAS motif. Moreover, we have previously shown that the bHLH motif is critical for dioxin receptor-Arnt interaction. Thus, deletion of the bHLH domain of Arnt abrogates heterodimerization with the ligand-activated dioxin receptor in vitro (23, 46) and functional interaction in vivo (46). We were therefore interested in studying the specificity in the bHLH-mediated dimerization process by substituting the bHLH domain of Arnt with that of the dioxin receptor, creating the swap mutant DRbHLH/Arnt (Fig. 5A).

DRΔPAS and DRbHLH/Arnt proteins were expressed in

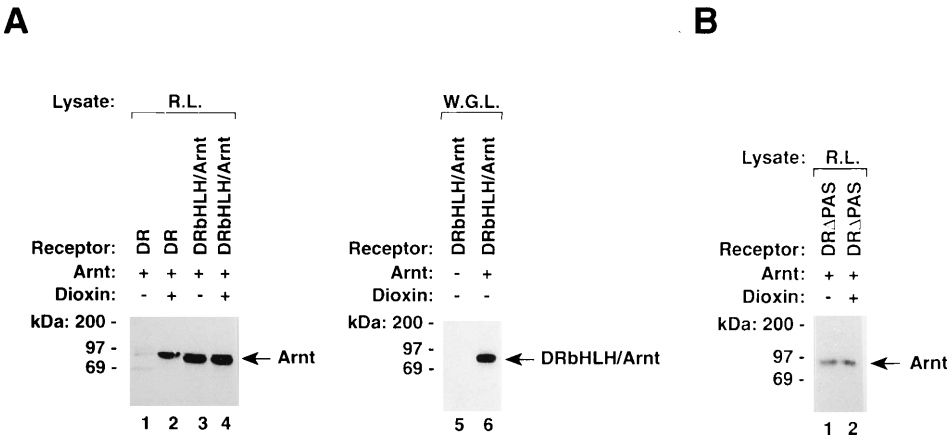

FIG. 6. Dimerization between Arnt and the dioxin receptor deletion mutant DRΔPAS or swap mutant DRbHLH/Arnt is ligand independent. (A) Arnt expressed in reticulocyte lysate (R.L.) in the presence of ^{35}S-methionine (10 μl) was incubated with the wild-type dioxin receptor (DR) (10 μl; lanes 1 and 2) or DRbHLH/Arnt (10 μl; lanes 3 and 4) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 10 nM dioxin (for 2 h at 25°C). Following immunoprecipitation with anti-dioxin receptor antibodies, immunoprecipitated proteins were visualized by SDS-PAGE and fluorography. The position of coprecipitated Arnt is indicated. The ^{35}S-methionine-labeled swap mutant DRbHLH/Arnt expressed in wheat germ lysate (W.G.L.) (10 μl) was incubated in the absence (lane 5) or presence (lane 6) of reticulocyte lysate-expressed Arnt and coimmunoprecipitated with anti-Arnt antibodies directed to the N terminus of Arnt. The positions of the coprecipitated swap mutant DRbHLH/Arnt and of the molecular mass standard proteins are indicated. (B) Reticulocyte lysate-expressed, ^{35}S-methionine-labeled Arnt (10 μl) was incubated with reticulocyte lysate (R.L.)-expressed DRΔPAS (10 μl) in the absence (lane 1) or presence (lane 2) of 10 nM dioxin (for 2 h at 25°C) and immunoprecipitated with anti-dioxin receptor antibodies. The positions of coprecipitated Arnt and of the molecular mass standard proteins are indicated.

reticulocyte lysate at levels that were very similar to if not slightly exceeding those of the wild-type dioxin receptor (data not shown). DNA binding analysis in the presence of Arnt and dioxin demonstrated that deletion of the PAS domain of the dioxin receptor led to a significant loss of XRE-binding activity, as compared with that of the wild-type receptor (Fig. 5B, compare lanes 5 and 7). In a similar fashion, substitution of the bHLH domain of Arnt with the corresponding domain of the dioxin receptor resulted in low levels of XRE-binding activity by DRbHLH/Arnt upon the addition of wild-type Arnt and dioxin (Fig. 5B, compare lanes 3, 5, and 6). Analogous to the case of the wild-type receptor, the DRΔPAS mutant did not exhibit any XRE-binding activity alone but required the presence of the Arnt partner factor to promote low but significant levels of XRE complex formation (Fig. 5B, compare lanes 4 and 7). Consistent with the low levels of XRE-binding activity in vitro of these heterodimeric complexes, expression in transient cotransfection experiments of Arnt together with either DRΔPAS or DRbHLH/Arnt did not significantly increase the low levels of dioxin induction of a minimal XRE-driven reporter gene that were produced by endogenous dioxin receptor and Arnt in HeLa cells (data not shown). In contrast, cotransfection of these cells with expression vectors encoding the wild-type dioxin receptor and Arnt results in strongly enhanced, ligand-inducible reporter gene activity (23).

The low levels of in vitro DNA-binding activity produced by DRΔPAS together with Arnt are consistent with the notion that the PAS domain may be important for the stability of the dioxin receptor-Arnt heterodimeric complex. By analogy, the low levels of DNA-binding activity of DRbHLH/Arnt in the presence of wild-type Arnt may also be due to an impaired dimerization efficiency. To address this issue, we performed coimmunoprecipitation experiments with [^{35}S]methionine-labeled proteins expressed in reticulocyte lysate. Interestingly, the bHLH swap mutant DRbHLH/Arnt showed very strong dimerization with labeled wild-type Arnt (Fig. 6A), as determined by coimmunoprecipitation with antibodies directed against an epitope located immediately N terminally in relation to the bHLH motif of the dioxin receptor (2, 46). In fact, very similar amounts of Arnt were coimmunoprecipitated by these antibodies upon exposure to either the ligand-stimulated wild-type dioxin receptor or the DRbHLH/Arnt mutant (Fig. 6A, compare lanes 2 to 4). However, whereas dimerization of Arnt with the wild-type dioxin receptor was dioxin inducible (Fig. 6A, lanes 1 and 2), physical interaction of Arnt with DRbHLH/Arnt was constitutive (Fig. 6A, compare lanes 3 and 4). Moreover, Arnt showed bona fide levels of dimerization with the bHLH swap mutant DRbHLH/Arnt following expression in wheat germ lysate. In agreement with deletion of the ligand-binding domain, the dimerization activity of DRΔPAS was ligand independent (Fig. 6B, compare lanes 1 and 2).

In the cases of both DRbHLH/Arnt and DRΔPAS, these results suggest that the low levels of XRE-binding activity produced by these proteins may not be due to an impaired stability of the heterodimeric complexes that were generated with wild-type Arnt. Deletion of the PAS domain or substitution of the bHLH motif appears rather to result in an altered DNA-binding affinity and/or specificity of the heterodimeric complexes. It is striking that Arnt (17) contains the Arg, His, and Glu residues that were shown in X-ray crystallographic studies to recognize specific bases in the E box target sequence for the bHLH-Zip factors Max and USF (11, 12), whereas only the Arg residue is conserved in the dioxin receptor (4, 10). Importantly, however, the DRΔPAS and the DRbHLH/Arnt mutants did not recognize the E box motifs μE3 and κE2 of immunoglobulin enhancers either individually or when coincubated with Arnt or other bHLH factors, such as E12 (data not shown). It will therefore be interesting to use expressed minimal hetero- and homodimeric complexes of the bHLH and bHLH-PAS domains of the dioxin receptor and Arnt in target site selection experiments in order to define optimal recognition motifs for this class of mutants.

Evidence that the bHLH domain of the dioxin receptor may be a target for the molecular chaperone hsp90. Given the striking differences in functional activities of the wild-type dioxin receptor following expression in reticulocyte versus wheat germ lysate and the correlation to interaction with hsp90 (see above), we next examined functional properties of the

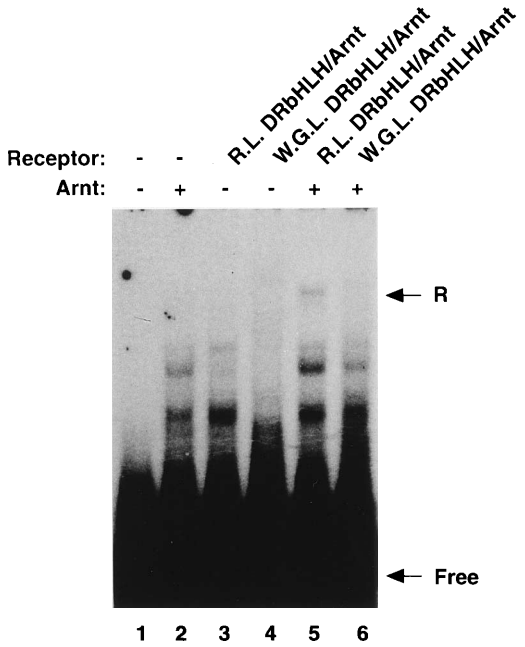

FIG. 7. The DRbHLH/Arnt swap mutant expressed in wheat germ lysate does not show XRE-binding activity. Gel mobility shift assays were performed with the ^{32}P-labeled XRE probe in the presence of Arnt expressed in reticulocyte lysate (10 μl; lane 2), the swap mutant DRbHLH/Arnt expressed in reticulocyte lysate (15 μl; lane 3) or in wheat germ lysate (15 μl; lane 4), a mixture of Arnt and DRbHLH/Arnt expressed in reticulocyte lysate (15 μl each; lane 5), and a mixture of Arnt expressed in reticulocyte lysate and DRbHLH/Arnt expressed in wheat germ lysate (15 μl each; lane 6). All reactions were incubated with 10 nM dioxin for 2 h at 25°C prior to gel shift analysis. Lane 1 shows the mobility of the free XRE probe. The positions of the reconstituted receptor-containing complex (R) and the free probe are indicated.

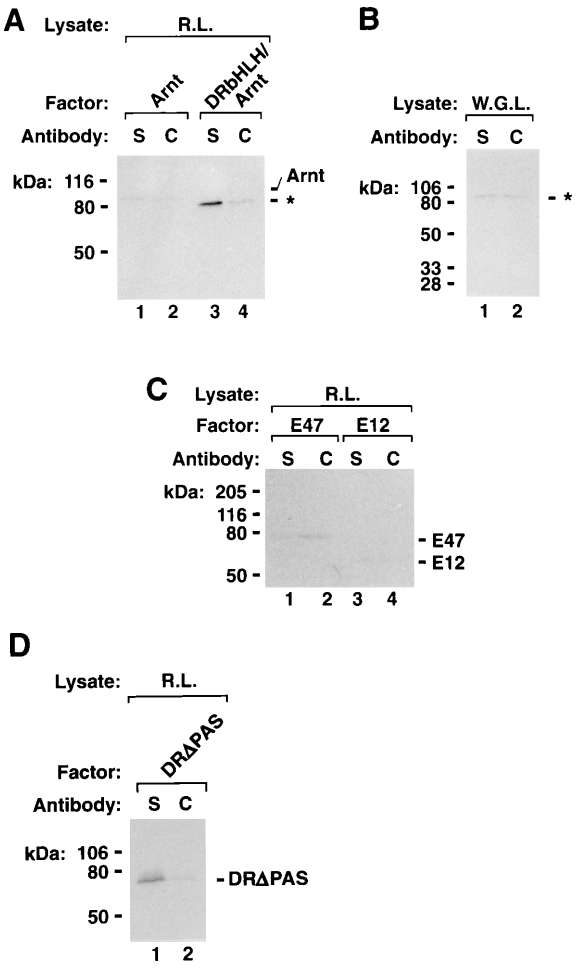

FIG. 8. Association of hsp90 with the bHLH motif of the dioxin receptor. Reticulocyte lysate (R.L.)-expressed, ^{35}S-methionine-labeled Arnt and DRbHLH/Arnt (A); E12 or E47 (C); DRΔPAS (D); or wheat germ lysate (W.G.L.)-expressed DRbHLH/Arnt (B) was immunoprecipitated with hsp90 specific antibody as described in the legend to Fig. 4. Lanes 2 and 4 show proteins immunoprecipitated by the control IgM antibody (C), while lanes 1 and 3 show proteins specifically coimmunoprecipitated with hsp90 (S). The positions of the in vitro-translated proteins are indicated. Asterisks indicate precipitated DRbHLH/Arnt. The positions of the molecular mass standards are also indicated.

DRΔPAS mutant expressed in wheat germ lysate. As observed with the wild-type dioxin receptor, this mutant receptor form did not exhibit any XRE-binding activity upon in vitro translation in wheat germ lysate (data not shown). In contrast, both reticulocyte lysate- and wheat germ lysate-expressed forms of the bHLH swap mutant DRbHLH/Arnt formed a strong constitutive complex with Arnt in coimmunoprecipitation assays (Fig. 6A, compare lane 4 with lanes 5 and 6). However, although wheat germ lysate-expressed DRbHLH/Arnt dimerized efficiently with wild-type Arnt, it did not show any detectable levels of XRE-binding activity in the absence or presence of Arnt in gel mobility shift assays (Fig. 7). In control reactions, reticulocyte lysate-expressed DRbHLH/Arnt together with Arnt generated low but significant levels of XRE-binding activity (Fig. 7, compare lanes 5 and 6). Thus, although the HLH dimerization motif of the dioxin receptor was functional when expressed in wheat germ lysate, this expression system did not appear to promote folding of the heterodimerized complex into a conformation that actively binds DNA.

The difference in XRE-binding activity between reticulocyte lysate- and wheat germ lysate-expressed forms of DRbHLH/Arnt prompted us to examine the abilities of these forms to associate with hsp90. It is noteworthy that the bHLH domains of the MyoD and E12/E47 transcription factors have been proposed to be conformationally modulated by hsp90. However, this process appears to involve transient interaction with hsp90, since it has not been possible to document any stable complexes between these proteins and hsp90 (39, 40). In coimmunoprecipitation experiments, [^{35}S]methionine-labeled, reticulocyte lysate-expressed wild-type Arnt is not precipitated by hsp90 antibodies (25) (Fig. 8A, compare lanes 1 and 2). Remarkably, however, labeled DRbHLH/Arnt was specifically immunoprecipitated by the hsp90 antibodies upon expression in reticulocyte lysate (Fig. 8A, compare lanes 3 and 4), indicating that the bHLH domain of the dioxin receptor mediates the formation of a stable complex with hsp90. In contrast, wheat germ lysate-expressed DRbHLH/Arnt was not associated with hsp90, as assessed by the hsp90 coimmunoprecipitation assay (Fig. 8B). Moreover, the hsp90 antibodies did not coimmunoprecipitate the E12 or E47 factors following expression in reticulocyte lysate (Fig. 8C), in agreement with the reported failure to observe any complexes between hsp90 and these factors by different experimental techniques (40). Given the result that wheat germ lysate-expressed, hsp90-free DRbHLH/Arnt maintained strong dimerization activity (Fig. 6A), these data suggest a role for hsp90 in chaperoning a DNA-binding conformation of the bHLH domain of the dioxin receptor. In the case of bHLH-PAS factors, this very function of hsp90 appears to be limited to the dioxin receptor, since

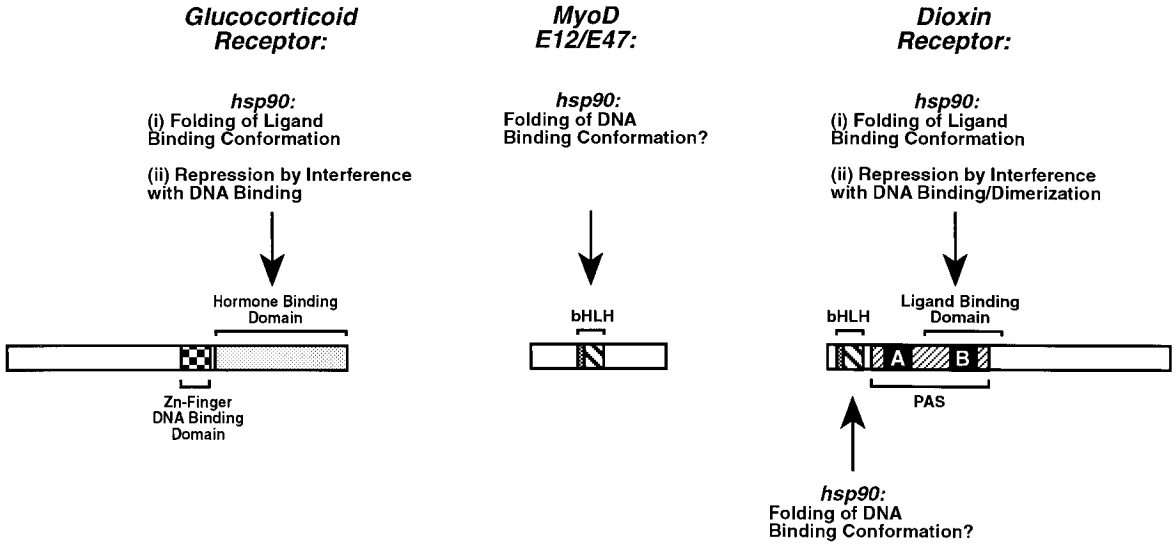

FIG. 9. Model of the different roles of hsp90 in modulating functions of the nuclear glucocorticoid and dioxin receptors and the bHLH factors MyoD and E12/E47. hsp90 interaction with the hormone- and ligand-binding domains of these nuclear receptors is essential for chaperoning a ligand-binding conformation as well as for maintaining them in their latent forms, while hsp90 interaction with the bHLH domains is thought to chaperone the DNA-binding conformation of these domains. See the text for details.

wild-type Arnt is not associated with hsp90 and exhibited heterodimerization and XRE-binding activities when expressed by in vitro translation in reticulocyte and wheat germ lysates.

DISCUSSION

Ligand-dependent activation of the dioxin receptor. The dioxin receptor is a ubiquitous bHLH transcription factor that appears to be confined to the cytoplasmic compartment (reference 34 and references therein) of nonstimulated target cells. In the absence of regulatory signals, the receptor is recovered in cytosolic extracts in a ligand binding-inducible conformation that is associated with the molecular chaperone hsp90 (7, 29). Importantly, ligand-induced derepression of the receptor involves the release of hsp90 (50) and nuclear translocation (34). Thus, ligand-dependent activation appears to distinguish the dioxin receptor from other bHLH factors. Although the bHLH-Zip factor SREBP-1 (51) is regulated by cellular sterol levels and nuclear transport, this activation process has recently been reported to involve proteolytic cleavage of the membrane-bound SREBP-1 precursor form (44).

By using in vitro expression assays, we have reconstituted ligand-dependent activation of the dioxin receptor in vitro and characterized functional domains involved in this process. The present results suggest a complex mechanism for activation of the receptor and the assembly of a functional receptor form.

The PAS domain distinguishes both Arnt and the dioxin receptor from the bHLH and bHLH/Zip classes of transcription factors (for a recent review, see reference 43). In the case of the dioxin receptor, the C-terminal half of this domain constitutes the ligand-binding domain (9, 45) and is associated with hsp90 (45). Interestingly, the corresponding region within the PAS domain of Arnt mediates neither of these activities (17, 25, 37). In contrast to the results of a recent study (9), we observed that our cell-free model system faithfully reconstituted repression of the ligand-free dioxin receptor. Thus, dioxin receptor expressed in vitro in reticulocyte lysate neither bound target DNA nor dimerized with Arnt. Consistent with the notion that PAS-hsp90 interaction is involved in the repression of dioxin receptor-Arnt heterodimerization (46), the fusion of the PAS domain with heterologous DNA-binding and transactivation domains results in the inhibition of these functions (45).

Deletion of the PAS domain, harboring the ligand-binding domain of the dioxin receptor, resulted in constitutive dimerization of the receptor with its partner factor Arnt. In agreement with this observation, deletion of the hormone-binding domain of the glucocorticoid receptor yields a mutant that exhibits constitutive functional activities (14). Remarkably, however, although the dioxin receptor PAS deletion mutant showed significant dimerization activity, the XRE-binding activity of the resulting heterodimeric complex was markedly reduced, as compared with that of the wild-type dioxin receptor-Arnt complex. In a similar fashion, deletion of the Zip motif of the bHLH-Zip factor USF results in a reduction in DNA-binding activity (22). Moreover, the Zip motif has been shown to determine dimerization specificity of bHLH-Zip factors (1, 18), in addition to stabilizing homo- and heterodimeric bHLH-Zip complexes. Our results indicate that deletion of the PAS domain of the receptor does not abrogate its dimerization activity with Arnt. Assuming that the generated heterodimeric complex maintains DNA-binding activity, it could therefore be interesting to examine by target site selection methods whether the PAS deletion mutant exhibits any altered specificity compared with that for the wild-type receptor-Arnt complex. Given the role of the PAS domain in the homodimerization processes of the *Drosophila* factor Per (19), it will also be interesting to perform a more detailed analysis of the importance of the PAS domain within both Arnt and the receptor for the relative stability of oligomeric complexes. Strikingly, the bHLH-Zip domain of USF has been shown to tetramerize in solution, whereas the minimal bHLH domain forms only a dimeric complex (11).

Modulation of dioxin receptor function by the molecular chaperone hsp90. In extracts from nontreated cells, the dioxin receptor is recovered as a complex with the molecular chaperone hsp90 (8, 30, 37, 50). Like the dioxin receptor, the glucocorticoid receptor belongs to a class of inducible tran-

scription factors that is stably associated with hsp90 in its latent, ligand-free form. As summarized in the model in Fig. 9, hsp90 appears to act as a chaperone, maintaining a high-affinity ligand-binding conformation of both the glucocorticoid (see references 36 and 41 for reviews) and dioxin receptors. Consistent with this model, neither the glucocorticoid receptor (6) nor the dioxin receptor is associated with hsp90 or exhibits ligand-binding activity upon expression in wheat germ lysate. Moreover, in strong support of the model mentioned above, the glucocorticoid receptor exhibits reduced ligand responsiveness when expressed in *Saccharomyces cerevisiae* cells containing low levels of hsp90 (31) or mutant forms of hsp90 (3).

It also appears that hsp90 is involved in the repression of both receptors in their ligand-free states. This notion is supported by the observation that fusion of the ligand-binding and hsp90-binding domains of the glucocorticoid receptor (32) or the dioxin receptor (45) inactivates an otherwise constitutive transcription factor in the absence of ligand. It remains unclear, however, whether hsp90, by binding to the ligand-binding domain, passively or actively inhibits function of the target factor (reviewed in reference 3).

hsp90 has also recently been reported to conformationally activate the DNA-binding activity of homodimers of the bHLH factors MyoD and E12 and MyoD/E12 heterodimers. Whereas bacterially expressed MyoD and E12 show bona fide levels of homo- and heterodimerization activities, hsp90 activates their DNA-binding activity in an ATP-independent manner but is not retained in the bHLH protein complex (39, 40). In striking analogy to this model, the DRbHLH/Arnt mutant of the dioxin receptor could heterodimerize with Arnt but not bind DNA or hsp90 upon expression in wheat germ lysate. In contrast, when expressed in reticulocyte lysate, the bHLH domain of the dioxin receptor was associated with hsp90 and showed dimerization and DNA-binding activities. As outlined in Fig. 9, two distinct chaperoning functions of hsp90 in modulating dioxin receptor function are therefore likely: (i) correct folding of the ligand-binding and, possibly, repressed receptor conformations and (ii) folding of a DNA-binding conformation of the bHLH domain. The dioxin receptor system may therefore provide an interesting model for the regulation of transcription factor function by molecular chaperones and for the investigation of the mechanism of action of hsp90. We have recently shown that a cellular factor, possibly Arnt, plays a role in enhancing the release of hsp90 from the ligand-bound receptor (25). It remains to be determined whether factors in addition to hsp90 and Arnt are necessary and sufficient for the two critical steps in the signaling pathway of the dioxin receptor: repression of the ligand-free receptor form and subsequent ligand-dependent activation.

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