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Functional Interference between Hypoxia and Dioxin Signal Transduction Pathways: Competition for Recruitment of the Arnt Transcription Factor

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Hypoxia-inducible factor 1α (HIF-1α) and the intracellular dioxin receptor mediate hypoxia and dioxin signalling, respectively. Both proteins are conditionally regulated basic helix-loop-helix (bHLH) transcription factors that, in addition to the bHLH motif, share a Per-Arnt-Sim (PAS) region of homology and form heterodimeric complexes with the common bHLH/PAS partner factor Arnt. Here we demonstrate that HIF-1α required Arnt for DNA binding in vitro and functional activity in vivo. Both the bHLH and PAS motifs of Arnt were critical for dimerization with HIF-1α. Strikingly, HIF-1α exhibited very high affinity for Arnt in coimmunoprecipitation assays in vitro, resulting in competition with the ligand-activated dioxin receptor for recruitment of Arnt. Consistent with these observations, activation of HIF-1α function in vivo or overexpression of HIF-1α inhibited ligand-dependent induction of DNA binding activity by the dioxin receptor and dioxin receptor function on minimal reporter gene constructs. However, HIF-1α- and dioxin receptor-mediated signalling pathways were not mutually exclusive, since activation of dioxin receptor function did not impair HIF-1α-dependent induction of target gene expression. Both HIF-1α and Arnt mRNAs were expressed constitutively in a large number of human tissues and cell lines, and these steady-state expression levels were not affected by exposure to hypoxia. Thus, HIF-1α may be conditionally regulated by a mechanism that is distinct from induced expression levels, the prevalent model of activation of HIF-1α function. Interestingly, we observed that HIF-1α was associated with the molecular chaperone hsp90. Given the critical role of hsp90 for ligand binding activity and activation of the dioxin receptor, it is therefore possible that HIF-1α is regulated by a similar mechanism, possibly by binding an as yet unknown class of ligands.

Hypoxia-inducible factor 1α (HIF-1α) has recently been reported to mediate transcriptional responses to hypoxia by binding to hypoxia-inducible enhancer motifs (hypoxia response elements [HREs]) of target genes (56). HRE core sequences are asymmetric E-box motifs that have been characterized in the erythropoietin (EPO) gene (50) and genes encoding vascular endothelial growth factor (VEGF) (14, 27) and a number of glycolytic enzymes (13, 14, 27, 49). HIF-1α binds HRE motifs as a heterodimeric complex, termed HIF-1, with the transcription factor Arnt (56). Both HIF-1α and Arnt are members of a novel subclass of the basic helix-loop-helix (bHLH) family of transcription factors. Members of this class of factors are characterized by a bHLH motif contiguous with a region, PAS (Per-Arnt-Sim), which is conserved between the *Drosophila* neuronal cell developmental regulator Sim, the *Drosophila* circadian rhythm regulatory protein Per, the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) (aryl hydrocarbon) receptor, and Arnt (for a recent review, see reference 29). In addition, the *Drosophila* developmental factor trachealess (22, 69) and a putative Down's syndrome-critical factor (11) have recently been shown to belong to the bHLH/PAS family of gene-regulatory factors.

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Whereas Arnt is a nuclear protein (20, 43) that constitutively activates promoters driven by the dyad symmetry CACGTG E-box element (2, 53), the intracellular dioxin receptor functions as a ligand-activated transcription factor that mediates the metabolic, toxic, and possibly carcinogenic effects of polycyclic aromatic hydrocarbons and halogenated aromatic hydrocarbons, most notably dioxin (reviewed in references 17 and 41). Upon exposure to ligand, the dioxin receptor dimerizes with Arnt, enabling both proteins to recognize an asymmetric E-box motif of xenobiotic response elements (XREs) that are distinct from the HRE motif (25, 47, 66).

In the absence of ligand, the dioxin receptor is recovered as a non-DNA-binding protein in cytosolic cellular extracts as a ~300-kDa heteromeric complex stably associated with the molecular chaperone hsp90 (reference 68 and references therein). hsp90 is critical for folding of a ligand-binding conformation of the receptor in vitro (3, 10, 44) and for ligand inducibility in vivo (8, 65). Dioxin and other known ligands induce nuclear translocation of the dioxin receptor (references 23 and 43 and references therein) and regulate dimerization with Arnt (66). In the ligand-induced receptor activation process, release of hsp90 appears to be necessary to unmask functional activities of the receptor protein (66, 68). Ligand (63) and hsp90 (63) binding activities are colocalized within the C-terminal half of the PAS domain of the dioxin receptor. In contrast, Arnt is not associated with hsp90 but may play an active role in release of hsp90 from the ligand-activated dioxin receptor (35, 36). All

known ligands of the dioxin receptor, e.g., the environmental pollutant dioxin and indolo[3,3-b]carbazole derivatives that represent very potent receptor agonists occurring in the diet (24, 46), are of xenobiotic origin. Thus, the existence of a physiological receptor ligand remains an enigma (reviewed in reference 42). Interestingly, however, targeted disruption of the mouse dioxin receptor gene has indicated that the receptor plays an important role in hepatic and possibly lymphoid development (12).

In our efforts to understand the mechanism of regulation of bHLH/PAS transcription factors, we demonstrate here that Arnt is critical for both dioxin receptor- and HIF-1α-mediated signalling pathways. Functionally HIF-1α dominated over the dioxin receptor, resulting in competition for recruitment of Arnt in vitro and negative regulation of dioxin receptor-mediated signalling in hepatoma cells. In contrast to the proposed model of activation of HIF-1α (56), HIF-1α and Arnt mRNA steady-state levels were not induced by exposure to hypoxia. Thus, HIF-1α may be conditionally regulated by a distinct mechanism, possibly by functioning as an orphan receptor binding an as yet unknown class of ligands. In support of this model, HIF-1α was found to be stably associated with the molecular chaperone hsp90 that is critical for ligand binding activity and conditional regulation of the dioxin receptor.

MATERIALS AND METHODS

Recombinant plasmids. Plasmids containing full-length Arnt (pArnt/GEM7) and the Arnt deletion mutants pArntΔbHLH/GEM7, pArntΔb/GEM7, and pArntΔPAS/GEM7 have been previously described (28, 36, 66). pDR/ATG/psp72 was obtained by subcloning an *Xho*I-*Hin*dIII fragment containing full-length murine dioxin receptor cDNA from pDR/ATG/BS (36) into *Xho*I-*Hin*dIII-digested psp72 (Promega). The reporter gene constructs pT81 (38), pTX.Dir (4), and pGLHIF1.3 (25) have been described previously. HIF-1α cDNA (56) was cloned by reverse transcription followed by PCR of total HeLa RNA and subcloned into pBS (Stratagene), generating pHIF-1α/BS. Nucleotide sequence was confirmed by sequencing. pHIF-1α/psp72 was obtained by inserting a *Bam*HI-*Cla*I fragment containing full-length HIF-1α cDNA into *Bam*HI-*Cla*I-digested psp72. pHIF-1α/CMV4 was generated by inserting a *Bam*HI-*Cla*I fragment of pHIF-1α/BS into *Bgl*II-*Cla*I-digested pCMV4 (1). pDR/CMV4, pArnt/CMV4, and pArntΔb/CMV4 have been described previously (28, 33).

Cell culture and treatments. The human hepatoma cell line HepG2 was grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) containing 10% fetal calf serum (Gibco-BRL), 100 μg of streptomycin per ml, 100 IU of penicillin (Gibco-BRL) per ml, and 0.25 μg of amphotericin B (Fungizone; Gibco-BRL) per ml. HeLa cells were cultured in high-glucose DMEM supplemented as described above. Cultures were treated with $CoCl_2$ or 2,3,7,8-tetrachlorodibenzofuran (TCDF; Cambridge Isotope Laboratories), both dissolved in dimethyl sulfoxide (DMSO), whereas control cultures received solvent only, not exceeding a final concentration of 0.1% (vol/vol). Hepa 1c1c7 (Hepa 1) cells and the Arnt-deficient subline Hepa 1C4 (19) were cultured in DMEM with the supplements described above and 1× minimal essential medium containing nonessential amino acids, 2 mM L-glutamine, and 1 mM sodium pyruvate. Oxygen tensions in the incubator were either 140 mm Hg (20% O_2 [vol/vol]; normoxia) or 7 mm Hg (1% O_2 [vol/vol]; hypoxia). Cells were subjected to hypoxic induction at 70 to 80% confluency. HeLaS3 (ATCC CCL-2.2), a subline of HeLa adapted to growth in suspension, was cultured in Ham's F-12 medium (Gibco-BRL) supplemented as described above. Hypoxic induction was achieved by incubation of HeLaS3 cells at a density of 10^7 cells per ml in an IL 237 tonometer (Instrumentation Laboratories) under continuous stirring for 4 h at 37°C, using a gas mixture of either 20% O_2, 5% CO_2, and 75% N_2 (normoxia) or 1% O_2, 5% CO_2, and 94% N_2 (hypoxia), respectively, at a flow rate of 500 ml/min. All chemicals, medium, and growth factors were purchased from Sigma unless stated otherwise.

RNA isolation and Northern (RNA) blot analysis. Total RNA was isolated by acid-phenol extraction as described previously (9), and mRNA levels were assayed by Northern blot analysis using standard procedures (48). cDNAs for fructose-1,6-bisphosphate aldolase A (60), VEGF (59), ribosomal protein L28 (59), EPO (60), mouse dioxin receptor (6), human Arnt (19), P-4501A1 (15), β-actin (Clontech), and HIF-1α were used as probes. The human multiple-tissue RNA blot was obtained from Clontech. ^{32}P-labeled probes were generated by a random priming procedure. RNA filters were prehybridized, hybridized, and washed according to standard procedures (48) prior to autoradiography. Two Arnt RNA species were detected as previously reported (19). The radioactive signals were recorded and quantified with a phosphor imager (Fuji).

Transfection and transient expression assays. HepG2 cells were incubated overnight in DMEM lacking serum with 1.2 μg of cationic liposomes (Lipofectin; Gibco-BRL) per cm^2 and plasmid DNA. The medium was then changed to DMEM supplemented with 10% serum (Gibco-BRL), and the cells were subsequently treated with either 150 μM $CoCl_2$, 50 nM TCDF, or DMSO alone for 36 to 48 h prior to harvest. In indicated experiments, HepG2 cells were transfected with 0.1 μg of pTX.Dir per cm^2 either alone or with 0.2 μg of pCMV/HIF-1α per cm^2. HeLa cells were transfected by a $CaPO_4$ precipitation method (15). The cells were incubated with the DNA-$CaPO_4$ solution for 16 to 20 h prior to treatment with 50 nM TCDF or DMSO alone for an additional 36 to 48 h. HeLa cells were cotransfected with either pTX.Dir (0.1 μg/cm^2) or pGLHIF1.3 (0.2 μg/cm^2) and a combination of pCMV4 expression vectors for the dioxin receptor, HIF-1α, Arnt, and ArntΔb. The luciferase assay was performed as described previously (48), with the modification that the cells were lysed on ice by a brief treatment in a sonicator. Luciferin was purchased from BioThema.

Cell extract preparation and electrophoretic mobility shift assay. Nuclear extracts were prepared from normoxic or hypoxic HeLa and Hepa 1 cells as described previously (25); 4 to 5 μg of nuclear extract was incubated with an EPO HRE probe (25) for 20 min at 4°C in a total volume of 20 μl containing 0.1 μg of sonicated, denatured calf thymus DNA in 10 mM Tris-HCl (pH 7.5)–50 mM KCl–50 mM NaCl–1 mM $MgCl_2$–1 mM EDTA–5 mM dithiothreitol–5% glycerol and separated on 4% polyacrylamide gels at 200 V in TBE buffer (89 mM Tris, 89 mM boric acid, 5 mM EDTA) at 4°C. Human HepG2 cells were treated with 50 nM TCDF in the absence or presence of increasing concentrations of $CoCl_2$ for the indicated period of time. Control cultures received DMSO for 1 h before harvest. Nuclei were prepared and protein was extracted as described previously (54). XRE binding reactions were assembled in a total volume of 20 μl with 10 μg of nuclear protein at final concentrations of 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 0.2 mM EDTA, 75 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin (Bayer) per ml, 10 μg of leupeptin per ml, 5 μM pepstatin (Boehringer), 5% glycerol, 4% Ficoll, 100 μg of poly(dI-dC) (Pharmacia) per ml, and 12.5 μg of poly(dA-dT) (Pharmacia) per ml, using a double-stranded, ^{32}P-labeled oligonucleotide as a probe, the sequence of which corresponded to positions −968 to −997 of the human *CYPIA1* gene (5′-CTCCGGTCCTTCTCACGCAA CGCCTGGGCA-3′, sense orientation). In competition experiments, a 100-fold molar excess of unlabeled oligonucleotide (unspecific competitor described in reference 15) was used. DNA-protein complexes were separated under nondenaturing conditions on a 4% polyacrylamide gel (29:1) run in 1× TGE buffer (50 mM Tris, 2.7 mM EDTA, 380 mM glycine) at 4°C.

In vitro protein expression and coimmunoprecipitation experiments. Full-length HIF-1α, dioxin receptor, and Arnt and the indicated Arnt deletion mutants were translated in vitro either in the presence or in the absence of [^{35}S]methionine in rabbit reticulocyte lysate as recommended by the manufacturer (Promega). [^{35}S]methionine-labeled in vitro-synthesized products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. Analysis of unlabeled in vitro translation products was determined by simultaneous incubation of an aliquot of the reaction in the presence of [^{35}S]methionine followed by SDS-PAGE and fluorography. Radiolabeled bands were analyzed on a phosphor imager (Fuji); when required, equal protein concentrations for competition and comparative coimmunoprecipitation experiments were calculated on the basis of incorporated [^{35}S]methionine. Arnt coimmunoprecipitation experiments were carried out as previously described (35). Briefly, [^{35}S]methionine-labeled in vitro-synthesized full-length HIF-1α or the dioxin receptor was incubated with equal concentrations of unlabeled in vitro synthesized Arnt or the Arnt deletion mutants as indicated in the figure legends. Reaction volumes were corrected with blank translation mix, and incubation was carried out at either 25°C for 2 h or 4°C overnight in the presence of 5 μg of each of the protease inhibitors aprotinin, leupeptin, and pepstatin per ml and 1 mM phenylmethylsulfonyl fluoride. Protein mixtures were precleared by incubation with 10 μl of preimmune serum on ice for 15 min followed by a further 15-min incubation with 50 μl of a 50% slurry of protein A-Sepharose in TEG buffer (20 mM Tris-HCl [pH 7.4], 1 mM EDTA, 10% [wt/vol] glycerol, 1 mM dithiothreitol) containing 150 mM NaCl, 0.2% Triton X-100, and 1 mg of bovine serum albumin per ml. Following rapid centrifugation, the resulting supernatants were incubated with 10 μl of Arnt-specific antiserum (33) for 1 h at room temperature, 100 μl of protein A-Sepharose (50% slurry as described above) was then added, and the reaction mixtures were incubated on ice for 45 min. After rapid centrifugation, the resulting Sepharose pellets were washed twice with 500 ml of supplemented TEG buffer and once with buffer minus bovine serum albumin. Coimmunoprecipitated proteins were analyzed by SDS-PAGE and fluorography. hsp90 coimmunoprecipitation was also carried out as previously described (35, 36). Briefly, [^{35}S]methionine-labeled in vitro-synthesized full-length HIF-1α was incubated with Sepharose containing preadsorbed hsp90-specific monoclonal immunoglobulin M antibody 3G3 (Affinity Bioreagents) or an equal concentration of control mouse immunoglobulin M antibody TEPC 183 (Sigma) in MENG buffer (25 mM morpholinepropanesulfonic acid [pH 7.5], 1 mM EDTA, 0.02% NaN_3, 10% glycerol) containing 20 mM molybdate, 2 mM dithiothreitol, and 2.5% (wt/vol) bovine serum albumin on ice for 90 min. After centrifugation, the resulting Sepharose pellets were washed four times with 1-ml aliquots of the above-described immunoprecipitation buffer without bovine serum albumin, and the coimmunoprecipitated products were analyzed by SDS-PAGE and fluorography.

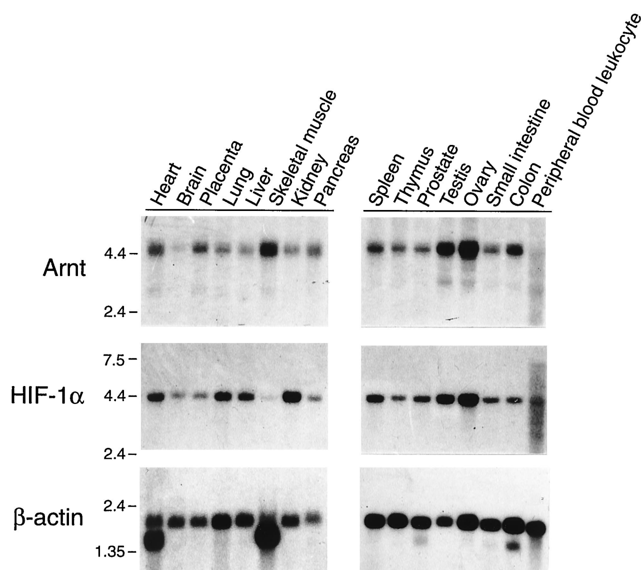

FIG. 1. Distribution of HIF-1α and Arnt mRNAs in human tissues. Total RNA from the indicated human tissues was used in RNA blotting experiments. The same filter was subsequently hybridized with HIF-1α and Arnt cDNA probes following ^{32}P labeling by random priming. For a loading control, the filter was analyzed for β-actin mRNA expression.

RESULTS

Ubiquitous and constitutive expression of HIF-1α and Arnt in human tissues. In rodents, the dioxin receptor and Arnt appear to be ubiquitously expressed transcription factors that show a largely coordinated manner of expression, as assessed by dioxin receptor ligand binding assays and analysis of dioxin receptor and Arnt mRNA expression levels (reference 7 and references therein). In the case of HIF-1α, it has been reported that hypoxia induces expression in human Hep3B cells of HIF-1α steady-state mRNA levels from very low background values (56). We therefore wished to examine constitutive expression levels of HIF-1α mRNA in a variety of human tissue samples and to compare these expression levels with those of Arnt, the partner factor of HIF-1α. In RNA blot experiments, we observed that Arnt mRNA was expressed in all tissues examined. Relative to β-actin control mRNA levels, the highest Arnt mRNA expression levels were detected in the testis, pancreas, ovary, and placenta, whereas low relative expression levels were found in total brain RNA (Fig. 1). In analogy to these results, HIF-1α mRNA was constitutively expressed in all tissues examined, with the highest relative expression levels in the kidney, testis, and ovary. Moreover, liver, pancreas, and lung tissues also showed high levels of HIF-1α mRNA expression. Thus, in contrast to human Hep3B cells (56), both Arnt and HIF-1α mRNAs were constitutively expressed at significant levels in human tissues, demonstrating a largely coordinated expression pattern in a number of these tissues, e.g., testis, ovary, and pancreas. A notable exception to this expression pattern was found for skeletal muscle, where HIF-1α mRNA levels were lower than Arnt mRNA levels (Fig. 1).

Arnt is critical for HIF-1α function. HIF-1α has been reported to recognize HREs of target promoters as a heterodimeric complex with Arnt (56). To characterize the mechanism of signal transduction by HIF-1α, we initially examined hypoxic regulation of target gene expression in wild-type Hepa 1 hepatoma cells and a subline thereof, Hepa 1C4, that expresses functionally deficient Arnt (19). Consequently, in the mutant Hepa 1C4 cells, the dioxin receptor cannot be converted into an XRE-binding form although it exhibits bona fide ligand binding properties (47, 66). In wild-type Hepa 1 cells, exposure to low oxygen resulted in about fourfold induction of VEGF mRNA levels, detected at 4 h after exposure, and a twofold up-regulation of aldolase A mRNA levels following 16 h of exposure, as assessed by RNA blotting experiments (Fig. 2A). Both the VEGF and aldolase A genes have been reported to be regulated under hypoxic conditions by the HIF-1α–Arnt complex via HRE motifs (49). In the case of the VEGF gene, hypoxic induction has been reported to involve mRNA stabilization (21, 26, 51), in addition to activation of transcription by HIF-1α (27, 30, 49). In contrast to wild-type Hepa 1 cells, exposure of the mutant Hepa 1C4 cells to hypoxia

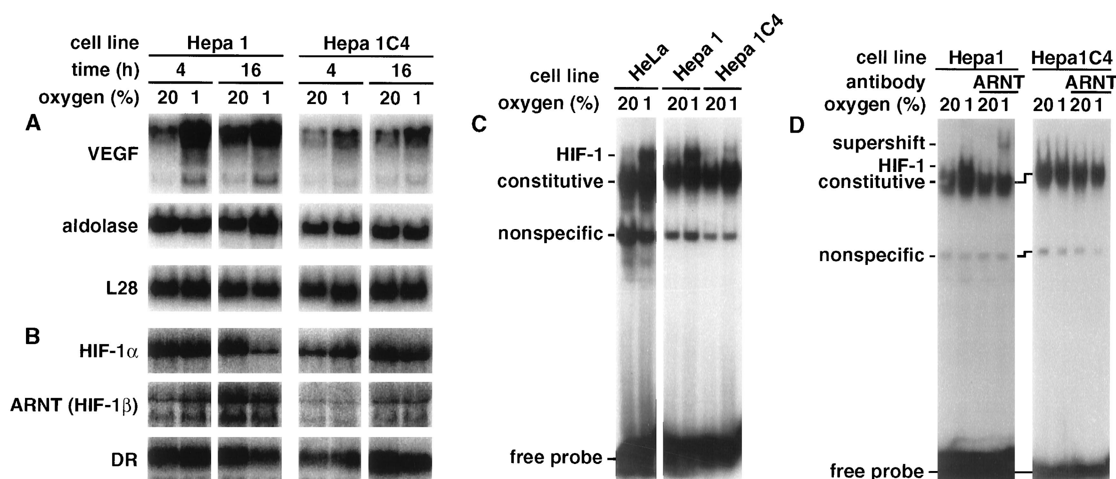

FIG. 2. Requirement of Arnt for hypoxic regulation of target genes. (A and B) RNA blot analysis of total RNA extracted from wild-type Hepa 1 or Arnt-deficient Hepa 1C4 cells following simultaneous cultivation at either 20 or 1% oxygen for 4 and 16 h. The same filter was subsequently hybridized with the indicated cDNA probes following ^{32}P labeling by random priming. The signal obtained with the ribosomal protein L28 probe was used to correct for differences in loading and blotting. (C) Electrophoretic gel mobility shift assay using nuclear extract prepared from HeLa, Hepa 1, or Hepa 1C4 cells cultured either in 20 or 1% oxygen for 4 h. An 18-bp HRE oligonucleotide from the EPO gene was used as a probe. (D) To identify Arnt in the HIF-1 complex, nuclear extracts from Hepa 1 and Hepa 1C4 cells were incubated with anti-Arnt antiserum prior to the DNA binding reaction. Note that the anti-Arnt antiserum recognized a single band in Hepa 1 cells.

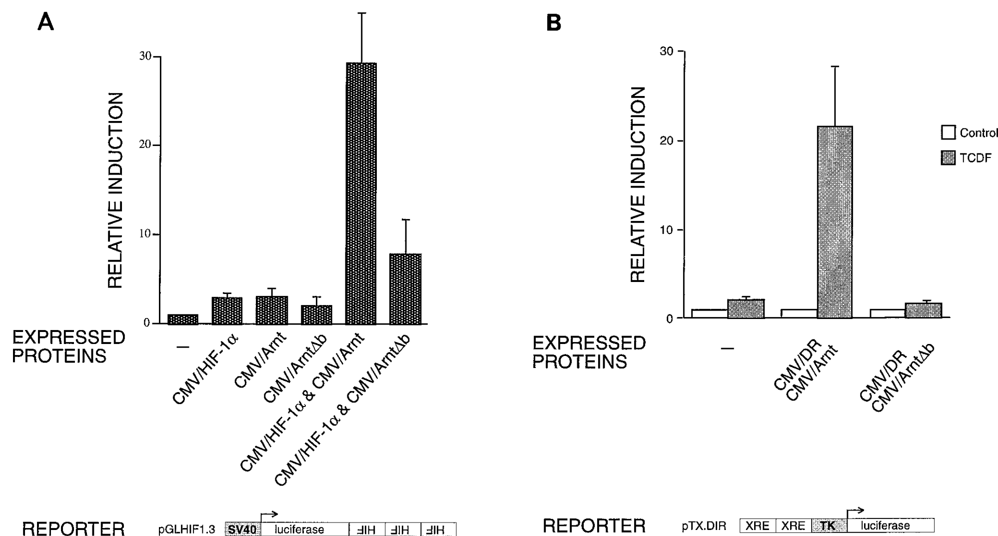

FIG. 3. Arnt is required for HIF-1α function. (A) HeLa cells were transiently cotransfected as described in Materials and Methods with pGLHIF1.3 and expression vectors for HIF-1α, Arnt, and ArntΔb. Luciferase activity was assayed after 48 h. (B) HeLa cells were transfected with the XRE-containing reporter gene pTX.DIR and indicated expression plasmids. Luciferase activity obtained was determined following treatment with 50 nM TCDF for 48 h. The results show relative induction obtained in cells transfected with expression plasmids relative to the activity obtained in cells transfected with the empty pCMV4 expression vector, and cell extracts were normalized on the basis of protein content. Each bar represents the standard deviation of the relative induction obtained from three independent experiments. SV40, simian virus 40; TK, thymidine kinase.

did not result in any induction of aldolase A mRNA expression levels (Fig. 2A), demonstrating that Arnt is required for this HIF-1α-mediated hypoxic response. Interestingly, however, hypoxia induction of VEGF mRNA levels was only partially impaired in mutant Hepa 1C4 cells relative to wild-type Hepa 1 cells, with an up to twofold induction response detected in the mutant cells (Fig. 2A), indicating that the HIF-1α–Arnt complex may only be partially involved in up-regulation of VEGF expression under hypoxic conditions.

It has previously been reported that exposure to hypoxia or $CoCl_2$ induces HIF-1α mRNA levels in human Hep3B hepatoma cells from very low background levels following 2 to 4 h of treatment (56). In contrast, in a number of human tissue samples, we have observed constitutive expression levels of HIF-1α mRNA that were comparable to those of Arnt (Fig. 1). Moreover, HIF-1α mRNA was constitutively expressed in Hepa 1 cells (Fig. 2B). Importantly, we detected no induction of HIF-1α mRNA expression by hypoxic treatment of these cells, but rather down-regulation of the mRNA levels following exposure to hypoxia (1% O_2) for 16 h (Fig. 2B). These results indicate that conditional regulation of HIF-1α function in Hepa 1 cells may be attributed to a mechanism that is distinct from induced HIF-1α expression levels observed in Hep3B cells. For reference, dioxin receptor mRNA was expressed at levels comparable to those of HIF-1α but unaffected by hypoxic treatment. Similarly, Arnt mRNA expression levels were not altered by hypoxic treatment of Hepa 1 cells. Although Hepa 1C4 cells are functionally Arnt deficient (19), Arnt mRNA was also constitutively expressed in these cells, albeit at levels much lower than those detected in wild-type cells (Fig. 2B). Interestingly, down-regulation of HIF-1α mRNA levels was not detected in hypoxic Hepa 1C4 cells (Fig. 2B), raising the possibility that negative feedback regulation of HIF-1α mRNA expression involves functional Arnt protein.

In analogy to the observations in Hepa 1 cells, hypoxia induction of HIF-1α mRNA levels was also not observed in a large number of other cell lines, including human HepG2, glioblastoma (LN229 and LN18) and B cells, mouse fibroblasts (L929), embryonic stem cells (CCE1), primary mouse astrocytes, and primary rat mesangial cells (data not shown). In summary, in contrast to the proposed model for regulation of HIF-1α function in Hep3B cells (56), hypoxia induction of HIF-1α and Arnt mRNA levels may not represent the primary mechanism of conditional regulation of HIF-1α in these cell lines.

As shown in electrophoretic mobility shift experiments (Fig. 2C; compare lanes 3 and 4), exposure of wild-type Hepa 1 cells to hypoxia induced formation of a nuclear complex on an HRE probe derived from the EPO gene (25). The induced complex was specific for the HRE motif, as assessed by oligonucleotide competition experiments (data not shown), and indistinguishable in relative mobility from the well-characterized (25, 57) HIF-1 complex generated in control nuclear extracts from hypoxic HeLa cells (Fig. 2C; compare lanes 2 and 4). Moreover, polyclonal anti-Arnt antibodies produced a supershift of this complex (Fig. 2D). Formation of this complex was not induced in nuclear extracts from mutant Hepa 1C4 cells grown under hypoxic conditions (Fig. 2C and D). For reference, the constitutive complex probably generated by the transcription factors ATF-1 and CREB-1 with the EPO HRE probe (25) remained unaffected upon exposure of the cells to hypoxia (Fig. 2C and D). In analogy to the inability of Arnt and the dioxin receptor to bind to the XRE motif alone (66), these results indicate that HIF-1α failed to recognize the HRE motif individually. In conclusion, Arnt-deficient, mutant Hepa 1C4 hepatoma cells are nonresponsive with regard to not only dioxin receptor- but also HIF-1α-mediated signalling pathways.

To investigate the functional activities of HIF-1α and Arnt individually and in combination with one another, and to further examine the role of Arnt in HIF-1α function, we performed transient-transfection experiments using a luciferase reporter gene, pGLHIF1.3 (25), that is driven by multimerized copies of the HRE motif of the EPO gene coupled to the simian virus 40 promoter (shown schematically in Fig. 3A). This reporter gene showed moderate (about twofold) inducibility by hypoxia (1% O_2) following transient tranfection into

wild-type Hepa 1 cells. This induction response was abrogated in mutant Hepa 1C4 cells, and it was not observed in wild-type Hepa 1 cells in assays using a reporter gene, pGLHIF1mt.3 (25), that contains three mutated copies of the HRE motif (data not shown). Upon transient expression of either HIF-1α or Arnt in HeLa cells, which express low levels of endogenous Arnt (13a), pGLHIF1.3 reporter gene activity was stimulated around two- to threefold under normoxic conditions (Fig. 3A), presumably as a result of constitutive interaction of these overexpressed factors with constitutively expressed endogenous Arnt or HIF-1α, respectively. Moreover, coexpression of HIF-1α and Arnt resulted in potent (around 30-fold) activation of the reporter gene (Fig. 3A). In control experiments, the low levels of Arnt and dioxin receptor endogenous to HeLa cells produced poor ligand inducibility of a transiently transfected XRE-driven reporter gene construct, pTX.DIR (Fig. 3B). A strong, ligand-dependent activation response can be reconstituted, however, upon coexpression of Arnt and dioxin receptor (Fig. 3B) (33). Deletion of the basic domain of Arnt yields a dominant negative mutant, ArntΔb (Fig. 4A), that, like the Id class of regulators of bHLH transcription factors (reviewed in reference 58), produces non-DNA-binding heterodimeric complexes with the ligand-activated dioxin receptor (28). Coexpression of ArntΔb and wild-type dioxin receptor in HeLa cells resulted in complete inhibition of ligand-induced activation of the XRE reporter gene (Fig. 3B). In a similar fashion, ArntΔb inhibited function of HIF-1α on the HRE-driven reporter gene (Fig. 3A), further demonstrating that Arnt is critical for the HRE-mediated induction response. Interestingly, we observed low levels of residual HIF-1α-dependent functional activity on the HRE-driven reporter gene (Fig. 3A) at a dose of the dominant negative ArntΔb mutant which completely inhibited dioxin receptor function (Fig. 3B), possibly suggesting that HIF-1α formed a stronger complex than the dioxin receptor with the low levels of endogenously expressed Arnt in these cells.

Arnt requires both the bHLH and PAS motifs to interact with HIF-1α. We next analyzed dimerization properties of HIF-1α with wild-type or mutant Arnt proteins (shown schematically in Fig. 4A) upon in vitro translation in rabbit reticulocyte lysate, and equal concentrations (Fig. 4B; compare lanes 7 to 10) of proteins were subsequently used in coimmunoprecipitation experiments. As expected, [^{35}S]methionine-labeled HIF-1α was coimmunoprecipitated with specific polyclonal antibodies against Arnt following incubation with nonlabeled, wild-type Arnt, whereas the Arnt antibodies precipitated only very low background levels of HIF-1α in the absence of Arnt (Fig. 4B; compare lanes 1 to 3). No dimerization between Arnt and HIF-1α was detected with the ArntΔPAS and Arnt ΔbHLH deletion mutants that lack the bHLH and PAS domains, respectively (Fig. 4B; compare lanes 2 to 5). Thus, consistent with the mode of interaction of Arnt with the ligand-activated dioxin receptor (28, 66), both the bHLH and and PAS domains of Arnt were critical for dimerization with HIF-1α. In contrast, deletion of the basic domain in the ArntΔb mutant did not affect the dimerization activity with [^{35}S]methionine-labeled HIF-1α (Fig. 4B; compare lanes 2 and 6).

In these coimmunoprecipitation experiments, HIF-1α exhibited very high affinity for Arnt. We therefore compared the dimerization activity of Arnt with HIF-1α with the interaction between Arnt and the ligand-activated dioxin receptor. Equal concentrations of either [^{35}S]methionine-labeled HIF-1α or [^{35}S]methionine-labeled, dioxin-activated dioxin receptor were incubated with identical concentrations of in vitro-translated unlabeled Arnt prior to immunoprecipitation with Arnt antibodies. Strikingly, up to 10-fold-higher levels of HIF-1α were recovered in these experiments in comparison with the coimmunoprecipitated levels of dioxin receptor (Fig. 4C), indicating a considerably higher affinity of Arnt for HIF-1α relative to the dioxin receptor.

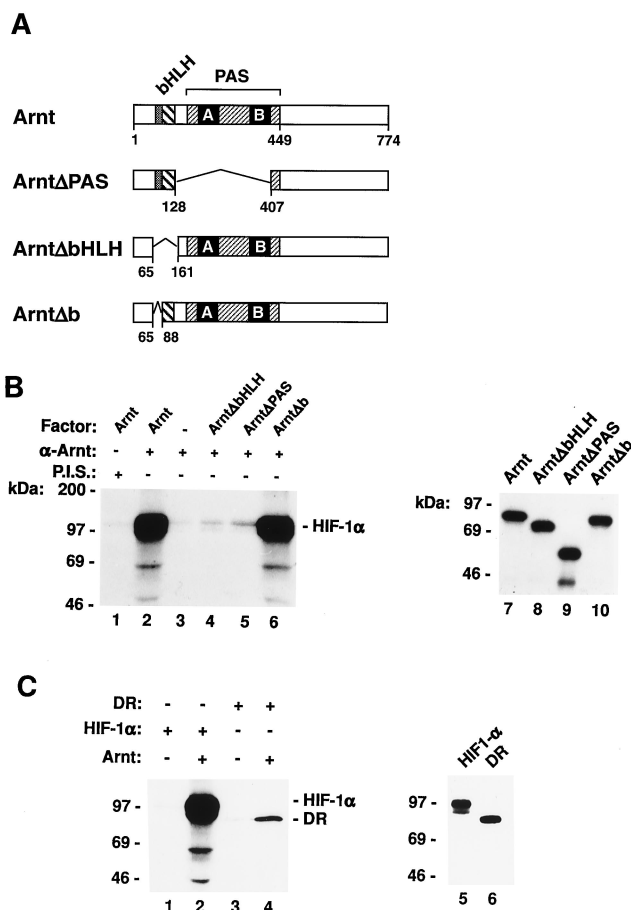

FIG. 4. Arnt requires both the bHLH and PAS motifs to interact with HIF-1α. (A) Schematic representation of full-length Arnt and Arnt deletion mutants. The bHLH and PAS motifs are indicated. (B) In vitro-translated [^{35}S]methionine-labeled full-length HIF-1α was incubated with equal concentrations of in vitro-translated unlabeled full-length Arnt (lanes 1 and 2), the indicated Arnt deletion mutants (lanes 4 to 6), or blank reticulocyte lysate (lane 3) as described in Materials and Methods. Coimmunoprecipitation was carried out with Arnt-specific antiserum (lanes 2 to 6) or preimmune control serum (P.I.S.; lane 1). Resulting coimmunoprecipitated products were visualized by SDS-PAGE and fluorography. Lanes 7 to 10 represent [^{35}S]methionine-labeled control translations of full-length Arnt and Arnt deletion mutants. (C) Equal concentrations of in vitro-translated [^{35}S]methionine-labeled full-length HIF-1α (lanes 1 and 2) and dioxin receptor (DR; lanes 3 and 4) were incubated with 5 μl in vitro-translated unlabeled full-length Arnt (lanes 2 and 4) or blank reticulocyte lysate (lanes 1 and 3) as described in Materials and Methods. Coimmunoprecipitation was carried out with Arnt-specific antiserum, and the precipitated material was analyzed as described above. Lanes 5 and 6 represent control in vitro translations of full-length HIF-1α and dioxin receptor in the presence of [^{35}S]methionine.

Functional interference between hypoxia and dioxin signalling pathways. Given the high affinity of HIF-1α for Arnt, we wished to examine whether activation of HIF-1α would affect functional properties of the ligand-induced dioxin receptor-Arnt complex. Both hypoxia and $CoCl_2$ induce DNA binding activity of the HIF-1α–Arnt complex and induce transcription of target genes, e.g., the EPO gene, via the HRE motif (reference 56 and references therein). Cells of hepatic origin are generally both dioxin and hypoxia inducible. For instance, human HepG2 hepatoma cells are highly dioxin responsive (ref-

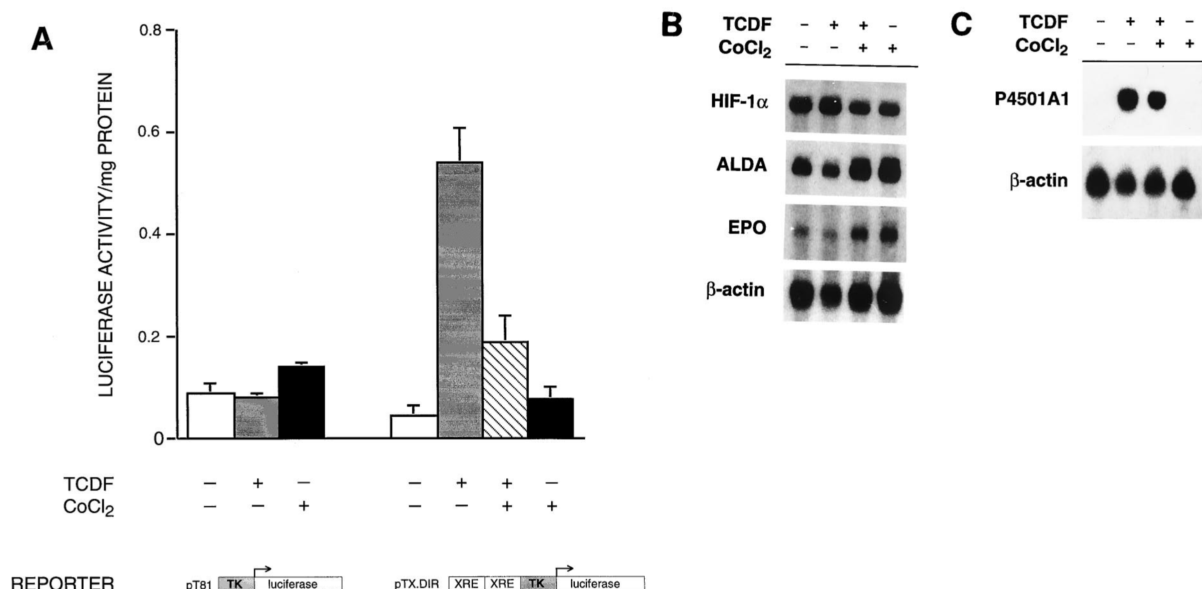

FIG. 5. Inhibition of dioxin responsiveness by $CoCl_2$ treatment of HepG2 cells. (A) HepG2 cells were transfected with the parental reporter gene pT81 or the XRE-containing reporter pTX.DIR, as indicated. Luciferase activity was monitored from cells treated with 50 nM TCDF in the absence or presence of 150 μM $CoCl_2$ for 48 h. Each error bar represents the standard deviation from three independent experiments. Cell extracts were normalized with regard to the protein content. TK, thymidine kinase promoter. (B) Northern blot analysis of HIF-1α, aldolase A (ALDA), EPO, and β-actin mRNA levels in HepG2 cells. The cells were treated with either vehicle alone (lane 1) or 50 nM TCDF for 8 h (lane 2). The cells were also pretreated with 150 μM $CoCl_2$ for 16 h prior to addition of TCDF (lane 3) and incubation for an additional 8 h or treated with $CoCl_2$ alone for 24 h (lane 4). (C) Inhibition of P-4501A1 mRNA induction by $CoCl_2$. HepG2 cells were treated with solvent alone (lane 1), 50 nM TCDF for 8 h (lane 2), or 150 μM $CoCl_2$ for 16 h (lane 4). In lane 3, cells were treated with 150 μM $CoCl_2$ for 16 h prior to addition of TCDF for an additional 8 h.

erence 15 and references therein) and show induction of EPO expression and DNA binding activity of HIF-1 by hypoxia and treatment with $CoCl_2$ (reference 60 and references therein). To study the effect of $CoCl_2$ treatment on dioxin receptor-mediated transcription, we transiently transfected HepG2 cells with a minimal reporter gene construct driven by a tandem of XREs. As shown in Fig. 5A, transcription of this reporter gene is strongly induced by exposure of the cells to the dioxin receptor ligand TCDF, whereas treatment of the cells with $CoCl_2$ did not affect promoter activity. Furthermore, neither treatment affected the basal activity of the parental reporter gene, pT81. Strikingly, however, dioxin receptor ligand responsiveness of the XRE-containing promoter was significantly repressed following coincubation of the cells with TCDF and $CoCl_2$ (Fig. 5A).

Consistent with the mode of regulation of HIF-1α mRNA expression levels by hypoxic treatment of Hepa 1 cells (Fig. 2B), HIF-1α mRNA steady-state levels were not induced following exposure of HepG2 cells to $CoCl_2$ but rather slightly (~2-fold) down-regulated (Fig. 5B). In control experiments, mRNA expression levels of the hypoxia-inducible aldolase A and EPO genes were elevated in response to $CoCl_2$ treatment of HepG2 cells (Fig. 5B). Relative to β-actin control mRNA levels, no effect of TCDF treatment on the basal expression level of either HIF-1α, aldolase A, or EPO mRNA was observed, nor did exposure to TCDF affect induction of aldolase A or EPO mRNA expression levels by $CoCl_2$ (Fig. 5B). Thus, although treatment of HepG2 cells with $CoCl_2$ impaired dioxin receptor function, the reverse effect was not observed; i.e., $CoCl_2$ responsiveness was not affected by the dioxin receptor ligand TCDF.

The cytochrome P-4501A1 gene is a well-characterized target gene of the dioxin receptor. Upon exposure to TCDF, a massive induction response is observed in HepG2 cells, as assessed by RNA blot analysis (Fig. 5C). Following pretreatment of the cells with $CoCl_2$ for 16 h prior to administration of TCDF for an additional 8 h, the TCDF induction response was reduced about twofold relative to β-actin control mRNA levels (Fig. 5C).

The inhibitory effect of $CoCl_2$ on ligand-dependent induction of the XRE-driven reporter gene and cytochrome P-4501A1 induction suggests that activation of HIF-1α may result in interference with dioxin receptor-mediated transcriptional regulation. To establish the role of HIF-1α in negative regulation of dioxin receptor function, we transiently expressed HIF-1α in HepG2 cells and monitored ligand-dependent activation of the cotransfected XRE-driven reporter gene construct. In the presence of the empty pCMV expression vector, we observed bona fide levels of induction of reporter gene activity by TCDF. Upon expression of HIF-1α, however, the ligand-dependent induction response was dramatically down-regulated (Fig. 6), demonstrating that HIF-1α interfered with dioxin receptor-mediated regulation of the reporter gene. In these experiments, $CoCl_2$ treatment was not required to inhibit TCDF responsiveness by HIF-1α. Thus, overexpression of HIF-1α resulted in constitutive HIF-1α activity. In agreement with these observations, overexpression of HIF-1α and Arnt individually or in combination resulted in constitutive activation of an HRE-driven reporter gene (Fig. 3A). In a similar fashion, transient overexpression of the dioxin receptor in certain cells yields constitutive active dioxin receptor-Arnt complexes (34).

The DNA binding activity of the dioxin receptor is repressed by $CoCl_2$ treatment. To further examine the effect of $CoCl_2$ on dioxin receptor function, we analyzed the XRE binding activity of the activated dioxin receptor-Arnt complex in nuclear extracts of HepG2 cells treated with TCDF in the absence or presence of $CoCl_2$. The DNA binding activity of the dioxin

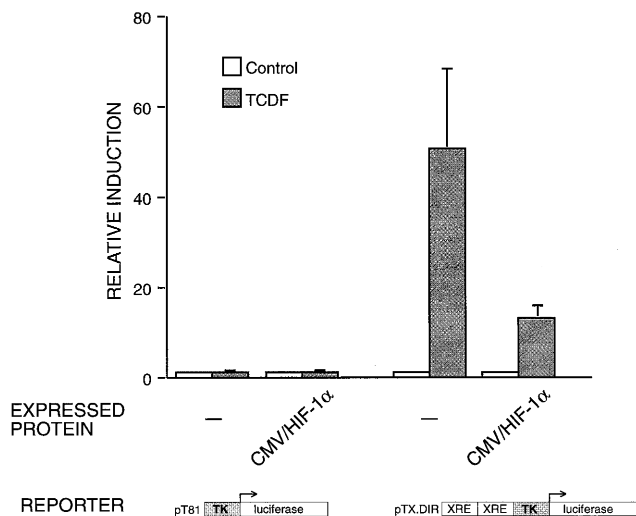

FIG. 6. Inhibition of dioxin receptor function by overexpression of HIF-1α. HepG2 cells were transiently cotransfected with the parental pT81 or the XRE-containing pTX.DIR reporter plasmids together with the HIF-1α expression plasmid pHIF-1α/CMV4 or an empty pCMV4 vector. Luciferase activity was assayed 48 h after transfection. Each bar represents the standard deviation of the relative luciferase activity from three independent experiments, with values normalized relative to the protein content of each extract.

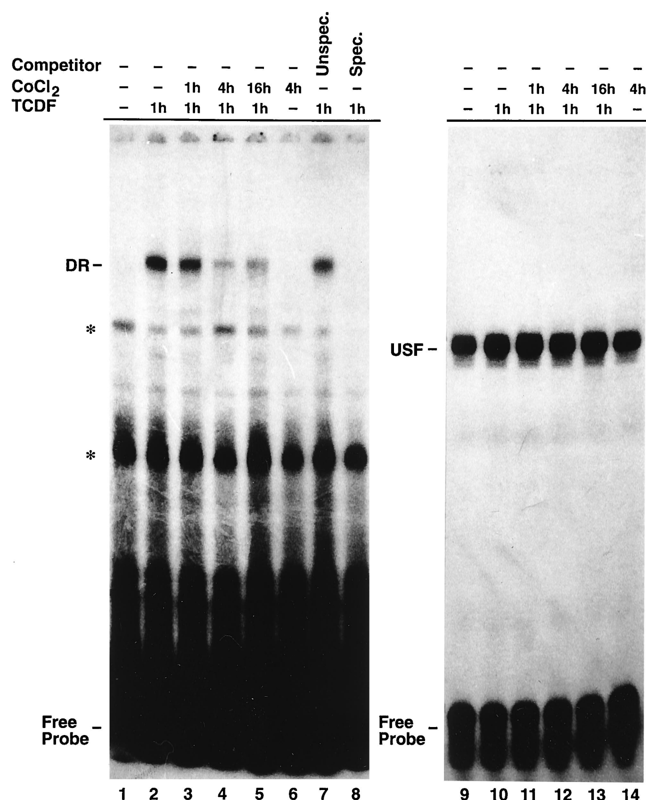

FIG. 7. The DNA binding activity of the dioxin receptor is down-regulated upon exposure to $CoCl_2$. An electrophoretic gel mobility shift assay was performed with nuclear extract prepared from either untreated HepG2 cells (lanes 1 and 9) or HepG2 cells treated with 50 nM TCDF alone for 1 h (lanes 2, 7, 8, and 10). Cells were also pretreated with 150 μM $CoCl_2$ at different time points prior to addition of TCDF for 1 h (lanes 3 to 5 and 11 to 13) or treated with 150 μM $CoCl_2$ alone for 4 h (lanes 6 and 14). Equal amounts of protein from the different nuclear extracts were incubated with ^{32}P-labeled oligonucleotides, and the extracts were analyzed for dioxin receptor-dependent (lanes 1 to 8) or USF-dependent (lanes 9 to 14) DNA binding activities. Competition experiments were performed by including a 100-fold excess of unlabeled probe (Spec.; lane 8) or an unrelated oligonucleotide (Unspec.; lane 7). The mobilities of the dioxin receptor (DR)- and USF-dependent DNA-protein complexes are indicated. Unbound probe is indicated as free probe, and the asterisks indicate complexes generated by constitutive XRE-specific factors.

receptor was monitored by an electrophoretic mobility shift assay. In nontreated cells, no XRE binding activity by the dioxin receptor was detected. Upon exposure of the cells to TCDF for 1 h, the receptor generated a distinct complex with the XRE probe (Fig. 7; compare lanes 1 and 2). This complex was specific for the receptor, since its formation could be inhibited by polyclonal dioxin receptor antibodies (data not shown) or by an excess of the unlabeled XRE probe but not by an excess of an unrelated sequence motif (Fig. 7; compare lanes 7 and 8). Treatment of the cells with $CoCl_2$ alone did not have any effect on the XRE binding activity of the receptor (lane 6). In contrast, pretreatment of HepG2 cells with $CoCl_2$ for increasing periods of time prior to addition of TCDF resulted in a marked decrease in XRE complex formation by the TCDF-activated dioxin receptor (Fig. 7; compare lanes 2 to 5). For reference, the DNA binding activity of the bHLH/leucine zipper transcription factor USF (16) to the symmetric E-box motif from the adenovirus major late promoter was unaffected by the TCDF or $CoCl_2$ treatment (Fig. 7; compare lanes 9 to 14) and therefore served as a control for the quality of the nuclear extracts. Thus, the XRE binding activity of the nuclear dioxin receptor was repressed in response to treatment of HepG2 cells with $CoCl_2$.

The ligand-activated dioxin receptor and HIF-1α compete for recruitment of the Arnt partner factor. We have previously demonstrated that the ligand-activated dioxin receptor requires recruitment of Arnt in order to recognize the XRE target sequence. Individually, neither the dioxin receptor nor Arnt binds to this sequence motif (66). Since XRE binding activity of the ligand-activated dioxin receptor was down-regulated by $CoCl_2$ treatment, and in view of the very stable complexes that were formed between HIF-1α and Arnt in vitro, we wished to investigate whether dioxin receptor and HIF-1α would compete for recruitment of the common partner factor Arnt. To this end, we performed coimmunoprecipitation experiments using Arnt, dioxin receptor, and HIF-1α proteins expressed in rabbit reticulocyte lysate. We first monitored dioxin receptor-Arnt dimerization by incubating [^{35}S]methionine-labeled dioxin receptor and in vitro-translated, unlabeled Arnt in the absence or presence of dioxin, followed by immunoprecipitation with polyclonal antibodies against Arnt. As expected, this assay reconstituted ligand-dependent interaction between the receptor and Arnt (66) (Fig. 8A; compare lanes 1 and 2). In the presence of increasing concentrations of in vitro-translated, unlabeled HIF-1α, however, coprecipitation of the ligand-activated, [^{35}S]methionine-labeled dioxin receptor was reduced in a concentration-dependent manner (Fig. 8A; compare lanes 2 to 5). Conversely, upon incubation of [^{35}S]methionine-labeled HIF-1α and unlabeled Arnt, precipitation of labeled HIF-1α by the Arnt antibodies was not affected by exposure to increasing concentrations of in vitro-translated, ligand-activated dioxin receptor (Fig. 8B; compare lanes 1 to 4). These concentrations of dioxin receptor were identical to the concentrations of HIF-1α that interfered with dioxin receptor-Arnt dimerization. These results indicate that HIF-1α competed with the ligand-activated dioxin receptor for recruitment of the Arnt partner factor. In agreement with the higher affinity of HIF-1α for Arnt, the ligand-activated dioxin receptor failed to disrupt the HIF-1α–Arnt complex at the

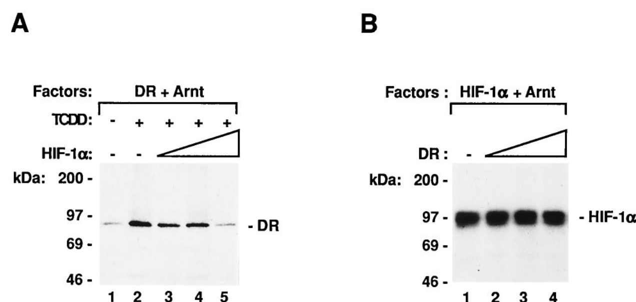

FIG. 8. HIF-1α competes with the ligand-activated dioxin receptor for recruitment of the Arnt partner factor. (A) In vitro-translated [^{35}S]methionine-labeled full-length dioxin receptor was incubated with 5 μl of unlabeled in vitro-translated full-length Arnt in the presence (lanes 2 to 5) or absence (lane 1) of 10 nM dioxin (TCDD). The ligand-activated dioxin receptor was also incubated without (lanes 1 and 2) or with a 0.5-fold (lane 3), 1-fold (lane 4), or 2-fold (lane 5) molar excess of unlabeled in vitro-translated full-length HIF-1α as described in Materials and Methods. Coimmunoprecipitation was carried out with Arnt-specific antiserum, and the coimmunoprecipitated products were visualized by SDS-PAGE and fluorography. (B) In vitro-translated [^{35}S]methionine-labeled full-length HIF-1α was incubated with 5 μl of unlabeled in vitro-translated full-length Arnt in the absence or presence of increasing concentrations of unlabeled dioxin receptor (DR) and then coimmunoprecipitated with Arnt-specific antiserum. The products were analyzed as described above. The concentration of [^{35}S]methionine-labeled HIF-1α was equivalent to that of [^{35}S]methionine-labeled dioxin receptor in panel A.

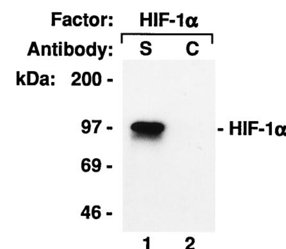

FIG. 9. HIF-1α is associated with the molecular chaperone hsp90. Full-length HIF-1α cDNA was in vitro translated in the presence of [^{35}S]methionine in rabbit reticulocyte lysate, coimmunoprecipitated with hsp90-specific (S; lane 1) or control immunoglobulin M (C; lane 2) antibody as described in Materials and Methods, and analyzed by SDS-PAGE and fluorography.

tested concentrations (up to a twofold molar excess of dioxin receptor over HIF-1α).

HIF-1α is associated with the molecular chaperone hsp90. We have previously observed that the non-DNA-binding, repressed form of dioxin receptor is associated with hsp90 (68) and that hsp90 is important for folding of a ligand-binding conformation of the receptor in vitro (10, 44). Importantly, ligand responsiveness of the dioxin receptor is abrogated in a yeast model system following down-regulation of hsp90 expression levels (8, 65). Taken together, these data strongly argue that hsp90 is critical for conditional regulation of dioxin receptor function. Moreover, we have recently demonstrated that hsp90 also is associated with the *Drosophila* bHLH/PAS factor Sim (35), to which HIF-1α shows a high degree of similarity (56). Given this background, we wished to test whether HIF-1α was associated with hsp90. HIF-1α was expressed and [^{35}S]methionine labeled by in vitro translation in reticulocyte lysate, and we performed coimmunoprecipitation experiments using a monoclonal hsp90 antibody, 3G3, that specifically recognizes both free hsp90 and hsp90 complexed with other proteins (39). These antibodies coimmunoprecipitate the dioxin receptor upon de novo synthesis in reticulocyte lysate (36, 63). We observed that the hsp90 antibodies also coimmunoprecipitated in vitro-translated, [^{35}S]methionine-labeled HIF-1α (Fig. 9, lane 1). In control reactions, only very low levels of labeled HIF-1α were detected following precipitation with irrelevant monoclonal antibodies (Fig. 9; compare lanes 1 and 2). Thus, these experiments demonstrated stable interaction of HIF-1α with hsp90. Although the functional significance of this interaction remains to be elucidated, the importance of hsp90 for conditional regulation of dioxin receptor activity suggests that a similar mechanism may modulate HIF-1α function. In support of this notion, the present data demonstrate that HIF-1α was constitutively expressed in a large number of tissues (Fig. 1), and, in contrast to the prevalent model of regulation of HIF-1α in Hep3B cells, HIF-1α mRNA expression levels were not upregulated in Hepa 1 following exposure to hypoxia (Fig. 2B) or in HepG2 cells in response to $CoCl_2$ treatment (Fig. 5B). Moreover, given the strict correlation between ligand and hsp90 binding activities of the dioxin receptor (10, 63), it will be interesting to examine the possibility that HIF-1α can bind an as yet unknown class of ligands and thus constitute a so-called orphan receptor.

DISCUSSION

In this study, we have demonstrated that Arnt is required for HIF-1α function. Thus, the ubiquitously expressed Arnt protein is involved in regulation of expression of multiple networks of target genes. Through heterodimerization with either the dioxin receptor or HIF-1α, it is required for recognition of cognate asymmetric E-box target sequences and thereby critical for conditional gene regulation of target genes by dioxin or hypoxia and $CoCl_2$, respectively. Moreover, Arnt has recently been reported to homodimerize in vitro (18, 53) and constitutively bind the E-box motif CACGTG and regulate reporter gene expression in vivo by the same E-box element (2, 53).

Consistent with a critical role of Arnt as a coregulator protein serving to target HIF-1α to its cognate response element, a dominant negative Arnt mutant inhibited HIF-1α function on minimal reporter genes driven by the EPO HRE motif. Moreover, induction of aldolase A mRNA levels was inhibited in hypoxic Hepa 1C4 cells expressing functionally deficient Arnt protein. Interestingly, however, hypoxic induction of VEGF mRNA expression was only partially inhibited in the mutant Hepa 1C4 subline. Thus, in agreement with the recently proposed posttranscriptional mechanism of hypoxic regulation of VEGF expression (21, 26, 51), there may exist an alternative pathway of hypoxic regulation that is independent of the HIF-1α–Arnt complex.

Cross-coupling of HIF-1α- and dioxin receptor-mediated regulatory pathways. The data presented here indicate that HIF-1α, once activated, dominates over the ligand-stimulated dioxin receptor for recruitment of Arnt, resulting in interference between hypoxia and dioxin signalling pathways. For example, in dioxin- and hypoxia-responsive human HepG2 cells, dioxin receptor function was impaired in cells cotreated with dioxin receptor ligand and $CoCl_2$. Under these conditions, Arnt therefore appeared to be limiting for dioxin responsiveness. This model is supported by the observation that the DNA binding activity of the dioxin receptor was inhibited in nuclear extracts of HepG2 cells exposed to both dioxin and $CoCl_2$. Moreover, HIF-1α exhibited an affinity for Arnt in vitro that was considerably higher than that of the ligand-activated dioxin receptor. HIF-1α could therefore efficiently compete with the ligand-activated dioxin receptor for dimerization with Arnt in vitro. Conversely, at all tested excess concentrations of activated dioxin receptor over HIF-1α, the dioxin receptor did not compete with HIF-1α for recruitment of Arnt. Thus, although

HIF-1α inhibited dioxin receptor function by sequestering Arnt, dioxin and hypoxia signalling pathways were not mutually exclusive. Inasmuch as HIF-1α mRNA expression patterns were largely correlated with Arnt mRNA expression levels in a number of human tissues, and, in turn, Arnt expression levels are largely correlated with those of the dioxin receptor (7), it will now be interesting to examine whether activation of the HIF-1α signalling pathway generally interferes with dioxin receptor function. Given the high affinity of HIF-1α for Arnt, the present data also raise the possibility that functional interaction of Arnt with other bHLH/PAS partner factors, in addition to the dioxin receptor, is negatively regulated by the activated form of HIF-1α. In this context, one interesting candidate partner factor is represented by mammalian homologs of *Drosophila* Sim, e.g., the putative Down's syndrome-critical factor (11). *Drosophila* Sim has recently been shown to dimerize with Arnt and to require Arnt for DNA binding activity (35, 53, 55, 62).

Combinatorial regulation of either HIF-1α or dioxin receptor activity by interaction with the Arnt partner factor may create the basis for very complex and possibly cell type-specific patterns of modulation of HIF-1α and dioxin receptor function. For instance, given the growing family of bHLH/PAS factors and the strikingly complex regulatory networks of other bHLH factors, it is possible that Arnt functionally interacts with additional, as yet unidentified partner factors. Since *Drosophila* Sim recently has been shown to dimerize with Arnt (35, 53, 55), among the interesting putative partner factors of Arnt are mammalian homologs of Sim, notably the Down's syndrome-critical factor (11). Thus, putative partner factors of Arnt may be found in developmental regulatory pathways, resulting in possibly novel phenomena of cross-coupling with dioxin receptor and HIF-1α-mediated signalling processes. The present experiments indicate that under conditions in which Arnt is limiting, the efficiency of Arnt-partner factor interaction constitutes a critical step in regulation of the activity of the two conditionally regulated bHLH/PAS factors HIF-1α and dioxin receptor. In conclusion, these data argue that the level of expression of Arnt or the pool of Arnt available for dimerization with the dioxin receptor represents an important mechanism for determining cellular sensitivity and responsiveness to dioxin.

Mechanism of activation of HIF-1α. What is the mechanism of conditional regulation of HIF-1α function by hypoxia or $CoCl_2$? In this study, we observed constitutive expression levels of HIF-1α mRNA that mirrored those of Arnt in a variety of tissues. In agreement with these data, HIF-1α mRNA levels remain unaltered in all organs examined of carbon monoxide-exposed mice, which show strong induction of serum EPO levels (61). Moreover, constitutive expression of HIF-1α mRNA was observed in several cell lines, including human HepG2 and murine Hepa-1 hepatoma cell lines. In contrast, both HIF-1α and Arnt mRNA have been reported to be expressed at very low constitutive levels in human Hep3B cells (56). Importantly, exposure of HepG2 or Hepa-1 cells to hypoxia or $CoCl_2$ did not induce HIF-1α mRNA expression levels but rather resulted in down-regulation of the mRNA levels. This result contrasts sharply with that for Hep3B cells, in which a dramatic induction of HIF-1α mRNA levels by hypoxia or $CoCl_2$ has recently been found (56). Induction of HIF-1α mRNA expression is therefore not likely to represent a more general mechanism for activation of HIF-1α function. Rather, the present data suggest that regulation of HIF-1α function involves a posttranscriptional mechanism.

The present experiments demonstrate that de novo-synthesized HIF-1α stably associates in vitro with the molecular chaperone hsp90. We do not yet know whether hsp90 is capable of mediating repression of HIF-1α function in nonstimulated target cells. It also remains to be established whether interaction with hsp90 is important for folding of a functional form of HIF-1α, consistent with the role of hsp90 as a molecular chaperone (37, 67). Previous data indicate that hsp90 is critical for ligand inducibility in vivo not only of the dioxin receptor (8, 65) but also of distinct nuclear receptors such as the glucocorticoid receptor (5, 40). In the cases of both the dioxin receptor (44, 68, 64) and the glucocorticoid receptor (reviewed in references 45 and 52), release of hsp90 appears to be required for unmasking of functional activity, since only the repressed, non-DNA-binding forms of the receptors show interaction with hsp90. In agreement with these findings, a domain of the dioxin receptor that mediates an inhibitory function in vivo is stably associated with hsp90 in vitro (31, 64). Moreover, artificial disruption of hsp90 in vitro results in constitutive DNA binding activity of the dioxin receptor (44), most probably as a result of constitutive dimerization with Arnt. It is therefore conceivable that dissociation of hsp90 results in unmasking of the dimerization interface of HIF-1α, subsequent recruitment of Arnt, and derepression of HIF-1α function.

In contrast to HIF-1α and the dioxin receptor, Arnt is not associated with hsp90 (36). These data reinforce the idea that only conditionally regulated transcription factors, e.g., dioxin receptor, HIF-1α, and certain members of the nuclear hormone receptor family, are associated with hsp90, whereas this interaction is not detected with constitutively active transcription factors. In the case of the dioxin receptor, hsp90 appears to chaperone a ligand-binding conformation of the receptor, since following release of hsp90, the dioxin receptor fails to bind ligand (2, 44). Consistent with these observations, the bacterially expressed minimal ligand binding domain of the receptor appears to be malfolded and shows no ligand binding activity (10). Given the correlation between association with hsp90 and ligand binding function of the dioxin receptor, and the importance of hsp90 for ligand binding activity of the glucocorticoid receptor (reviewed in references 45 and 52), it is possible that HIF-1α represents an intracellular orphan receptor for an as yet unknown class of ligands. Thus, in addition to the growing number of orphan receptors within the steroid receptor superfamily (see reference 32 for a review), certain bHLH/PAS factors may represent a novel and distinct class of orphan receptors. Such receptors offer the potential for accurate conditional regulation of functional activities. In summary, it is tempting to speculate that hsp90 may be important for the maintenance and/or folding of a repressed form of HIF-1α and that this provides both the regulation and signal-sensing mechanism necessary for the hypoxia-controlled process.

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