Repression of Dioxin Signal Transduction in Fibroblasts

IDENTIFICATION OF A PUTATIVE REPRESSOR ASSOCIATED WITH Arnt

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Heterodimeric complexes of basic helix-loop-helix/PAS transcription factors are involved in regulation of diverse physiological phenomena such as circadian rhythms, reaction to low oxygen tension, and detoxification. In fibroblasts, the basic helix-loop-helix/PAS heterodimer consisting of the ligand-inducible dioxin receptor and Arnt shows DNA-binding activity, and the receptor and Arnt are able to activate transcription when fused to a heterologous DNA-binding domain. However, fibroblasts are nonresponsive to dioxin with regard to induction mediated by the DNA response element recognized by the receptor and Arnt. Here we demonstrate that Arnt is associated with a fibroblast-specific factor, forming a complex that is capable of binding the dioxin response element. This factor may function as a repressor since negative regulation of target gene induction appears to be abolished by inhibition of histone deacetylase activity by trichostatin A. Finally, the negative regulatory function of this factor appears to be restricted for dioxin signaling since Arnt was able to mediate, together with hypoxia-inducible factor-1α, transcriptional activation in hypoxic cells. Taken together, these data suggest that fibroblast-specific inhibition of dioxin responsiveness involves recruitment by Arnt of a cell type- and signaling pathway-specific corepressor associated with a histone deacetylase.

There is ample evidence for tissue-specific differences in response to environmental contaminants such as halogenated polycyclic aromatic hydrocarbons. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD; dioxin) is a prototype compound for this class of compounds (for review, see Ref. 1). Induction of the dioxin response gene CYP1A1 shows cell-type dependence as demonstrated both in cell culture and in transgenic mice (2). The locus of the mammalian homologue (Sim2) coincides with the Down’s syndrome chromosomal region (15). Moreover, the hypoxia-inducible factor-1α (HIF-1α) (16) is also a bHLH/PAS protein that regulates inducible expression of genes such as erythropoietin, vascular endothelial growth factor, and a number of glycolytic enzymes in hypoxic cells (reviewed in Ref. 17). Under conditions of low oxygen tension, recruitment of Arnt is essential to enable HIF-1α to bind its target DNA sequences (hypoxia response elements). The mechanism of activation of this physiologically important class of transcription factors is not yet understood.

It has become increasingly apparent that histone acetyltransferases and histone deacetylases (HDACs) play important roles in transcriptional regulation. Recent studies suggest that acetylation and deacetylation of histones are involved in the process of chromatin assembly (reviewed in Refs. 18 and 19). Moreover, histone acetyltransferases and HDACs have been found to be components of some transcriptional coactivator and corepressor complexes (for recent reviews, see Refs. 20 and 21), respectively, which suggests that they modulate transcriptional activity at specific promoters by locally perturbing chromatin structure. Several of these histone acetyltransferase-containing proteins complexes have been identified. For example, CBP/p300, P/CAF, and SRC-1 interact with a variety of DNA-binding transcription factors and have been shown to function as transcriptional coactivators (reviewed in Ref. 21). Moreover, repression by nuclear receptors has been correlated to binding of corepressors mouse Sin3, NCoR/SMRT, and the HDAC Rpd3/HDAC1 (22). Furthermore, the Mad/Max heterodimer recruits an HDAC via mouse Sin3 and NCoR/SMRT,
Trichostatin A Derepresses CYP1A1 Induction in Fibroblasts

Most studies of mechanisms underlying induction of the CYP1A1 gene encoding cytochrome P4501A1 as well as cytochrome P4501A1 enzymatic activity have been performed using hepatic cells since liver is the tissue that has the highest concentration of P4501A1 (reviewed in Ref. 25). Studies of, for example, transgenic mice carrying the CYP1A1 promoter in front of the lacZ reporter gene show that inducibility varies in different adult tissues (2), but the mechanism behind this phenomenon is not known. Moreover, consistent with reports on P4501A1 levels in various tissues after in vivo exposure, we have found normal fibroblasts in culture to be nonresponsive to dioxin with regard to CYP1A1 inducibility (26, 27).

Analyses of nuclear extracts from fibroblasts show that the dioxin receptor can be activated to its DNA-binding form by ligand (27). Moreover, two constitutively expressed XRE-binding protein species that are not found in responsive cells, i.e. keratinocytes and HepG2 hepatoma cells, are present in fibroblast extracts. We report that this constitutive fibroblast-specific XRE-binding factor(s) is a heteromeric complex, in which one of the factors is Arnt, whereas the other protein(s) that directly contacts the XRE remains to be identified. More important, we also demonstrate that the HDAC inhibitor trichostatin A (TSA) (28) in combination with dioxin can overcome transcriptional repression in fibroblasts. These findings suggest that a cell type-restricted partner factor possibly harboring HDAC activity modulates Arnt function in fibroblasts, resulting in efficient interference with the dioxin receptor signaling pathway.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—The human hepatoma cell line HepG2 was grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% (v/v) fetal calf serum (Life Technologies, Inc.), 100 µg/ml streptomycin, 100 IU/ml penicillin, and 0.25 µg/ml fungizone. Fibroblasts were prepared from neonatal foreskin by placing the proteinase-treated dermis in a cell culture dish in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented as described above until the fibroblasts had adhered and were subsequently treated as described previously (29). Cells at the third to fifth passages were routinely used. Cultures were treated with 2.3,7,8-tetrachlorodibenzofuran (TCDF; Cambridge Isotope Laboratories), TCDD (Cambridge Isotype Laboratories) dissolved in Me2SO, cycloheximide (Sigma), trichostatin A (TSA) (28) or SHH in phosphate-buffered saline, and the cells were subsequently treated with 10 nM TCDD or Me2SO alone for an additional 36–48 h. The DNA/cell mixture was incubated for 5 min at room temperature prior to electroporation (13 ohms, 600 microfarads, and 400 V) with an Electro Cell Manipulator (TecHturn). After electroporation, cells were put on ice for 10 min before seeding out 100 × 10^6 cells in 25-cm^2 cell culture flasks. Fibroblasts were grown for 48 h prior to treatment with 50 nM TCDF or Me2SO alone for an additional 36–48 h. For the experiments shown in Fig. 7A, both fibroblasts and HepG2 cells were transfected with Fugene 6 transfection reagent (Roche Molecular Biochemicals). Three microliters of Fugene 6 was combined with the indicated plasmid, and the cells (10^6/cm^2) were incubated with the Fugene 6/DNA mixture for 6 h prior to treatment with 10 nM TCDD and/or TSA for 36–40 h. Luciferin was purchased from BioTrend.

RNA Isolation and RNA Blot Analysis—Total RNAs were isolated using acid-phenol extraction as described (33). Polyadenylated RNA was prepared by using streptavidin-conjugated paramagnetic oligo(dt) particles (Promega) according to the manufacturer's protocol. RNA was fractionated through formaldehyde-agarose gels, blotted onto nylon filters, and hybridized, and the filters were subsequently prehybridized, hybridized, and washed according to standard procedures (34) prior to autoradiography. 32P-Labeled probes for the cDNAs of cytochrome P4501A1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (27), cytochrome P4501B1 (4), murine dioxin receptor (7), human Arnt (9), human HIF-1α, erythropoietin, aldolase A (35), and β-actin (CLONTECH) were generated by a random priming procedure. A complementary synthetic oligonucleotide specific for the extra exon in the alternatively spliced N terminus of Arnt (5'-AGTCTCTTCT TATCCCGCAG GCTGCTGCTA TC-3') (9) was synthesized and 32P-end-labeled with T4 polynucleotide kinase as described (34). 

Immunoblot Analysis—Cellular protein was prepared by lysing the cells (20 nM sodium phosphate (pH 7.2), 50 mM β-glycerophosphate, 100 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1 mM phenylmethylsulfonfluoride, 5 µg/ml peptatin (Roche Molecular Biochemicals), 10 µg/ml leupeptin (Sigma), 10 µg/ml aprotenin (Bayer), and 0.1% Nonidet P-40, and the lysates were cleared by centrifugation at 15,000 × g for 45 min at 4 °C. Proteins were separated by 9% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes, and the membranes were subsequently stained by ECL Western blotting detection (Amersham Pharmacia Biotech) using anti-dioxin receptor or anti-Arnt antisera at a 1:200 dilution. Preimmune serum did not, under these conditions, give rise to any background reactivity (27). The antisera were raised as described (36, 37) against the N-terminal part of the mouse dioxin receptor and the human Arnt protein, respectively.

Preparation of the 5-Bromo-2′-deoxyuridine-substituted DNA and DNA Cross-linking—A synthetic oligonucleotide spanning the XRE1 element of the rat cytochrome P4501A1 upstream promoter region (3) (5'-gaacctctgaa ggcgtcttctt acggaaacatt ccggggacg-3') was synthesized and 32P-end-labeled as described (34). The DNA contains three bromodeoxyuridine residues within the recognition sequence for the receptor as well as a fourth residue near one end of the oligonucleotide (underlined bases). EMSA DNA-binding reactions (contained in an open 1.5-ml Eppendorf tube on ice) were irradiated for 5 min with UV (Stratagene; emission wavelength = 254 nm; intensity = 4000 microwatts/cm 2) at a distance of 4–5 cm from the source. The proteins were separated by SDS-polyacrylamide gel electrophoresis (9%), dried, and subjected to autoradiography. For SDS-polyacrylamide gel electrophoresis analysis, prestained and 14C-labeled molecular mass marker proteins were purchased from Bio-Rad and Amersham Pharmacia Biotech, respectively.

Nuclear Extract Preparation and EMSA—Cells were treated with 50 nM TCDD for 1 h or 10 µg/ml cycloheximide for 4 h before harvest. Nuclei and protein extract were prepared as described previously (38). DNA-binding reactions were assembled in a total volume of 20 µl with 10 µg of nuclear proteins at a final concentration of 25 µg HEPS (pH 7.9), 0.2 mM EDTA, 75 mM KCl, 2 mM MgCl 2, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonfluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 5 µg/ml pepstatin, 5% glycerol, 4% Ficoll, 100 µg/ml poly(dC-dC) (Amersham Pharmacia Biotech), and 12.5 µg/ml poly(dA-dT) (Amersham Pharmacia Biotech) using a double-stranded, 32P-labeled oligodeoxynucleotide as a probe, which is the sequence of which corresponded to residues 968–979 of the human CYP1A1 gene (5'-CTCCGCTGCTT CTCACGCAG CCAGGAGAAG CAG) was used as nonspecific competitor DNA, whereas unlabeled probe DNA served as specific competitor. When using anti-dioxin receptor or anti-Arnt antisera, 10 µg of nuclear protein was preincubated with the diluted antisera (1:10) for 20 min at room temperature prior to the DNA-binding reaction. Protein-DNA complexes were separated under nondenaturing conditions

which seems to repress genes that normally are activated by the Myc/Max heterodimer (23, 24).

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on a 3% polyacrylamide gel (29:1) run in 50 mM Tris, 380 mM glycine, and 2.7 mM EDTA at 4 °C.

RESULTS

Lack of Induction of Dioxin Target Genes in Human Fibroblasts—We have previously demonstrated cell type-specific differences in P4501A1 inducibility and shown that normal human fibroblasts are nonresponsive to dioxin with regard to induction of P4501A1 mRNA levels (27). To investigate if the nonresponsive phenotype of fibroblasts is a more generalized phenomenon, we analyzed regulation in fibroblasts of another dioxin response gene, cytochrome P4501B1 (4). Very low levels of constitutive P4501B1 mRNA expression were observed by RNA blot analysis in untreated fibroblasts, and more important, this message was not induced upon exposure of cells to TCDF (Fig. 1, compare lanes 1 and 2). In control experiments analyzing primary human keratinocytes, P4501B1 mRNA levels were barely detectable by RNA blot analysis, being very similar to the low levels of constitutive P4501B1 mRNA expression observed in primary human fibroblasts. Treatment of keratinocytes with TCDF, however, resulted in significant induction of P4501B1 mRNA (Fig. 1, compare lanes 4 and 5). Cycloheximide treatment is known to induce P4501A1 expression in hepatic cells, keratinocytes, and fibroblasts in the absence of ligand (27). Interestingly, also P4501B1 mRNA expression was induced following cycloheximide treatment of fibroblasts (Fig. 1, lane 3). Thus, although TCDF induction response is inhibited, both CYP1A1 and CYP1B1 expression appear to be regulated by a similar, if not identical, cycloheximide-sensitive mechanism, demonstrating that both promoters are not irreversibly repressed in fibroblasts.

To examine any differences in expression levels of the dioxin receptor and Arnt between nonresponsive and responsive cells, polyadenylated RNA from fibroblasts and HepG2 cells was analyzed. RNA blot analysis demonstrated that the ~6-kilobase dioxin receptor mRNA species was expressed at similar levels in both cell types (Fig. 2A). Two Arnt mRNA transcripts of 2.6 and 4.2 kb were present at comparable levels in both fibroblasts and HepG2 cells, respectively (Fig. 2A). In addition, an alternatively spliced Arnt mRNA has been described (9) that contains an extra exon of 45 nucleotides encoding 15 amino acids of unknown relevance. No cell type-specific differences in the presence of this alternatively spliced Arnt mRNA were observed (Fig. 2A). Moreover, immunoblot analysis with anti-serum directed against the dioxin receptor or Arnt showed no differences between HepG2 cells and fibroblasts in expression levels of these proteins (Fig. 2B). Thus, these results suggest that nonresponsiveness of fibroblasts to dioxin is not attributable to altered expression levels of the dioxin receptor or its partner factor Arnt.

Dioxin Receptor and Arnt Fusion Proteins That Are Functionally Uncoupled from One Another Are Active in Fibroblasts—To investigate if the dioxin receptor and Arnt by themselves could function as independent activators in fibroblasts, we used expression plasmids coding for fusion proteins. These constructs contain the N-terminal DBD fragment (amino acids 1–500) of the human glucocorticoid receptor that spans the major transactivating (α) and DNA-binding (DBD) domains, but lack the large C-terminal hormone-binding domain (Fig. 3A). This DNA-binding glucocorticoid receptor derivative replaced the dioxin receptor DNA-binding N-terminal bHLH motif (construct pDBD/DR), generating a dioxin receptor with affinity for glucocorticoid response elements. In addition, Arnt devoid of its N-terminal bHLH domain was fused to the DBD motif (pDBD/Arnt) (Fig. 3A). Fibroblasts and HepG2 cells were transiently transfected with the chimeric constructs, and activity was assayed as ability to induce glucocorticoid response element-dependent expression of a cotransfected reporter gene (pT199(GRE)Luc). Upon expression of pDBD/DR in HepG2 cells, a 5-fold induction of reporter gene activity by TCDF was obtained, whereas the activities of pDBD and pDBD/Arnt constructs were not altered by this treatment (Fig. 3B). Interestingly, expression of pDBD/DR produced a 3-fold induction response in fibroblasts (Fig. 3B), and moreover, pDBD/Arnt showed high constitutive activity in the same cells. Taken
together, these results indicate that the dioxin receptor and Arnt are functionally active in fibroblasts when analyzed in a transcription-activating context not involving their usual response element. This suggests that repression in fibroblasts is mediated by the XRE sequence element, but not directly through negative regulation of the transactivating domains of the dioxin receptor/Arnt heterodimer.

**Endogenous Arnt Is Functional as a Partner Factor to HIF-1α in Fibroblasts**—Low oxygen tension, CoCl$_2$, and desferroxamine induce expression of different genes such as erythropoietin, vascular endothelial growth factor, and some glycolytic enzymes (17). This effect is mediated by the XRE sequence element, but not directly through negative regulation of the transactivating domains of the dioxin receptor/Arnt heterodimer. Moreover, HIF-1α mRNA levels were not affected in either of the cell lines. These results indicate that both endogenous Arnt and HIF-1α are functional in fibroblasts since the HIF-1α/Arnt heteromer is able to regulate hypoxia-inducible genes.

**Identification of a Novel Partner Factor for Arnt in Fibroblasts**—Nuclear extracts from fibroblasts and HepG2 cells were analyzed by EMSA to characterize proteins binding to a $^{32}$P-labeled XRE oligonucleotide probe. Fibroblast extracts gave rise to two constitutive, distinct, and specific protein-XRE complexes (Fig. 5A) that were not formed if nuclear extracts from HepG2 cells were used (Fig. 5, compare A and B) (27). Formation of these two complexes was not affected by cycloheximide treatment (Fig. 5A, compare lanes 1 and 3). Thus, two critical...
parameters distinguish these complexes from a labile factor(s) that has been implicated in negative regulation of \( CYP1A1 \) expression in a number of cell lines (39, 40): (i) cell-type specificity and (ii) insensitivity to cycloheximide treatment.

Three protein-XRE complexes were detected by EMSA using extracts from TCDF-treated fibroblasts (Fig. 5B). These were specific as assessed by competition with unlabeled specific and nonspecific oligonucleotides (Fig. 5, A, compare lanes 1 and 2; and B, compare lanes 1–3). We have presently no indications that the smaller fibroblast-specific complex detected represents a degradation product of the larger, and thus, it cannot formally be excluded that the small species (complex 2 in Fig. 5, A and B) represents a distinct endogenous form. The dioxin receptor-containing complex was identified by preincubating extracts with anti-dioxin receptor antiserum. As shown in Fig. 5B (compare lanes 4 and 9), this antiserum inhibited formation of the dioxin-inducible complex by extracts from fibroblasts while leaving the two additional constitutive XRE-specific complexes unaffected. Interestingly, these two constitutive fibroblast-specific complexes were supershifted to a slower mobility in EMSA following incubation of extracts with anti-Arnt antiserum (Fig. 5B, compare lanes 5 and 10).

In parallel experiments, UV-induced cross-linking of proteins binding to XRE was performed. To cross-link nuclear proteins to the XRE, we generated a double-stranded, bromodeoxyuridine-substituted XRE probe. Nuclear extracts from TCDF-treated fibroblasts and HepG2 cells were incubated with this oligonucleotide for 20 min prior to UV irradiation for 5 min. Analysis of covalently cross-linked proteins from HepG2 cells by SDS-polyacrylamide gel electrophoresis revealed a single radiolabeled band of \( \sim 120 \) kDa. Formation of this complex was inhibited by preincubation with anti-dioxin receptor antiserum (Fig. 6, compare lanes 1 and 2). Moreover, 120 kDa was consistent with the size of the human dioxin receptor as determined by immunoblot analyses (27), whereas Arnt immunoreactivity was observed as a 90-kDa species using extracts from either HepG2 cells or fibroblasts (Fig. 2B).

In contrast to results obtained with extracts from HepG2 cells, three specific protein-XRE complexes of \( \sim 125, 120, \) and 90 kDa were detected upon UV cross-linking using nuclear extracts from fibroblasts (Fig. 6, lane 3). Consistently, the appearance of the \( \sim 120 \)-kDa protein-DNA complex was abolished when fibroblast extracts were preincubated with anti-

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**Fig. 4.** RNA blot analysis of hypoxia-inducible genes. Total RNA was prepared from cultures of fibroblasts and HepG2 cells that were treated with increasing concentrations of CoCl\(_2\) (50, 100, and 150 \( \mu \)M) for 24 h. Subsequently, cells were analyzed for expression of HIF-1\(\alpha\), erythropoietin (EPO), aldolase A, and \( \beta \)-actin mRNAs by RNA blot analysis. Five micrograms of RNA was loaded in each lane.

**Fig. 5.** EMSA of nuclear extracts from fibroblasts and HepG2 cells. A, fibroblasts were treated for 4 h with vehicle alone (lanes 1 and 2) or 10 \( \mu \)g/ml cycloheximide (CHX; lanes 3 and 4), and nuclear extracts were prepared. The protein extracts were analyzed for the presence of specific XRE-binding activity. Competition was performed by addition of a 100-fold molar excess of unlabeled XRE oligonucleotide (lanes 2 and 4). B, EMSA was performed with nuclear proteins extracted from fibroblasts or HepG2 cells treated for 1 h with 50 nM TCDF. Protein extracts were analyzed for the presence of specific XRE-binding activity by competition experiments with a 100-fold molar excess of an unrelated sequence motif (lanes 2 and 7) or the XRE sequence motif (lanes 3 and 8). To identify XRE-binding proteins, nuclear extracts were preincubated with diluted (1:10) anti-dioxin receptor antiserum (aDR; lanes 4 and 9) or anti-Arnt antiserum (aA; lanes 5 and 10) prior to the DNA-binding reaction. Note that anti-dioxin receptor and anti-Arnt antisera recognized a common single complex generated by both fibroblasts and HepG2 cell extracts (denoted Rec), whereas formation of fibroblast-specific complexes 1 and 2 was only affected by anti-Arnt antiserum. In both A and B, the asterisk indicates a nonspecific protein-XRE complex.
dioxin receptor antiserum (Fig. 6, compare lanes 3 and 6). The ~125- and ~90-kDa UV-cross-linked protein-DNA complexes were specific for the XRE (Fig. 6, compare lanes 3 and 5) as assessed by DNA competition experiments. Formation of these two complexes was, however, not affected either by antiserum against the dioxin receptor or by antiserum against the Arnt protein (Fig. 6, compare lanes 3, 6, and 7). It is conceivable that these two novel cross-linked proteins are part of the two complexes detected by EMSA when using fibroblast extracts. In conclusion, these data indicate that the constitutive XRE-binding factor(s) detected in fibroblasts are heteromeric complexes containing, in addition to an as yet unidentified partner factor(s), the bHLH/PAS factor Arnt.

**Treatment with Trichostatin A Derepresses P4501A1 Induction by Dioxin**—It was recently shown that transcriptional repression by sequence-specific DNA-binding factors can involve recruitment of an HDAC to the promoter region (reviewed in Ref. 20). Important tools to study the role of histone acetylation are specific inhibitors of HDAC activity such as sodium butyrate and the more potent and specific inhibitor TSA (28). Fibroblasts and HepG2 cells were transfected with a minimal XRE-luciferase reporter construct, and as a control, the reporter construct without XRE was used. Cells were then treated with dioxin in the absence or presence of TSA for 36–40 h. As shown in Fig. 7A, dioxin did not induce the reporter gene in fibroblasts. However, a 3-fold induction of luciferase activity was observed when fibroblasts were treated with both dioxin and TSA. In control experiments, high ligand-dependent inducibility of the XRE-luciferase reporter construct was only marginally reduced upon exposure to TSA.

To further clarify that TSA could relieve a repressive effect on dioxin inducibility in fibroblasts, P4501A1 mRNA levels were determined by RNA blot analysis. Fibroblasts and HepG2 cells were treated with dioxin and various concentrations of TSA. As expected, dioxin alone did not induce P4501A1 mRNA in fibroblasts. Cotreating fibroblasts with dioxin and increasing concentrations of TSA, however, resulted in induction of P4501A1 mRNA levels as compared with those detected in cells treated with TSA alone (Fig. 7B, compare lanes 3–8). In addition, when fibroblasts were treated with both TCDD and sodium butyrate, an induction of P4501A1 mRNA levels was detected (Fig. 7B, lanes 9 and 10). However, HepG2 cells showed no significant difference in dioxin inducibility after cotreatment with TSA (Fig. 7B, compare lanes 12–14).

**DISCUSSION**

Normal fibroblasts do not respond to dioxin with increases in target gene expression, and an XRE sequence is not capable of mediating ligand-dependent activation of a minimal promoter in this cell type (27). This lack of response is seen despite the fact that both the dioxin receptor and its partner factor Arnt are expressed in these cells. The data presented here suggest that nonresponsiveness is not due to lack of transactivating capacity of these two proteins. Instead, we have identified in fibroblasts a novel Arnt-interacting partner factor(s) that, in association with Arnt, directly contacts DNA. The expression of this factor correlates with the phenomenon that inducibility can be restored by inhibition of HDAC activity. Taken together, these data have interesting implications for the negative and positive regulation of bHLH/PAS proteins exerted at the level of recruitment of corepressors/activators.

Although the endogenous dioxin receptor can be activated to its DNA-binding form upon exposure to dioxin receptor ligands, this does not lead to induction of transcription of either CYP1A1 or CYP1B1 target genes or to increased expression of XRE-containing minimal promoter constructs in fibroblasts (27). Thus, it was important to establish that the functional properties of the dioxin receptor and Arnt, other than their DNA-binding activity, were not inhibited by post-translational mechanisms. By fusing the dioxin receptor and Arnt, devoid of their bHLH motifs, to a heterologous DNA-binding domain, the independent function of these two factors could be studied in transfected cells. More important, these experiments show that the chimeric dioxin receptor is conditionally regulated by dioxin and that the constitutive transcriptional activation function of Arnt is similar in both fibroblasts and dioxin-responsive HepG2 cells. In addition, endogenous Arnt appears to be functional when targeting a different response element together with another partner factor, HIF-1α, since the hypoxia response genes erythropoietin and aldolase A are induced under hypoxic conditions also in fibroblasts.

Using fibroblast protein extracts, three distinct XRE-binding complexes can be visualized after UV cross-linking to an XRE probe. One of these complexes represents the activated dioxin receptor. The other two complexes are detected only when using fibroblast extracts, and these are not related to either the dioxin receptor or the Arnt protein. Notably, analyses of protein-XRE complexes in vitro by EMSA show that the fibroblast-specific protein-XRE complexes harbor Arnt. Thus, these results strongly indicate the presence of a fibroblast-specific, DNA-contacting factor interacting with Arnt, the expression of which correlates with the nonresponsive phenotype of the cells.

This putative repressor protein is clearly distinct from a previously described P4501A1-repressing activity in a mutant hepatoma cell line (41) in that the complex described here is a novel DNA-binding species. Furthermore, repression by a heteromeric DNA-binding factor containing the bHLH/PAS factor Arnt represents a mechanism of negative regulation that is distinct from the action of the negative regulatory HLH factor Id that forms abortive, non-DNA-binding heteromers with bHLH partner factors of the myogenic family (42).

The identification of mechanisms underlying nonresponsiveness of cells in the presence of a functional activator is inter-
esting from a general perspective in that less is known about negative than positive regulation of gene expression. Several models have been proposed for how repressors mediate their negative regulatory activity. For example, the Ying-Yang-1 transcription factor can either repress or activate transcription depending on promoter context (43, 44), whereas Max can heterodimerize with either Myc or Mad and then function as a transcriptional activator or repressor, respectively (45). Furthermore, nuclear hormone receptors such as thyroid hormone receptors alternate in their function as repressors or activators depending on occupation of the hormone-binding domain (46). When bound to DNA, these heterodimers recruit the corepressors mouse Sin3 and/or NCoR, which are associated with an HDAC (22). This complex is believed to deacetylate adjacent chromatin structures, which results in transcriptional silencing. Using the HDAC inhibitor TSA, we show that repression of CYP1A1 mRNA induction was abrogated, resulting in ligand-dependent induction responses. This mechanism most likely is mediated through the XRE since a minimal XRE construct also was induced when fibroblasts were cotreated with dioxin and TSA. These results indicate that the fibroblast-specific XRE-binding factor recruits an HDAC activity, which subsequently silences transcription of CYP1A1. Recently, it has been shown that only MeCP2 (methylcytosine-binding protein-2) interacts with mouse Sin3 and maintains transcriptional repression (47). To exclude the possibility that cell type-specific inhibition of CYP1A1 is caused by DNA methylation, we used the demethylating agent 5-aza-2'-deoxycytidine. However, we could not detect any induction of CYP1A1 following cotreatment of fibroblasts with 5-aza-2'-deoxycytidine and dioxin (data not shown).

If Arnt is repressed in dioxin signal transduction, why is Arnt functional in partnership with HIF-1α in mediating hypoxia-inducible gene expression in fibroblasts? At present, we have no mechanistic data to explain this phenomenon. Interestingly, however, it has been shown that both dimerization with distinct partner factors (48) and binding to specific DNA response elements (49) may allosterically alter functional properties of certain steroid receptors. It is therefore possible that the conformation of the Arnt-HIF-1α complex in hypoxic cells is not permissive for interaction with corepressors and/or the fibroblast-specific factor. Recently, it has been reported that CBP/p300 plays a critical role in signal transduction by HIF-1α (50, 51). Moreover, CBP and the CBP-associated factors P/CAF and SRC-1 have been demonstrated to harbor histone acetyltransferase activity. Thus, when Arnt is interacting with HIF-

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**Fig. 7.** Inducibility of cytochrome P4501A1 is derepressed by treatment with trichostatin A. A, fibroblasts and HepG2 cells were transfected with 2 and 1 μg of pT81Luc and pTX.Dir, respectively. Luciferase activity after treatment with 10 nM TCDD in the presence or absence of either 0.3 or 3 μM TSA was determined. Results are representative of several independent experiments, and values have been normalized to protein content of cellular extracts. B, fibroblasts and HepG2 cells were cotreated with 10 nM TCDD and increasing concentrations of TSA (1.0, 1.5, 2.0, 2.5, and 3.0 μM) or 25 mM sodium butyrate (NaBu) for 24 h. Cytochrome P4501A1 and GAPDH mRNA levels were assayed by RNA blot analysis of 20 μg of total cellular RNA/lane. TK, thymidine kinase.
1α, histone acetyltransferase activity may therefore modulate the acetylation pattern of histones, which in turn will alter chromatin structure. However, when Arnt constitutively binds to the XRE together with the fibroblast-specific factor, the complex seems to function as a repressor, which indicates that, in the context of XRE binding, Arnt is involved in recruitment of corepressors. Thus, exchange of HIF-1α for the fibroblast-specific factor switches the Arnt heterodimer from transcriptional activator to repressor, suggesting that the cell type-specific fibroblast factor is needed for the recruitment of conceivable corepressors. Moreover, if the repressor in fibroblasts associates with a corepressor that harbors HDAC activity, do positive transcriptional effects of the dioxin receptor depend on recruitment of histone acetyltransferases? Although this issue has not been conclusively elucidated, it has been shown that induction of CYP1A1 transcription is dependent on the C-terminal region of the dioxin receptor, which has transcriptional capacity and mediates functional promoter communication with the transcription machinery (52). In addition, Kobayashi et al. (53) have observed that the histone acetyltransferase-containing coactivator CBP/p300 interacts with the C-terminal domain of Arnt. Together with our results, these observations are consistent with the model that the Arnt partner in fibroblasts may determine dominant-negative regulation of dioxin responsiveness by recruitment of a corepressor harboring HDAC activity. To further elucidate this mechanism of repression of dioxin signal transduction in fibroblasts, it will now be important to identify the Arnt-associated factor and to examine whether it is associated with a corepressor harboring HDAC activity.

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