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Maximal Aryl Hydrocarbon Receptor Activity Depends on an Interaction with the Retinoblastoma Protein

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ABSTRACT

The aryl hydrocarbon receptor (AhR) belongs to the basic helix-loop-helix/periodicity/AhR nuclear translocator/simple-minded (Per-Arnt-Sim) family of transcription factors that regulate critical functions during development and tissue homeostasis. Within this family, the AhR is the only member conditionally activated in response to ligand binding, typified by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). We recently demonstrated that the AhR interacts with the retinoblastoma protein (pRb). This report presents evidence that a LXCXE motif in the AhR protein confers pRb binding, which is necessary for maximal TCDD induced G_1 arrest in rat 5L hepatoma cells. The data support a mechanism whereby pRb seems to regulate G_1 cell cycle progression distinct from the direct repression of E2F-mediated transcription. Furthermore, the results indicate that the AhR-pRb interaction regulates TCDD induction of *CYP1A1*, suggesting that pRb may be a general AhR coactivator.

The aryl hydrocarbon Receptor (AhR) belongs to a growing family of transcription factors characterized by a basic helix-loop-helix (bHLH) DNA-binding domain and a PAS homology domain involved in protein dimerization (Schmidt and Bradfield, 1996). Interest in the AhR dates back to the mid-1970s because of its involvement in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxicity (Poland and Knutson, 1982). Among the various bHLH/PAS members, the AhR is the only protein conditionally activated in response to ligand binding. AhR ligands include the halogenated aromatic hydrocarbons typified by TCDD, polycyclic aromatic hydrocarbon (e.g., polychlorinated biphenyls), and related nonphysiological xenobiotics (e.g., heterocyclic amines). Several natural compounds are known to bind the AhR (Bjeldanes et al., 1991; Sinal and Bend, 1997; Schaldach et al., 1999), but whether they constitute physiologically relevant endogenous ligands requires further study. The structural diversity among the ligands, however, attests to the promiscuous nature of AhR ligand binding.

The unliganded AhR is a cytosolic protein bound to the chaperone 90-kDa heat shock protein 90 (hsp90) and an immunophilin-like molecule (reviewed by Whitlock, 1999). Upon ligand binding, the AhR translocates into the nucleus concomitant with dissociation from hsp90 and binds to regulatory *cis* elements in partnership with the Arnt protein, itself a member of the bHLH/PAS family. TCDD-induced AhR/Arnt DNA binding results in transcriptional regulation of target genes, including several genes encoding for the drug metabolizing enzymes *CYP1A1*, *1A2*, *1B1* and glutathione *S*-transferase Ya (Poland and Knutson, 1982; Whitlock, 1999). Two lines of evidence implicate the AhR in physiological processes germane to normal development and tissue homeostasis. First, TCDD exposure in animals induces teratogenesis, immunosuppression, reproductive defects, and tumor promotion. Second, AhR-null mice are resistant to TCDD toxicity, displaying instead certain abnormal phenotypes, including liver defects, a hyperproliferative phenotype in organs such as the stomach and vasculature, and decreases in circulating T and B cells (Schmidt et al., 1996; Gonzalez and Fernandez-Salguero, 1998). These observations suggest that the AhR influences normal cell proliferation and differentiation. Studies on growth rates of AhR-positive and -negative mouse and rat hepatoma cell lines revealed that the AhR modulates G_1 cell cycle progression (Ma and Whitlock, 1996; Weiss et al., 1996). The pronounced TCDD-induced G_1 arrest in the rat 5L hepatoma cell line involves an AhR-mediated induction of the cyclin-dependent kinase inhibitor, $p27^{Kip1}$ (Kolluri et al., 1999). Likewise, TCDD is known to inhibit

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ABBREVIATIONS: AhR, aryl hydrocarbon receptor; bHLH, basic helix-loop-helix; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PAS, Per-Arnt-Sim (periodicity/aryl hydrocarbon receptor nuclear translocator/simple-minded); hsp90, 90-kDa heat shock protein; Arnt, aryl hydrocarbon receptor nuclear translocator; pRb, retinoblastoma protein; CDK, cyclin-dependent kinase; bp, base pair(s); PCR, polymerase chain reaction; GFP, green fluorescent protein; WT, wild-type; RT, reverse transcription (or transcriptase); Me_2SO, dimethyl sulfoxide; TAD, transactivation domain; EMSA, electrophoretic mobility shift assay.

DNA synthesis in primary hepatocytes and hepatocyte proliferation in vivo after partial hepatectomy (Bauman et al., 1995; Hushka and Greenlee, 1995).

We recently showed that the AhR and Retinoblastoma tumor suppressor protein (pRb) interact directly (Ge and Elferink, 1998). Mechanistically, hypophosphorylated pRb regulates transition through the G_1/S restriction point by binding to and repressing several members of the E2F transcription factor family, thus preventing expression of genes required for S phase (Dyson, 1998). pRb and the related "pocket" proteins p107 and p130, contain a conserved A/B pocket domain that interacts with proteins harboring an LXCXE motif. These include the viral oncoproteins E1A and the simian virus 40 large T-antigen. Binding of pRb to E2F requires additional C-terminal residues composing the "large A/B pocket" (Qin et al., 1992). Hyperphosphorylation of pRb by the sequential action of the cyclin-dependent kinases 4/6 and 2 (CDKs) respectively, inactivates pRb culminating in entry into S phase (Sherr and Roberts, 1999). D-type cyclins control CDK4/6 activity and cyclins A and E regulate CDK2. In turn, CDK inhibitors comprise the INK4 proteins (p15, p16, p18, and p19) that bind to and inhibit the cyclin D-associated CDKs, and the more promiscuous Cip/Kip inhibitors ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$), which suppress most cyclin-CDK complexes, particularly cyclin E-CDK2 and cyclin A-CDK2 (Sherr and Roberts, 1999).

The documented importance of pRb in controlling E2F-mediated transcription notwithstanding, pRb interacts with many other proteins, raising the possibility that its action as a tumor suppressor depends in part on these interactions. Previously, we speculated that the LXCXE motif in the AhR contributes to the AhR-pRb interaction (Ge and Elferink, 1998). Yeast two-hybrid experiments confirm that this motif is indeed involved in the AhR-pRb interaction, which in subsequent functional studies is shown to be necessary for maximal TCDD-induced G_1 arrest in rat 5L hepatoma cells. *CYP1A1* expression studies suggest that the AhR-pRb interaction is required for maximal AhR transcriptional activity implying that pRb may be an AhR coactivator. The ramifications of these observations are considered in the context of cell cycle control.

Experimental Procedures

Materials. Restriction endonucleases and other DNA modifying enzymes (T4 DNA ligase, calf intestinal alkaline phosphatase) were purchased from Life Technologies Inc. (Gaithersburg, MD) and New England Biolabs (Beverly, MA). The MATCHMAKER LexA Two-Hybrid System used and yeast culture media were from CLONTECH (Palo Alto, CA). The *Taq* and KlenTaq DNA polymerases were obtained from Qiagen (Santa Clarita, CA) and Sigma (St. Louis, MO), respectively. The pRb antibody (G3-245) was from Pierce (Rockford, IL). TCDD was from the National Cancer Institute Chemical Carcinogen Reference Standard Repository. [^{3}H]TCDD was from the Cambridge Isotope Laboratories (Andover, MA). Western-Star and Galacton-Star kits were purchased from Tropix (Bedford, MA). Radioactive compounds were acquired from Amersham Pharmacia Biotech (Piscataway, NJ). Protein-G coupled Sepharose resin, custom synthesized oligonucleotides, LipofectAMINE Plus and G418 were from Life Technologies Inc. The pTRE and pTet-Off vectors were from CLONTECH and the hygromycin B was from Sigma. The hydroxylapatite was obtained from Bio-Rad (Richmond, CA).

Oligonucleotides. Sequences of the oligonucleotides used are presented in Table 1. The underlined nucleotide doublets denote the mutated bases introduced to generate the alanine-substituted constructs.

Yeast Plasmid Construction and the Two-Hybrid Assay. All AhR LexA-fusion proteins were constructed using pLexA vector (MATCHMAKER system) and were derived from a series of yeast 'bait' constructs described elsewhere (Ge and Elferink, 1998). Creation of the bHLH deletion constructs took advantage of two conveniently located *Eco*RI sites, one in the pLexA vector polylinker and the second at position 346 in the human AhR coding sequence. *Eco*RI digestion of pYAhRFL, pYAhR1–672, pYAhR1–589 and pYAhR1–528, followed by religation of the arms recovered the reading frame and removed a 381-bp fragment encoding the bHLH domain of the human AhR, to yield the constructs pYAhR117–853, pYAhR117–672, pYAhR117–589 and pYAhR117–528. Generation of the alanine-substituted constructs involved subcloning the *Not*I inserts from pYAhRFL, pYAhR1–672, pYAhR1–589, and pYAhR1–528 into a modified pLexA vector engineered to remove the vector's *Eco*RI and *Bam*HI sites by *Eco*RI/*Bam*HI digestion, end-filling with Klenow and religation. Site-directed mutagenesis involved a two-stage PCR protocol using KlenTaq polymerase in all the PCR intended for DNA cloning. The first round of PCR used the primer pairs oligonucleotides 3:2, 5:2, 7:2, 4:1, 6:1, and 8:1 as six individual PCR reactions (25 cycles). The second round of PCR combined DNA from reactions 3:2 and 4:1; 5:2 and 6:1; 7:2 and 8:1, and used oligonucleotides 1 and 2 to amplify the PCR products, resulting in 1052-bp PCR products that were digested with *Eco*RI and *Bam*HI, generating 925-bp fragments encoding the L331A, C333A, or E335A alanine substitutions. These fragments were used to replace the corresponding DNA fragment in pYAhRFL, pYAhR1–672, and pYAhR1–589 to generate the series of alanine substituted constructs. The pRb prey construct (pB42ADpRb) encodes amino acids 374 to 928 of the human pRb (Ge and Elferink, 1998). Constructs were checked by DNA sequencing using the dideoxynucleotide chain termination method. Yeast cells (leucine auxotrophic strain EGY48) were transformed using the LiAc method as described in the MATCHMAKER manual (CLONTECH). The two-hybrid assay uses two reporters (*LEU2* and *lacZ*), under the control of LexA operators.

Expression Plasmids. A full-length, wild-type rat AhR cDNA was generated from a cDNA clone (Elferink and Whitlock, 1994) by PCR amplification using oligonucleotides 13 and 14, and subcloned into the *Xba*I site of pTRE (CLONTECH) to yield pTRErAhR. Generation of the E333A mutation involved PCR amplification of pTRErAhR with primer pairs, oligonucleotides 9:12 and 10:11 (25 cycles), followed by a second round of PCR with oligonucleotides s11 and 12. The 654-bp PCR product was digested with *Eco*47III and *Spe*I, and the resultant 533 fragment cloned into pTRErAhR replac-

TABLE 1

Oligonucleotides used.

The underlined nucleotide doublets denote the mutated bases introduced to generate the alanine-substituted constructs

Oligonucleotide	Sequence
1	5′-GGAGGCCAGGATAACTGTAG-3′
2	5′-GAGTGGATGTGGTAGCAGA-3′
3	5′-CATGCTGCAGATATGGCTTATTGTGCCGAGTCCCA-3′
4	5′-TGGGACTCGGCACAATAAGCCATATCTGCAGCATG-3′
5	5′-CATGCTGCAGATATGCTTTATGCTGCCGAGTCCCA-3′
6	5′-TGGGACTCGGCAGCATAAAGCATATCTGCAGCATG-3′
7	5′-CATGCTGCAGATATGCTTTATTGTGCCGCTTCCCA-3′
8	5′-TGGGAAGCGGCACAATAAAGCATATCTGCAGCATG-3′
9	5′-ATGCTTCACTGCGCAGCATCCCAC-3′
10	5′-GTGGGATGCTGCGCAGTGAAGCAT-3′
11	5′-CTGGCAATGAATTTCCAAGGGAGG-3′
12	5′-GGTGGAAAGAATCCTTACTCGGGG-3′
13	5′-GCTCTAGAGGCACCATGAGCAGCGGCGCCAACATC-3′
14	5′-GCTCTAGACTACAGGAATCCGCTGGGTGTGAT-3′
15	5′-CCACACCTGTCACTGACAAAA-3′
16	5′-TCAATGAGGCTGTCTGTGATG-3′
17	5′-ACCAGGGCTGCCTTCTCTTGTGACAAAGTG-3′
18	5′-TGAGGTCCACCACCCTGTTGCTGTAGCCAT-3′

ing the *Eco*47III/*Spe*I fragment encompassing the LXCXE motif generating pTRErAhRE333A. Successful cloning was confirmed by sequencing. Synthesis of the recombinant adenoviral clone involved subcloning the rAhR sequence from pTRErAhR into the *Xba*I site of the pAdTrack-CMV shuttle vector (He et al., 1998). Recombination and analysis of the recombinants was performed as described by He et al. (1998). Viral stocks were prepared by recovering the viruses from infected human embryonic kidney 293 packaging cells, purified by CsCl banding and frozen at −80°C as single-use aliquots. The virus AdrAhRFL expresses the full-length rat AhR and the green fluorescent protein (GFP) from separate but identical CMV promoters. Viral titers were determined as GFP expression forming units/ml on 293 cells. We found that the number of expression forming units in BP8 cells is ≈200-fold lower than that of 293 cells, thus requiring a higher multiplicity of infection for AhR expression in BP8 cells.

Cell Culture, Transfections and Infections. Wild-type rat hepatoma 5L cells and AhR-defective BP8 variants were grown as monolayers in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 atmosphere at 37°C. All transfections were performed using LipofectAMINE Plus (Life Technologies) in accordance with the manufacturer's recommendations. For the stable transfections BP8 cells were transfected with the plasmid pTet-Off (CLONTECH) and grown in medium containing 500 μg/ml G418 for 14 days. A stable transfectant expressing the Tet repressor was clone purified before transfection with pTRErAhR or pTRErAhRE333A, and pTK-Hyg at a 10:1 ratio, respectively. Cells resistant to G418 and hygromycin B (100 μg/ml) selection were clone purified and screened for inducible AhR expression after removal of 1 μg/ml doxycycline. The BP8-WT and BP8-E333A lines used in this study express at a physiological level, the wild-type and alanine-substituted AhRs, respectively. Although the reason remains unclear, repeated attempts to isolate inducible cell lines proved unsuccessful. The lines are maintained in medium containing 500 μg/ml G418 and 100 μg/ml hygromycin B. Cells infected with the virus AdrAhRFL were seeded at 10^6 cells per 100-mm dish and cultured overnight before infection at a multiplicity of infection of 100:1. TCDD treatment was initiated 24 to 36 h after infection.

Flow Cytometry and Microscopy. Cells were trypsinized, washed twice in PBS containing 1 g/l glucose, and 5 mM EDTA, fixed in ice-cold 70% ethanol at 3×10^6 cells/ml, and stored at 4°C for at least 18 h. Cells were stained with 50 μg/ml propidium iodide and 1 mg/ml RNase A for 30 min in the dark at room temperature in the PBS buffer. DNA content analyses were performed on a Becton Dickinson FACSCalibur cytometer using CellQuest and ModFit software. For microscopy, cells were grown on chambered glass slides (Nunc, Naperville, CT) and infected with AdrAhRFL. Cells were fixed in 4% (w/v) paraformaldehyde for 10 min, washed three times in PBS, and imaged on a Nikon Microphot-SA fluorescence microscope fitted with a GFP filter and SPOT CCD camera. These studies were performed in the NIEHS Center for Molecular and Cellular Toxicology Imaging and Cytometry Facility Core at Wayne State University.

Immunoprecipitation and Western Blots. Subconfluent cultures were harvested by scraping on ice in 1 ml per 100-mm plate of buffer composed of 25 mM HEPES, pH 7.6, 1.5 mM EDTA, 5 mM dithiothreitol, and 10% glycerol containing 1 mM sodium vanadate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). Cells were lysed by Dounce homogenization (20 strokes) and the cell debris was removed by centrifugation at 18,000*g* for 30 min at 4°C. Protein from 200×10^3 cells was treated with Me_2SO or 10 nM TCDD for 2 h/20°C before immunoprecipitation with a primary antibody (IPn Ab) against pRb (anti-Rb) or the AhR (anti-AhR) for 4 h on ice, followed by precipitation with Protein-G resin. Beads were washed four times in radioimmunoprecipitation assay buffer, and the bound proteins were fractionated by 7.5% SDS/polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, blocked with 4% bovine lacto transfer optimizer in Tris-buffered saline containing 0.1% Tween 20. Filters were probed with the anti-pRb antibody and antibodies against mouse AhR and Arnt (kindly provided by Dr. R. Pollenz (Medical University of South Carolina, Charleston, SC) for 4 h at room temperature, followed by an alkaline phosphatase-conjugated secondary antibody for 1 h. Detection was by chemiluminescence using Western-Star and imaging on a GS-525 Molecular Imager.

RT-PCR. Total RNA was extracted from the cells using the method of Chomczynski and Sacchi (1987). First strand cDNA was generated from 1 μg of total RNA using a dT primer and Superscript II reverse transcriptase. PCR (30 cycles) using *Taq* polymerase was performed using oligonucleotide 15 and 16 (rat *CYP1A1*) and 17 and 18 (rat GAPDH) in the same reaction tube. The PCR products were fractionated on a 1.2% agarose gel and visualized by ethidium bromide staining. Images were captured digitally using a gel documentation system (Alpha Innotech, San Leandro, CA) and the bands quantitated using Molecular Analyst software (Bio-Rad).

Hydroxylapatite Binding Assay. Cytosol from the 5L and BP8 stable transfectants was prepared as described previously (Reiners et al., 1997). Cytosol (2 mg/ml, 0.1 ml) was incubated with 1% (v/v) Me_2SO or increasing concentrations of [^{3}H]TCDD (100 pM–10 μM, 40 Ci/mmol) in the absence or presence of 100-fold excess 2,3,7,8-tetrachlorodibenzofuran for 2 h at 20°C. [^{3}H]TCDD binding to hydroxylapatite followed the conditions described by Gasiewicz and Neal (1982). Specific [^{3}H]TCDD binding was determined by subtracting the scintillation counts for nonspecific binding ([^{3}H]TCDD+2,3,7,8-tetrachlorodibenzofuran) from total [^{3}H]TCDD binding for each ligand concentration.

Electrophoretic Mobility Shift Assay. 5L, BP8, BP8-WT, and BP8-E333A cells were grown to 80 to 90% confluence. The cytosolic fraction was prepared and the mobility shift assay performed as described in detail by Reiners et al. (1997). The complementary oligonucleotides 5′-GATCCGGCTCTTCTCACGCAACTCCGAGCTCA-3′ and 5′-GATCTGAGCTCGGAGTTGCGTGAGAAGAGCCG-3′ contain an AhR DNA binding site (underlined) were annealed and used as the DNA probe in the assay.

Results

We recently showed that the human AhR makes direct contact with the pRb through two distinct domains in the AhR (Ge and Elferink, 1998). One site is located within the N-terminal 364 amino acids of the AhR, whereas the second is confined to a glutamine (Q)-rich region located in the receptor's C-terminal transactivation domain (TAD). Within the N-terminal region are two potential pRb binding sequences: one is the bHLH domain and the second is a LXCXE motif. Both elements in other proteins are known to bind pRb (Gu et al., 1993; Lee et al., 1998). Using the yeast two-hybrid system, we first examined whether the AhR bHLH domain binds pRb (Fig. 1). We generated a series of "bait" constructs fusing the LexA repressor DNA binding domain to the AhR missing the bHLH domain (Fig. 1A). In addition, the constructs contain various C-terminal deletions because previous studies showed that the full-length AhR possesses a potent TAD within this region capable of driving yeast reporter expression in the absence of a "prey" construct (Ge and Elferink, 1998). Removal of the TAD was necessary to detect a two-hybrid interaction. Except for the yeast transformed with the AhR117–853 bait construct (containing the entire AhR TAD), the absence of pB42ADpRb prey construct prevented the leucine auxotrophic cells from growing on the leucine deficient X-gal plates (Fig. 1B, -pRb). Growth of cells

containing the C-terminal truncated AhR constructs required cotransformation with pB42ADpRb (Fig. 1B, +pRb). Quantitative β-galactosidase activity measuring LacZ reporter gene expression corroborates the plate assay detecting *LEU* reporter expression (results not shown). Hence, the evidence shows that the bHLH domain is not required for the AhR-pRb interaction.

Next, we examined whether the LXCXE motif is responsible for the physical interaction with pRb (Fig. 2). We introduced alanine substitutions by site-directed mutagenesis at each of the three conserved residues in the human AhR LXCXE motif (Fig. 2A; L331A, C333A, and E335A). Figure 2B shows the growth properties of the full-length AhR and two C-terminal truncation constructs (1–672 and 1–589) on leucine deficient X-gal plates in the absence or presence of pRb. Consistent with previous results examining growth on leucine deficient plates (Ge and Elferink, 1998), cells expressing the truncated wild-type AhR (WT) grow only in the presence of pB42ADpRb (Fig. 2B, +pRb). In contrast, coexpression of pRb fails to support cell growth of cells containing the mutated AhR constructs implying that the AhR-pRb interaction requires each of the conserved amino acids within the LXCXE motif. Our observations agree with the well-documented role for the LXCXE motif as a consensus pRb binding sequence (Weinberg, 1995). Quantitative β-galactosidase activity measurements support the plate assay results (results not shown). It is worth noting that the alanine substitutions in the full-length AhR (AhRFL) do not disrupt TAD activity, suggesting that the receptor's C-terminal region is not noticeably affected by the mutations.

To explore the functional significance of the AhR-pRb interaction we initiated studies in the 5L (AhR positive) and BP8 (AhR negative) rat hepatoma cell lines (Weiss et al., 1996). Asynchronous 5L cells exposed to 10 nM TCDD for 24 h undergo a pronounced G_1 arrest, marked by a shift from 48.8 ± 2.5% to 84.1 ± 3.3% of the cells in G_1 (Fig. 3). Unlike the 5L cells, the BP8 clonal variant, selected for its resistance to benzpyrene toxicity, fails to arrest after TCDD treatment. Pharmacological evidence for the role of the AhR in the TCDD-induced G_1 arrest comes from a dose-response study (Fig. 4). The EC_{50} concentration for G_1 arrest is 30 to 100 pM and is maximal by 300 pM, closely approximating the dose-response for *CYP1A1* induction (Nebert, 1989). Furthermore, 1 μM 3′methoxy-4′-nitroflavone, a high-affinity AhR antagonist that competes with TCDD for AhR binding and prevents receptor activation (Henry et al., 1999) completely suppresses the G_1 arrest induced by 150 pM TCDD. This result implies that the G_1 arrest requires AhR activation to a transcriptionally competent form. Using a recombinant adenovirus expression system (He et al., 1998) to express the wild-type rat AhR in BP8 cells, we obtained genetic evidence for the AhR's role in TCDD-induced G_1 arrest (Fig. 5). Approximately 20 to 30% of the BP8 cells were infected with the adenovirus 24 h before TCDD treatment as monitored by GFP expression (Fig. 5A). Infected cultures were treated with 10 nM TCDD for 24 h and sorted by flow cytometry using GFP as a marker (Fig. 5B). Noninfected (BP8-GFP) and infected (BP8+GFP) cells were collected and analyzed for DNA content by flow cytometry using propidium iodide (Fig. 5C). DNA content reveals that 86.8% of the AhR expressing cells (BP8+GFP) are in G_1 whereas only 65.1% of the uninfected cells (BP8-GFP) from the same culture were in G_1. This result agrees with the observations reported by Weiss et

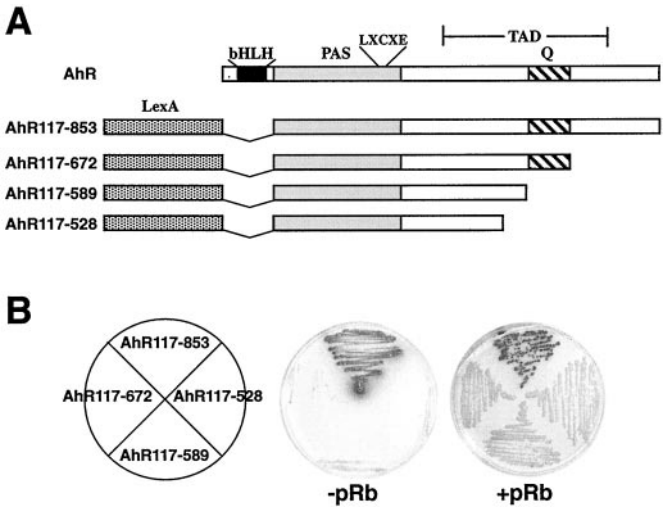

Fig. 1. The AhR-pRb interaction does not depend on the bHLH domain. A, the schematic shows the domain structure of the AhR and AhR-LexA fusion proteins, depicting the LexA DNA binding (stippled), bHLH (black), PAS (shaded), and glutamine-rich (hatched), and TAD domains. Also indicated is the location of the LXCXE motif within the PAS domain. B, yeast cells containing a LacZ reporter plasmid (p8op-LacZ) were transformed with the various bHLH-deleted AhR bait constructs in the absence (−pRb) and presence (+pRb) of plasmid pB42ADpRb and grown on leucine-deficient X-gal plates at 30°C for three to five days.

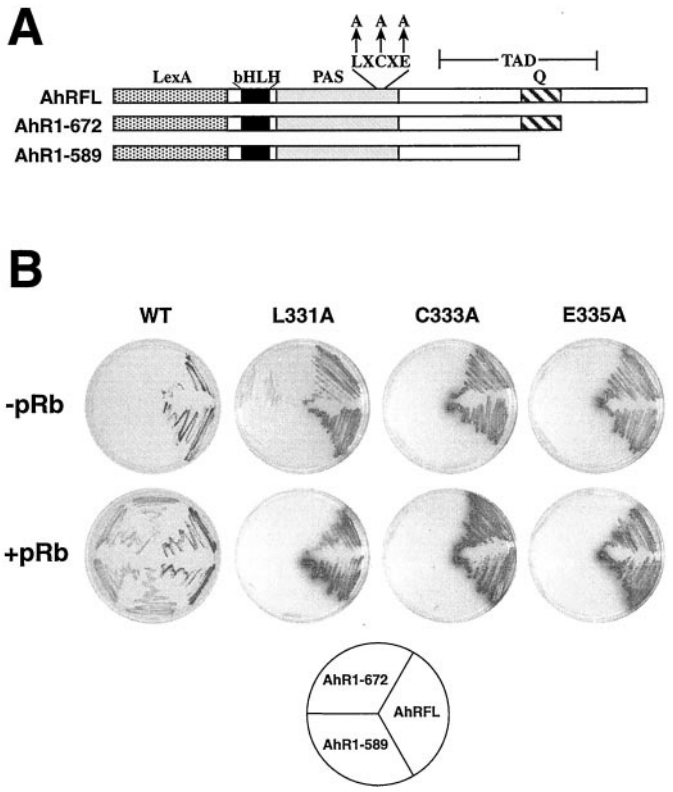

Fig. 2. Point mutations in the LXCXE motif disrupt the AhR-pRb interaction. Site-directed mutations were introduced into the AhR to substitute each of the conserved residues in the LXCXE motif with an alanine. A, yeast cells containing a LacZ reporter plasmid (p8op-LacZ) were transformed with the wild-type (WT) or various alanine-substituted AhR bait constructs (L331A, C333A, E335A) in the absence (−pRb) and presence (+pRb) of plasmid pB42ADpRb and grown on leucine-deficient X-gal plates at 30°C for 3 to 5 days. Synthesis of the LexA-AhR fusion construct encoding a wild-type AhR was described in Ge and Elferink (1998).

al. (1996). Control experiments using an adenovirus expressing LacZ confirm that the TCDD-induced G_1 arrest is specific for AhR expression (data not shown).

Given the AhR's involvement in TCDD-induced G_1 arrest in 5L cells and the well-documented role of pRb in blocking G_1 cell cycle progression (Dyson, 1998), we asked whether the AhR-pRb interaction is necessary for the TCDD-induced G_1 arrest. We generated stably transfected BP8 cell lines, one expressing the wild-type rat AhR constitutively (BP8-WT), and the second line expressing constitutively a mutated AhR harboring an alanine substitution at position 333 in the rat receptor's LXCXE motif (BP8-E333A). This change corresponds to the E335A mutation in the human AhR examined in the yeast two-hybrid system (see Fig. 2). Western blotting confirms expression of the AhR proteins in the BP8-WT and BP8-E333A lines at levels ≈30% and ≈100% of that in 5L cells, respectively (Fig. 6A). TCDD induced G_1 arrest in the 5L, BP8, and stable transfectant lines was examined by flow cytometry (Fig. 6B). The results show that TCDD induced a pronounced G_1 arrest in the BP8-WT line increasing the G_1 population by 27% from 56 to 83%, comparable with that seen with the 5L cells. In contrast, TCDD shifts the percentage of BP8-E333A cells in G_1 by only 9% from 54 to 63%. This represents a statistically significant decline in the TCDD inducible G_1 arrest (**$p < 0.01$), although the modest response seen in the BP8-E333A cells is siginificant (*$p < 0.05$). This residual G_1 arrest may reflect pRb binding to the Q-rich region in the AhR (Ge and Elferink, 1998), or represent AhR activity that is independent of pRb. Notwithstanding, the evidence suggests that the AhR mediated G_1 arrest in 5L cells is at least partially dependent upon the AhR-pRb interaction through the LXCXE motif.

Coimmunoprecipitation experiments were performed to confirm that the AhR's E333A point mutation in BP8-E333A cells indeed disrupts the AhR-pRb interaction (Fig. 7). Because AhR expression in the BP8-WT cells is too low to reliably detect the AhR in immunoprecipitates, we infected BP8 cells with the AdrAhRFL adenovirus to obtain AhR expression comparable with that in BP8-E333A cells. We and others have previously documented AhR-pRb coprecipitation in 5L cells (Ge and Elferink, 1998) and MCF-7 cells (Puga et al., 2000). We show here that an anti-AhR antibody can coprecipitate pRb and the Arnt protein in a TCDD-dependent manner from the virally infected BP8 cells (compare lanes 3 and 4). In contrast, although the mutant AhR in BP8-E333A cells binds the Arnt protein, it failed to associate with pRb. Hence, in keeping with the yeast two-hybrid observations, the E333A substitution in the LXCXE motif disrupts the AhR-pRb interaction in the hepatoma cells. Because Arnt binds to the mutant AhR, the conformation of the receptor's HLH domain (amino acids 28-80) seems to be unaltered by the alanine substitution at position 333.

The LXCXE motif lies within the AhR ligand-binding domain (Burbach et al., 1992). Hence, it is formally possible that the diminished G_1 arrest response in the BP8-E333A cells is caused by a mutation-induced conformational change in the AhR affecting ligand binding and receptor activation to a DNA binding form. Saturation-binding analyses were performed by incubating cytosols from 5L, BP8-WT, and BP8-E333A cells with [^{3}H]TCDD over a range of concentrations, and specific binding at each concentration determined using the hydroxylapatite assay (Gasiewicz and Neal, 1982). The overlapping saturation binding curves obtained with AhR from the various cell lines indicates that TCDD binding affinities for the wild-type and mutant receptor are essentially identical (Fig. 8). Therefore, ligand binding does not account for the functional difference between the wild-type and mutant AhR. Because ligand binding affinity is critically dependent upon hsp90 binding to this region of the AhR (Coumailleau et al., 1995), these data also infer that the E333A mutation does not disrupt the AhR-hsp90 interaction. Hence,

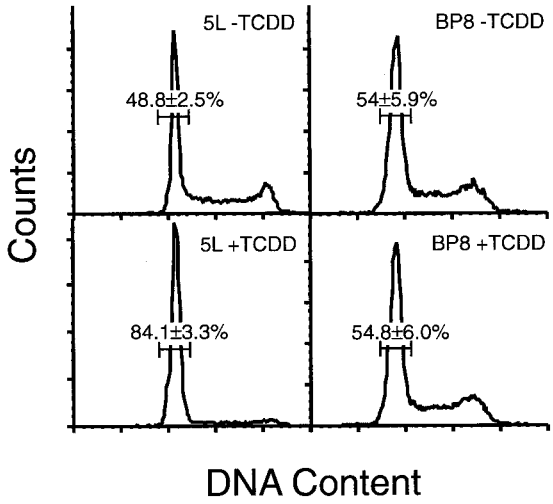

Fig. 3. TCDD induced G_1 arrest in rat 5L cells. Asynchronous 5L and BP8 cell cultures were grown in the presence of Me_2SO (−TCDD) or 10 nM TCDD (+TCDD) for 24 h, fixed in ethanol and stained with propidium iodide. DNA content in 20×10^3 cells was determined using a FACSCalibur cytometer equipped with CellQuest and ModFit software. The percentage of cells in G_1 is indicated and represents the mean ± S.D. from at least four experiments.

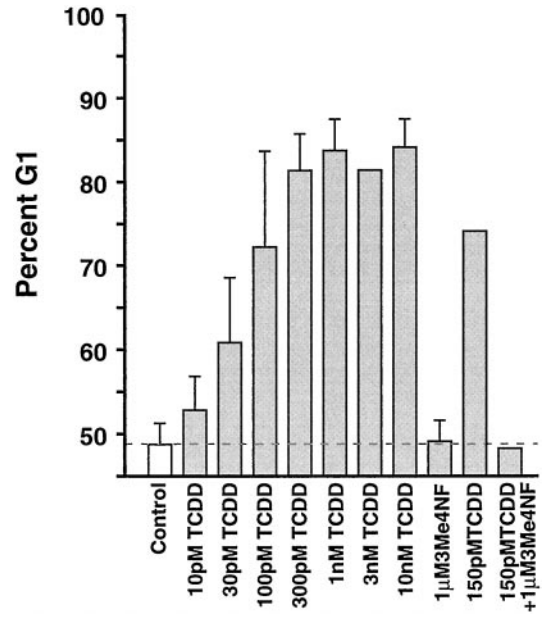

Fig. 4. Effect of AhR activation on G_1 arrest. Asynchronous 5L cells were grown in the absence (Control) or presence of TCDD (10 pM-10 nM), or the presence of 1 μM 3′methoxy-4′-nitroflavone (3 Me4NF) ± 150 pM TCDD for 24 h before ethanol fixation and propidium iodide staining. DNA content analyses were performed on 20×10^3 cells using the FACSCalibur flow cytometer equipped with CellQuest and ModFit software. The percentage of cells in G_1 is presented as the mean ± S.D. The dashed line denotes the G_1 content in untreated (control) asynchronous cultures.

this result provides compelling evidence that the E333A mutation—located within the ligand-binding domain—does not impart a conformational change upon the receptor protein. Ligand binding triggers AhR activation to a DNA binding form. Thus we also examined AhR DNA binding in vitro using the electrophoretic mobility shift assay (EMSA) (Fig. 9). EMSA detected the formation of TCDD-inducible AhR-DNA complexes in extracts from each of the cell lines expressing the AhR (Fig. 9, arrow). The evidence shows that the mutated AhR in BP8-E333A cells retains the capacity to bind DNA. However, comparing AhR protein levels (Fig. 6A) with the DNA binding properties (Fig. 10) in each cell line suggests that pRb may be contributing to AhR DNA complex formation. Because the E333A mutation specifically disrupts the pRb interaction, the identical migratory behavior of the DNA complex obtained with the wild-type or mutant AhR suggests that pRb is ordinarily not part of the in vitro complex detected by the EMSA. This agrees with our inability to "supershift" the AhR-DNA complex immunologically with an antibody against pRb (data not shown). Failure to detect protein components in the EMSA that contribute to DNA binding is not uncommon. Nguyen et al. (1999) could not detect the coactivator ERAP 140 in the AhR-DNA complex by EMSA, despite showing that it contributed to AhR DNA binding and function. Likewise, Gu et al. (1993), studying the MyoD-pRb interaction, failed to detect pRb in the MyoD-DNA complex in the EMSA, and estrogen receptor DNA binding and function is enhanced by cyclin D1 without forming a detectable ternary complex in the EMSA (Zwijsen et al., 1997). These reports illustrate that physiologically significant interactions in vivo are not necessarily recapitulated by the EMSA in vitro.

Kolluri et al. (1999) recently showed that the TCDD induced G_1 arrest depends on AhR-mediated induction of $p27^{Kip1}$. The evidence presented here indicates that the AhR-pRb interaction is required for maximal TCDD induced G_1 arrest. This suggests that pRb binding may be needed for full AhR transcriptional activity. Because TCDD inducible *CYP1A1* expression is a hallmark of AhR transcriptional activity, we examined *CYP1A1* expression in the various cell lines (Fig. 10). Total RNA was isolated from the 5L, BP8, BP8-WT, and BP8-E333A cell lines treated with vehicle or 10 nM TCDD for 24 h, and subjected to RT-PCR using primers specific for *CYP1A1* and GAPDH (as an internal PCR control for quantitative purposes). The *CYP1A1* expression profile in BP8-WT cells is nearly identical to that detected in 5L cells, whereas the induction response in BP8-E333A cells is significantly weaker ($*p < 0.05$) being only about 40% of that detected in the cells expressing a wild-type AhR. This resembles the difference in the G_1 arrest response (Fig. 6B, 27% in the BP8-WT versus 9% in the BP8-E333A cells). Given that the AhR in BP8-WT and BP8-E333A cells exhibits a comparable level of DNA binding (Fig. 9), the difference in AhR-mediated transcriptional activity implies that pRb may be functioning as a coactivator.

Discussion

This article provides evidence that maximal TCDD induced G_1 arrest and *CYP1A1* expression in rat 5L and BP8-WT cells relies on the AhR-pRb interaction involving the receptor's LXCXE motif. Furthermore, the evidence suggests that pRb functions as an AhR coactivator. Recently, Lu and Danielsen (1998) demonstrated that pRb binding to the androgen receptor is essential for its transcriptional activity, confirming that pRb can function as a transcriptional coactivator. Overexpression of pRb also potentiates glucocorticoid receptor transcriptional activity in concert with hBrm, a homolog of the *Saccharomyces cerevisiae* SWI2/SNF2 protein involved in chromatin remodeling (Singh et al., 1995). Because hBrm binds to pRb through a LXCXE motif however, pRb potentiation of AhR activity probably involves a mechanism distinct from that used by the glucocorticoid receptor. Instead, AhR coactivation may rely upon the interaction of pRb with $TAF_{II}250$, an integral component of the TFIID basal-transcription complex (Brehm and Kouzarides, 1999).

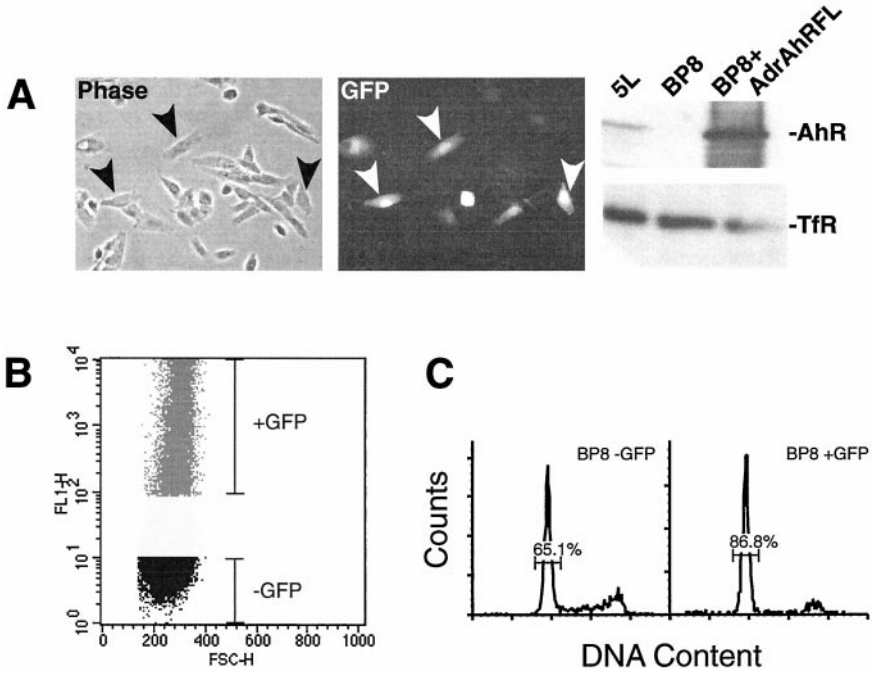

Fig. 5. TCDD induced G_1 arrest is an AhR-mediated event. A, BP8 cells (Phase) were infected with a recombinant adenovirus expressing the full-length, wild-type AhR (AdrAhRFL) for 48 h, resulting in approximately 20% infection as monitored by GFP expression (GFP). Arrowheads depict infected cells. AhR expression in the 5L, BP8, and virally infected BP8 cells (BP8+AdrAhRFL) was analyzed by Western blotting. Analysis of the transferrin receptor (TfR) is used as a loading control. B, the AdrAhRFL-infected BP8 cells were treated for 24 h with 10 nM TCDD, and the cells harvested and sorted on the basis of GFP fluorescence. Uninfected cells were gated between 10^0 and 10^1 FL1-H and infected cells between 10^2 and 10^4 FL1-H. C, sorted cells were ethanol fixed, stained with propidium iodide and the DNA content of the uninfected (BP8-GFP) and infected cells (BP8+GFP) expressing the AhR was determined.

It is noteworthy that drug-inducible expression of a CAT reporter construct under the control of the rat *CYP1A1* promoter could be repressed by cotransfection of a construct expressing the E1A viral oncoprotein (Sogawa et al., 1989). Futhermore, the effect of E1A was on XRE-mediated enhancer activity, suggesting that AhR-driven *trans*-activation was repressed by E1A. Because E1A contains a LXCXE motif and functions as an oncoprotein by sequestering pRb (Whyte et al., 1989), E1A's repression of the *CYP1A1* promoter may involve a mechanism denying the AhR access to pRb.

We previously showed that the AhR-pRb interaction occurs through two distinct AhR domains (Ge and Elferink, 1998). Yeast two-hybrid experiments (Fig. 2) and the coprecipitation results with the hepatoma cells (Fig. 7) confirm that the LXCXE motif makes up one of the AhR-pRb interaction domains. Binding to the LXCXE motif is negatively regulated by phosphorylation at pRb residues Thr 821 and Thr 826 (Zarkowska and Mittnacht, 1997), suggesting that pRb binding to the AhR is restricted to the hypophosphorylated, active form of pRb. Coprecipitation data presented here and else-

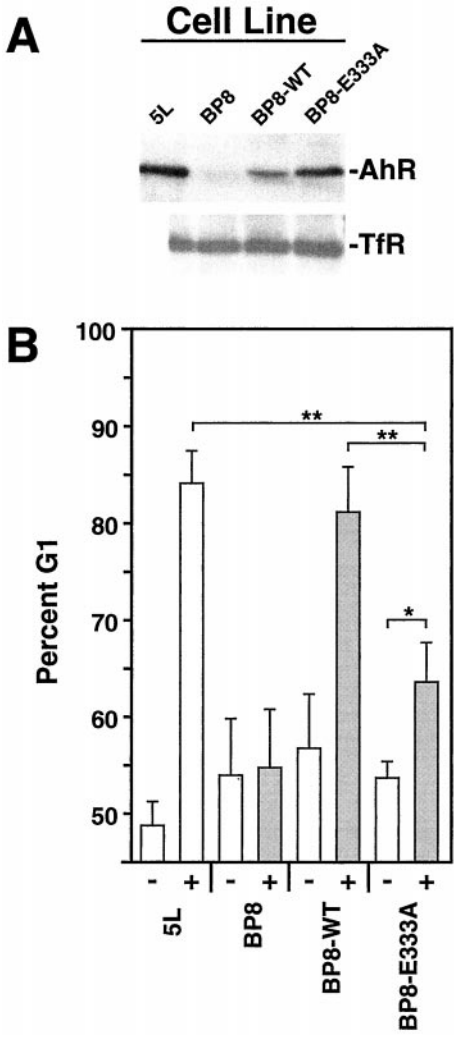

Fig. 6. A point mutation in the AhR LXCXE motif disrupts AhR-mediated cell cycle arrest. A, AhR protein levels in the 5L, BP8, and the two stable transfectants expressing constitutively a wild-type (BP8-WT) and a mutated (BP8-E333A) AhR, were determined by Western blot analysis. Probing for the transferrin receptor (TfR) was used as a loading control. B, asynchronous 5L, BP8, BP8-WT, and BP8-E333A cell cultures were grown in the presence of Me_2SO (−) or 10 nM TCDD (+) for 24 h, fixed in ethanol and stained with propidium iodide. DNA content in 20×10^3 cells was determined using a FACSCalibur cytometer equipped with CellQuest and ModFit software. The percentage of cells in G_1 is indicated and represents the mean ± S.D. from at least three experiments. $^{**}p < 0.01$ for the G_1 population difference between TCDD-treated 5L or BP8-WT cells and the BP8-E333A cells, $^{*}p < 0.05$ for the G_1 population difference between control and TCDD-treated BP8-E333A cells (determined using an unpaired Student's *t* test).

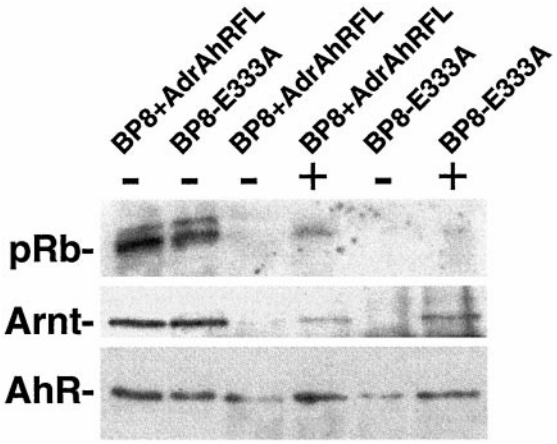

Fig. 7. A point mutation in the AhR LXCXE motif disrupts the AhR-pRb interaction. Coimmunoprecipitations were performed to assess pRb and Arnt protein binding with the wild-type AhR in virus-infected BP8 cells and the mutated AhR in BP8-E333A cells. BP8 cells were infected with AdrAhRFL at a multiplicity of infection of 20. Total cell lysates from 200×10^3 cells were treated with Me_2SO (−) or 10 nM TCDD (+) for 2 h at 20° C before immunoprecipitation with a primary antibody (Ipn Ab) against the AhR for 4 h on ice and precipitation with Protein-G resin. Total cell lysate (15 μg) (lanes 1 and 2) and immunoprecipitates (lanes 3-6) were analyzed by Western blotting after fractionation by SDS-polyacrylamide gel electrophoresis, transfer to membrane, and probing for the AhR, pRb, and Arnt.

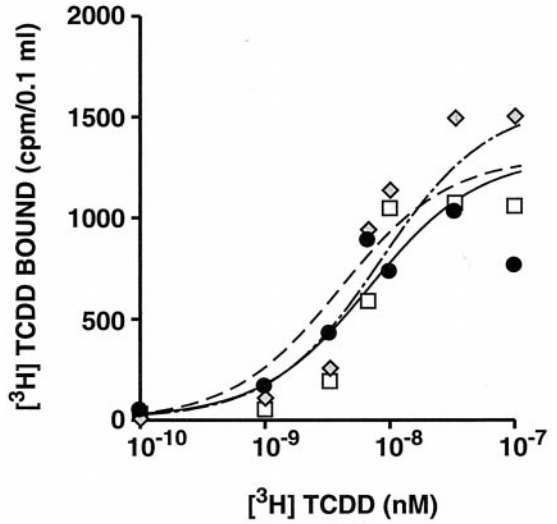

Fig. 8. Mutagenesis of the glutamic acid at position 333 in the rat AhR does not affect TCDD binding affinity. Saturation binding curves with [^{3}H]TCDD were generated using cytosolic extracts from the 5L (circles with solid line), BP8-WT (squares with dashed line), and BP8-E333A (diamonds with dot and dash line) cell lines. Cytosols (2 mg/ml, 0.1 ml) were incubated with [^{3}H]TCDD over a wide range of concentrations. Specific binding of [^{3}H]TCDD to the AhR was determined at each ligand concentration by the hydroxylapatite assay (Gasciewicz and Neal, 1982). Each value represents the mean of two to five independent determinations. Data was plotted using Biosoft Ultrafit software with the error bars omitted for clarity.

where supports this interpretation (Fig. 7; Ge and Elferink, 1998; Puga et al., 2000). Given that hypophosphorylated pRb is limited to the G_0 and G_1 phases of the cell cycle, it predicts that pRb-dependent AhR activity will be cell-cycle dependent. Efforts are currently underway to examine this prediction. The second pRb-binding domain is confined to an 83-amino-acid, Q-rich region within the TAD (Ge and Elferink, 1998). In vivo studies indicate that in the native chromosomal setting, TCDD-induced *CYP1A1* gene expression is heavily dependent upon the receptor's TAD (Ko et al., 1996). In this context, the residual *CYP1A1* expression (Fig. 10) and G_1 arrest (Fig. 6) seen in BP8-E333A cells may reflect pRb binding through the Q-rich domain that is undetectable under in vitro condition (Fig. 7) but nevertheless persists in vivo. Alternatively, this activity may be independent of pRb, relying instead on a direct AhR interaction with components of the transcriptional machinery such as transcription factor IIB (Swanson and Yang, 1998) or another coactivator such as RIP140, which was recently shown to bind the AhR Q-rich region (Kumar et al., 1999).

Kolluri et al. (1999) demonstrated that the TCDD-induced cell cycle arrest in 5L cells involves AhR-mediated induction of the CDK inhibitor $p27^{Kip1}$, although the precise induction mechanism remains unclear. $p27^{Kip1}$ is a potent inhibitor of cyclinE-CDK2 and cyclinA-CDK2 activity and the amount of "free" $p27^{Kip1}$ responsible for inhibiting CDK2 activity is tightly regulated (Sherr and Roberts, 1999). By suppressing CDK2 activity, pRb remains active as an inhibitor of E2F function and cells are prevented from entering S phase. We propose a model (Fig. 11) wherein AhR-mediated G_1 arrest results from $p27^{Kip1}$ induction preventing pRb phosphorylation, thereby keeping E2F repressed. Because our data suggest that hypophosphorylated pRb can function as an AhR coactivator, we envision establishment of a positive feedback loop sustaining AhR transcriptional activity—conditional upon the presence of an AhR agonist. In contrast, transition through the G_1/S checkpoint relies on CDK4/6 and CDK2-mediated pRb hyperphosphorylation to derepress E2F-regulated gene expression. In turn, E2F facilitates its own transcriptional activity by controlling expression of the cyclin E gene (Ohtani et al., 1995), thereby increasing cyclin E-CDK2 activity and hastening pRb hyperphosphorylation. Hence, E2F activity also establishes a positive feedback mechanism driving entry into S phase. Collectively, the opposing actions of $p27^{Kip1}$ and cyclin E-CDK2 function as a "binary switching mechanism" wherein G_1/S phase transition seems to require not only the emancipation of E2F transcriptional activity, but also AhR inactivation to terminate synthesis of $p27^{Kip1}$. As a regulatory component common to both pathways, pRb hyperphosphorylation meets both endpoints simultaneously. Hy-

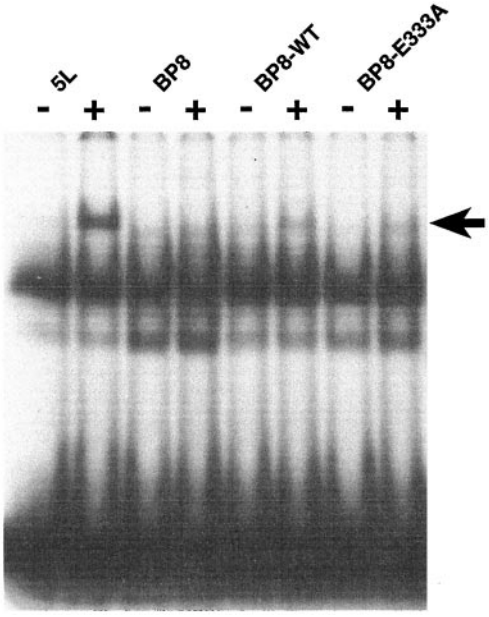

Fig. 9. Electrophoretic mobility shift assay on the AhR. Cytosolic extracts were prepared from the 5L, BP8, BP8-WT, and BP8-E333A cell lines as described previously (Reiners et al., 1997). The AhR was transformed in vitro with Me_2SO (−) or 10 nM TCDD (+) for 2 h/20°C and used in the mobility shift assay with a ^{32}P-labeled oligonucleotide probe containing an AhR binding site. The arrow denotes the position of the AhR-DNA complex. The data shows the result from a typical experiment repeated three times.

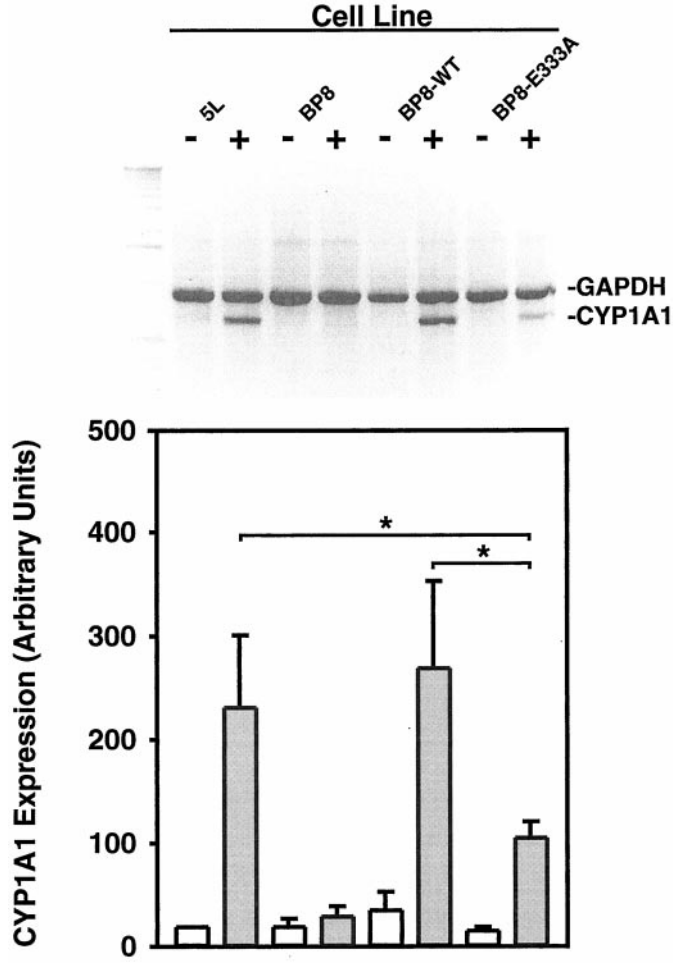

Fig. 10. The AhR-pRb interaction contributes to *CYP1A1* induction by TCDD. Total RNA was extracted from 5L, BP8, BP8-WT, and BP8-E333A treated with Me_2SO (−) or 10 nM TCDD (+) for 24 h. RT-PCR was performed on dT-primed, reverse-transcribed RNA (1 μg) followed by PCR using primers specific for rat CYP1A1 and rat GAPDH. PCR products were fractionated on a 1.2% agarose gel and visualized by ethidium bromide staining. Gel images were captured digitally using a CCD camera and the band signals were quantified using Molecular Analyst software (Bio-Rad). Quantification of the *CYP1A1* PCR products (normalized against GAPDH) showing the mean ± S.D. from three independent experiments is presented graphically. *$p < 0.05$ for the difference in *CYP1A1* PCR products between TCDD-treated BP8-E333A cells and the 5L or BP8-WT cells (determined using an unpaired Student's t test).

perphosphorylation of pRb may also explain why oncogenic *ras* suppresses AhR activity in MCF-10A human breast cancer cells (Reiners et al., 1997). Activation of *ras* and the downstream mitogen-activated protein kinase cascade in response to mitogens induces cyclin D1 expression (Peeper et al., 1997), triggering the activation of cyclin D-CDK4/6. CDK4/6 directly phosphorylates pRb but also sequesters $p27^{Kip1}$ away from the cyclin E-CDK2 complex, thus increasing CDK2 activity and further pRb phosphorylation (Sherr and Roberts, 1999). Hence, the inhibitory effect of *ras* on AhR activity may be due to pRb hyperphosphorylation.

An alternative mechanism for how the AhR might contribute to cell cycle arrest involves the formation of a ternary complex between E2F, pRb, and the AhR that represses E2F regulated gene expression. Puga et al. (2000) recently proposed such a mechanism based on the expression of E2F-driven reporter constructs. Repression of E2F activity by pRb has been shown to involve recruitment of histone deacetylase 1 by binding with the LXCXE motif in the deacetylase (Brehm et al., 1998), promoting histone deacetylation and formation of a transcriptionally less active chromatin conformation. However, the existence of the E2F/pRb/AhR ternary complex has not been demonstrated, nor has the AhR been shown to possess histone deacetylase activity. How, then, does TCDD suppress the E2F-driven reporter expression in mouse Hepa 1 cells (Puga et al., 2000)? In our hands, TCDD induces a rapid (4 h) and sustained (at least 48 h) increase in $p27^{Kip1}$ protein in the Hepa 1 cells (C. J. Elferink and A. Levine, unpublished observations). Moreover, the increase requires a functional AhR and Arnt protein. Discerning between a $p27^{Kip1}$ mediated mechanism and one involving a ternary complex will require further study, but it is worth noting that the published data favoring the ternary complex is entirely compatible with the model outlined in Fig. 11.

Evidence indicates that AhR ligand binding is necessary for nuclear translocation, dimerization with Arnt, and maintenance of a transcriptionally active AhR complex (Lees and Whitelaw, 1999). Although our study dealt with the effects of TCDD on cell cycle progression, the impact of AhR function in normal cell cycle progression in vivo cannot be fully gauged until a physiological (endogenous) ligand is identified. It will be interesting to see if the ligand is cell-cycle regulated.

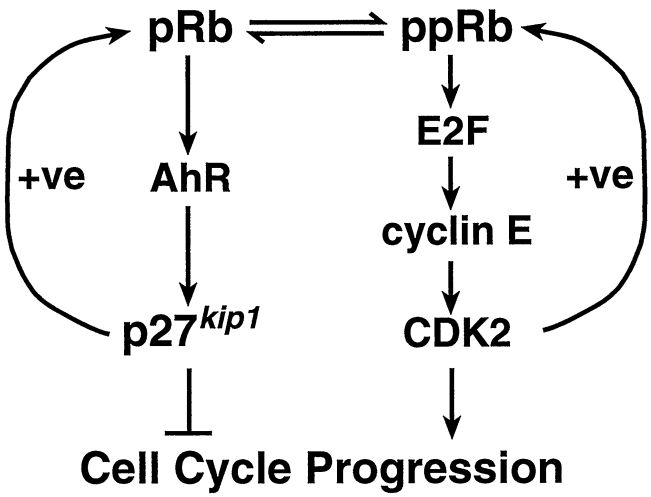

Fig. 11. A binary switch model for cell cycle progression. The diagram groups the components of the growth stimulatory and inhibitory positive feedback mechanisms (shaded) discussed in the text. AhR-mediated expression of $p27^{Kip1}$ inhibits cell cycle progression by repressing CDK2 activity thus preventing pRb phosphorylation, whereas E2F-regulated expression of cyclin E facilitates CDK2 activity, pRb hyperphosphorylation (ppRb), and entry into S phase. Not shown are the numerous other signaling molecules such as *ras*, the cyclin D-CDK4/6 complex, and pRb phosphatases that also influence the activity of these molecular components.

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