0026-895X/05/6701-88–96$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2005 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 67:88–96, 2005

Vol. 67, No. 1
2410/1189811
Printed in U.S.A.

Multiple Mechanisms Are Involved in Ah Receptor-Mediated Cell Cycle Arrest

Gengming Huang and Cornelis J. Elferink

Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, Texas

Received May 6, 2004; accepted October 14, 2004

ABSTRACT

The liver is the only solid organ that can respond to major tissue loss or damage by regeneration to restore liver biomass. The aryl hydrocarbon receptor (AhR) agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) can disrupt the regenerative process, as evidenced by suppression of DNA synthesis in rat primary hepatocytes in culture and in vivo liver regeneration after partial hepatectomy. Independent observations demonstrated that AhR-mediated G_1 phase cell cycle arrest depends on an interaction with the retinoblastoma tumor suppressor protein (pRb), but differences exist regarding proposed mechanisms of action. Two distinct models have been proposed, one supporting the AhR-pRb interaction functioning in corepression of E2F activity and the other favoring an AhR-pRb interaction participating in transcriptional coactivation of genes encoding G_1 phase regulatory proteins. In the present study, experiments in rat hepatoma cells using dominant-negative DNA-binding-defective AhR and Ah receptor nuclear translocator (Arnt) mutants provided evidence that TCDD-induced AhR-mediated G_1 arrest is only partially regulated by direct AhR transcriptional activity, suggesting that both coactivation and corepression are involved. Studies using a small interfering RNA to down-regulate Arnt protein expression revealed that TCDD-induced G_1 arrest is absolutely dependent on the Arnt protein.

The liver is the only solid organ that can respond to major tissue loss or damage by regeneration. A variety of insults, including physical injury, viral hepatitis, drug-induced toxicity, or ischemia, trigger the regenerative process. Although this unique ability for the liver to repair itself has been recognized since antiquity, exploration of the mechanisms began only fairly recently. Central to this process is hepatocyte proliferation, which requires normally quiescent cells to enter the cell cycle and divide until liver mass is restored. Recent findings examining aryl hydrocarbon receptor (AhR) activity in G_1 phase cell cycle control suggest a role for this protein in liver regeneration. The relationship between the AhR and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxicity is now well established, underscoring the significance of past observations that TCDD can suppress DNA synthesis in rat primary hepatocytes in culture (Hushka and Greenlee, 1995) and rat liver regeneration after partial hepatectomy (Bauman et al., 1995). This evidence is consistent with a cell cycle inhibitory role for the AhR. Several other reports describe a role for the AhR in cell cycle control; most of these reports identified G_1 phase as the cell cycle period influenced by AhR activity (Ma and Whitlock, 1996; Weiss et al., 1996; Ge and Elferink, 1998; Kolluri et al., 1999; Elizondo et al., 2000; Puga et al., 2000; Tohkin et al., 2000; Elferink et al., 2001; Levine-Fridman et al., 2004). The precise mechanism(s) involved, however, remains obscure.

The AhR is a member of the eukaryotic basic helix-loop-helix (bHLH)-PAS domain protein family that functions as sensors of extracellular signals and environmental stresses affecting growth and development (Gonzalez and Fernandez-Salguero, 1998; Gu et al., 2000), and evidence is mounting that the AhR contributes to normal physiological processes in growth and differentiation (Lahvis et al., 2000). The AhR is a soluble cytosolic protein in a complex with the chaperone proteins hsp90 (Perdew, 1988) and hsp23 (Kazlauskas et al.,

This work was supported by the National Institutes of Environmental Health Sciences (NIEHS) grant R01-ES07800 and, in part, by NIEHS Center grant ES06676.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.104.002410.

ABBREVIATIONS: AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; bHLH, basic helix-loop-helix; PAS-B, C-terminal Per/Arnt/Sim domain of human HIF-2; hsp, heat shock protein; XRE, xenobiotic response element; Arnt, Ah receptor nuclear translocator; pRb, retinoblastoma tumor suppressor protein; CDK, cyclin-dependent kinase; GFP, green fluorescent protein; CMV, cytomegalovirus; PCR, polymerase chain reaction; HEK, human embryonic kidney; siRNA, small interfering RNA; RFP, red fluorescent protein; MOI, multiplicity of infection; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; FACS, fluorescence-activated cell sorting; EMSA, electrophoretic mobility shift assay; RNAi, RNA interference; CBP, cAMP response element-binding protein-binding protein; BRG, brahma-related gene; ORF, open reading frame.

1999) and an immunophilin-like protein (Carver and Bradfield, 1997; Ma and Whitlock, 1997; Meyer et al., 1998). Upon ligand activation, the AhR translocates into the nucleus, dissociates from the hsp proteins, and binds to DNA response elements (known as a dioxin responsive element or XRE) with the Arnt protein, a heterodimerization partner and member of the PAS protein family (Lees and Whitelaw, 1999). The DNA-bound AhR/Arnt dimer recruits cofactors, and the complex modulates expression of target genes (Kumar et al., 1999; Elferink et al., 2001; Beischlag et al., 2002; Wang and Hankinson, 2002).

Independent findings demonstrated that the AhR interacts directly with the retinoblastoma tumor suppressor protein pRb (Ge and Elferink, 1998; Puga et al., 2000). The AhR-pRb interaction seems to be restricted to the hypophosphorylated "active" form of pRb (Puga et al., 2000; Elferink et al., 2001), and because hypophosphorylated pRb is confined to the G_0 (quiescence) and G_1 phase of the cell cycle, the AhR-pRb interaction—and functional consequences of this interaction—are likely to be cell cycle-dependent. Given that pRb is a major G_1 cell cycle checkpoints, controlling protein functionally inactivated by the G_1 cyclin-dependent kinases CDK4 and CDK2 (Sherr, 1996; Sherr and Roberts, 1999), it stood to reason and subsequent scientific scrutiny that the AhR-pRb interaction could suppress entry into S phase in various cell lines (Puga et al., 2000; Elferink et al., 2001; Levine-Fridman et al., 2004). However, interpretation of the data engendered two very different proposed mechanisms of action for AhR-mediated growth arrest. Puga et al. (2000) examined AhR and pRb activity in two human cancer cell lines, SAOS-2 and C33A. Ectopic expression of the AhR and pRb in these cell lines (which both lack endogenous expression of the proteins) and subsequent TCDD treatment blocked entry into the S phase of the cell cycle and repressed E2F-dependent gene expression of a luciferase reporter gene. As depicted in Fig. 1, these researchers suggested that the AhR forms a quaternary complex with pRb, E2F, and DP (the E2F-binding partner in transactivation) to directly corepress E2F-mediated expression of genes encoding proteins necessary for S phase (DNA synthesis). In contrast, we observed that maximal TCDD-induced G_1 arrest and induction of the *CYP1A1* gene in the 5L hepatoma cells depended on the AhR-pRb interaction (Elferink et al., 2001). Maximal *CYP1A1* induction by TCDD relies on pRb binding to the AhR, suggesting that pRb can function as a coactivator protein, a finding supported by previous reports (Singh et al., 1995; Lu and Danielson, 1998). Because TCDD promotes AhR-dependent expression of the CDK2 inhibitor $p27^{Kip1}$ in 5L cells (Kolluri et al., 1999), we proposed an indirect mechanism for TCDD-induced G_1 arrest involving $p27^{Kip1}$ inhibition of CDK2-mediated pRb inactivation. The experiments reported here show that AhR and Arnt protein DNA binding and heterodimerization, activities required for AhR transcriptional activation, only partially account for the G_1 arrest response. When viewed in conjunction with other recent findings (Marlowe et al., 2004), the evidence supports the conclusion that both modes of action (i.e., coactivation and corepression) are involved.

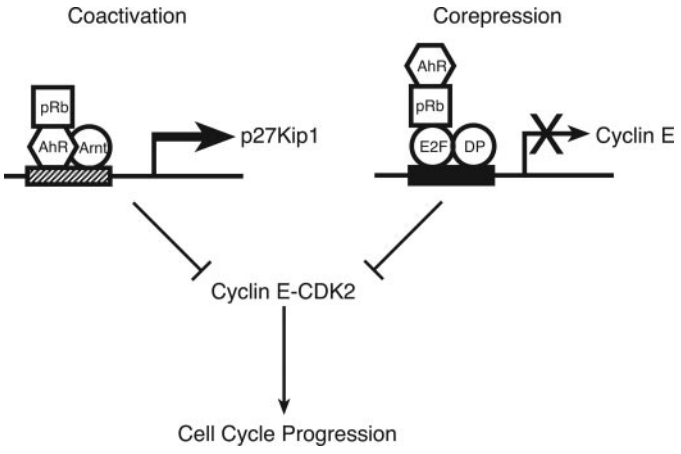

Fig. 1. Two models for AhR-mediated cell cycle arrest. The diagram depicts the components and proposed mechanisms of action for the coactivation (induction of $p27^{Kip1}$ expression) (see Kolluri et al., 1999; Marlowe et al., 2004) and corepression (repression of cyclin E expression) (see Marlowe et al., 2004) models. Both mechanisms inhibit CDK2 activity and prevent pRb inactivation, thereby suppressing cell cycle progression. To emphasize the salient distinctions between the models, documented interactions by other cofactors such as p300/CBP are not depicted.

Materials and Methods

Materials. Anti-AhR (rabbit polyclonal antibody) was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Hypoxia-inducible factor-1β/Arnt1, $p27^{Kip1}$, and pRb antibodies were purchased from BD Biosciences (San Jose, CA). Mouse anti-actin monoclonal antibody was obtained from Chemicon (Temecula, CA), [γ-^{32}P]ATP was acquired from Amersham Biosciences Inc. (Piscataway, NJ), and anti-rat *CYP1A1* was obtained from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Permanox chamber slides were obtained from Lab Tech (Naperville, IL). Alexa Fluor 594 goat anti-rabbit IgG(H+L) was obtained from Molecular Probes (Eugene, OR). Species-specific horseradish peroxidase-conjugated secondary antibodies were purchased from Zymed Laboratories (South San Francisco, CA). TCDD was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Kansas City, MO). Protein G-coupled Sepharose resin was purchased from Invitrogen (Carlsbad, CA). Propidium iodide, ribonuclease A, Dulbecco's modified Eagle's medium, and poly-L-lysine were purchased from Sigma-Aldrich (St. Louis, MO). Oligonucleotides were purchased from Sigma-Genosys (The Woodlands, TX).

Production of Adenoviruses. Generation of the virus AdrAhRFL (encoding a full-length wild-type rat AhR and GFP expressed from separate but identical CMV promoters) was described previously (Elferink et al., 2001). Generation of AdΔbArnt (encoding a dominant-negative Arnt protein lacking the basic region of the DNA-binding domain) involved a two-stage PCR using the primer pairs 1:3 and 2:4 in separate PCRs, combining the reactions and repeating the PCR with primer pair 1:4 to generate an Arnt protein-coding sequence with nucleotides 276 to 322 (encoding amino acids 90–105) of the ORF deleted. The PCR product was cloned into the XbaI site of pAdTrack-CMV (He et al., 1998), and DNA sequence was verified.

Recombination with the adenoviral genome encoded by pAdEasy1 and analysis of the recombinants were performed as described by He et al. (1998). The adenovirus AdAhR-R39/41A was generated by two-step PCR using AdrAhRFL DNA as a template and primer pairs 5:7 and 6:8 in separate reactions, followed by PCR with primers 5:8 on the combined DNA in a second PCR. The resultant PCR product was subcloned into the XbaI site of pAdTrack-CMV and recombined with pAdEasy1 to produce a virus encoding an AhR with arginines 39 and 41 mutated to alanine residues. The control adenoviruses AdGFP and AdRFP were generated by recombining pAdTrack-CMV (GFP) or pAdRFP—generated by replacing the GFP ORF with the ORF from pHcRed as described below—with pAdEasy1. PCR routinely involved a hot-start at 94°C, followed by 35 cycles oscillating

between 94°C for 15 s, 55°C for 30 s, and 72°C for 1 to 2 min, with a final extension reaction at 72°C for 6 min. Viral stocks were prepared by recovering the virus particles from infected human embryonic kidney (HEK) 293 packaging cells, purified by $CsCl_2$ banding, and frozen at −80°C as single-use aliquots. Viral titers were determined as GFP or RFP expression forming units per milliliter in 293 cells using a Zeiss Axiovert 200 fluorescence microscope equipped with filter sets for GFP (excitation at 489 nm; emission at 508 nm) and RFP (excitation at 588 nm; emission at 618 nm). Based on expression forming units in 5L or BP8 cells, viral infection requires an MOI of ~100 to obtain 100% infection.

Construction of Viral Shuttle Vectors for Expression of the Small Interfering RNA. The CMV promoter in pAdTrack-CMV (He et al., 1998) was altered through site-directed mutagenesis to introduce a BglII site at position 5 relative to the transcription start site. This facilitates cloning small interfering RNA (siRNA)-encoding double-stranded oligonucleotides so that transcription begins at the first residue of the siRNA sequence. PCR using primers 9 and 10 (Table 1) generated a 1.7-kilobase fragment that was directionally subcloned into the BstXI and KpnI site of pAdTrack-CMV and renamed pAdiGFP. To substitute the GFP ORF in pAdiGFP with the RFP from pHcRed (BD Biosciences), PCR on pHcRed using primers 11 and 12 generated a 1.5-kilobase fragment subsequently subcloned into the HpaI sites flanking GFP in pAdiGFP to generate pAdiRFP. The siRNA sequence targeting the rat and mouse Arnt protein transcript was generated by PCR using *Taq* polymerase, a template (oligonucleotide 13), and primers 14 and 15. The resultant 68-base pair PCR product was subcloned into pDriveTA vector (QIAGEN, Valencia, CA), and DNA was sequence-verified. The PCR product was subsequently subcloned into the BglII and KpnI sites of pAdiRFP. Recombination of the pAdiArnt shuttle vector with pAdEasy1 in BJ5183 cells and production of the AdiArnt adenoviruses in HEK293 cells was performed as described previously (He et al., 1998; Elferink et al., 2001).

Cell Culture, Infections, and Transfections. HEK293, AhR-negative BP8, and AhR-positive 5L cells were grown as monolayers in medium consisting of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 atmospheres at 37°C. For viral infection, BP8 and 5L cells were seeded at 2×10^6 cells per 100-mm dish and cultured overnight before infection with an MOI designed to obtain either partial or complete infection of the culture as indicated. Transfections of 293 cells with the recombined viral constructs were performed using LipofectAMINE Plus (Invitrogen) in accordance with the manufacturer's recommendations.

Indirect Immunofluorescence. BP8 cells were plated at a density of 2×10^4/ml on Permanox chamber slides (Lab-Tek, Naperville, IL) coated with 0.01% (w/v) poly-L-lysine and infected with AdrAhRFL, AdAhR-R39/41A, or AdGFP at an MOI of 100. Cells were maintained for 24 h at 37°C and subsequently treated with 6 nM TCDD (+) or Me_2SO (−) for 1 h. Cells were washed with PBS at room temperature and fixed for 30 min at room temperature in PBS containing 4% (w/v) paraformaldehyde, pH 7.4. Paraformaldehyde was removed by washing once in 20 mM PBS/glycine and twice in PBS for 5 min, and cells were permeabilized in PBS containing 0.2% (v/v) Triton X-100 for 5 min at room temperature, followed by three washes in PBS. Cells were preblocked with 10% (w/v) goat serum in PBS for 1 h at room temperature, followed by 3 μg/ml rabbit anti-AhR antibody in 10% (w/v) goat serum for 1 h. After five washes in PBS, cells were incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG(H+L) (1:600) in 10% (w/v) goat serum for 1 h and washed four times in PBS, and the slides were mounted with Fluoromount-G for visualization using a Zeiss Axiovert 200 fluorescence microscope.

Immunoprecipitation and Western Blots. For immunoprecipitations, the nuclear extract (500 μg) was incubated with the primary antibody (anti-AhR or anti-Arnt) for 4 h on ice followed by binding to protein G resin (preblocked with nuclear extract) for 1 h on ice. The resin was washed four times in radioimmunoprecipitation assay buffer [150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, and 5 mM EDTA] containing 0.5 mM phenylmethylsulfonyl fluoride, and the bound protein recovered in SDS-PAGE loading buffer.

Immunoprecipitated protein and total cell lysates were fractionated by 10% SDS-PAGE, transferred to the polyvinylidene difluoride membrane, and blocked with 5% bovine lacto transfer optimizer in Tris-buffered saline containing 0.1% Tween 20. Filters were probed with antibodies against the AhR, Arnt protein, P4501A1, $p27^{Kip1}$, pRb, or actin for 4 h at room temperature, followed by the appropriate horseradish peroxidase-conjugated secondary antibody at room temperature. Immunoreactivity was visualized at room temperature with the enhanced chemiluminescence Western blot detection reagent (Amersham Biosciences Inc.).

Flow Cytometry. Cells were collected by trypsinization and washed three times in Ca^{2+}/Mg^{2+}-free PBS containing 1g/l glucose and 5 mM EDTA before being fixed in 70% (v/v) ethanol at 10^6 cells/ml. Cells were stained with 50 μg/ml propidium iodide and 1 mg/ml RNase A for 30 min in the dark at room temperature and subjected to flow cytometry using CellQuest software on a BD Biosciences FACSCalibur cytometer. DNA content was determined using ModFit (Verity Software House, Topsham, ME).

Electrophoretic Mobility Shift Assay. Subconfluent BP8 cells cultures were infected with the adenoviruses AdAhR-R39/41A, AdrAhRFL, or AdGFP at an MOI of 100 for 24 h. The cytosolic extract

TABLE 1
Oligonucleotides used

Oligonucleotide	Sequence
1	5′-GCTCTAGAGGCACCATGGCGGCGACTACAGCTAAC-3′
2	5′-GAGAGACTTAACAAGATGACA-3′
3	5′-CATCTTGTTAACTCTCTCTTT-3′
4	5′-CGAGATCTCTATTCAGAAAAAGGGGGAAA-3′
5	5′-GCTCTAGAGGCACCATGAGCAGCGGCGCCAACATC-3′
6[a]	5′-TAAACGACACGCAGACGCGCTGAACAC-3′
7[a]	5′-GTGTTCAGCGCGTCTGCGTGTCGTTTA-3′
8	5′-GCTCTAGACTACAGGAATCCGCTGGGTGTGAT-3′
9	5′-CGCCCAGGCCGCCGCCCGTGGGCCC-3′
10	5′-GACGGTACCAGATCTCTAGCGGATCTGACAGATCTCTAAACCAGC-3′
11	5′-CGCGTTAACAATAAAAACCGTATTACCGCCATGCAT-3′
12	5′-ACCACAACTAGAATGCAGTGA-3′
13	5′-GGAAGATCTGTTACATCACAGAACTGTCATTCAAGAGATGACAGTTCTGTGATGTAATTGGTACCCCG-3′
14	5′-GGAAGATCTGTTACATCACAGAACTGTCAT-3′
15	5′-CGGGGTACCAATTACATCACAGAACTGTCAT-3′
16[b]	5′-GATCTGAGCTCGGAGTTGCGTGAGAAGAGCCG-3′ 3′-ACTCGAGCCTCAACGCACTCTTCTCGGCCTAG-5′

[a] Underlined nucleotides denote the substitutions changing the arginine codons to alanine codons.
[b] Underlined nucleotides identify the AhR DNA binding site.

fraction was prepared, and electrophoretic mobility shift assay (EMSA) was performed as described in detail by Shen et al. (1991). The AhR was transformed in vitro with Me_2SO (vehicle) or 6 nM TCDD for 2 h at 20°C, and EMSA was performed using a [γ-^{32}P]ATP end-labeled double-stranded DNA probe containing an AhR-binding site (Table 1, oligonucleotide 16).

Results

Published studies from independent laboratories showed that the AhR can inhibit G_1 phase cell cycle progression (Kolluri et al., 1999; Puga et al., 2000; Elferink et al., 2001). Although the findings concur that the growth arrest depends on a direct interaction between the AhR and hypophosphorylated (active) form of pRb, different mechanisms of action have been proposed. Puga et al. (2000) suggested that the AhR functions as a corepressor of E2F-mediated gene expression in conjunction with pRb. This model places the AhR in a quaternary complex bound to pRb but not directly to DNA nor in association with the Arnt protein (see Fig. 1). An alternative scenario suggests that transcriptional activity by the AhR/Arnt heterodimer—augmented by the pRb coactivation—promotes expression of the cyclin-dependent kinase inhibitor p27^{Kip1}, thus preventing inactivation of pRb and facilitating continued pRb-mediated repression of E2F. Published studies determined that the AhR-pRb interaction occurs primarily through an LXCXE motif located within the receptor's ligand-binding domain, although a second site in the C-terminal transactivation domain also contributes to pRb binding (Ge and Elferink, 1998; Puga et al., 2000; Elferink et al., 2001; Marlowe et al., 2004). It is significant that the AhR-pRb interaction does not require the bHLH domain necessary for AhR-DNA binding and dimerization with the Arnt protein (Elferink et al., 2001). Hence, experiments using targeted mutations in the AhR and Arnt proteins' DNA-binding domain (i.e., basic region) that specifically disrupt AhR or Arnt XRE binding ought to distinguish between the two models.

Initial efforts used a dominant-negative mutant of the Arnt protein (ΔbArnt) lacking the entire basic (DNA-binding) region in the bHLH domain, generated by deleting amino acids 90 to 102. Deletion of the basic region specifically abolishes DNA binding without affecting AhR dimerization through the HLH domain (Reisz-Porszasz et al., 1994; Bacsi and Hankinson, 1996) or its nuclear import (Eguchi et al., 1997). Hence, dimers between the receptor and ΔbArnt protein are unable to function as transcriptional complexes. Asynchronous cultures of 5L rat hepatoma cells infected with the adenovirus (AdΔbArnt) reveal a marked decline in both constitutive and TCDD-induced P4501A1 protein expression compared with cells infected with the control virus (AdGFP), consistent with overexpression of the dominant-negative ΔbArnt protein (Fig. 2A). The residual, albeit muted, induction response detected in cells infected with AdΔbArnt is attributed to the formation of a few functional AhR complexes resulting from incomplete suppression of endogenous Arnt protein. On the other hand, the residual response may reflect a normal induction in a small percentage of uninfected 5L cells despite using an MOI intended to achieve 100% infection. ΔbArnt expression also abolished the ~2-fold induction of p27^{Kip1} detectable after TCDD treatment in either uninfected or AdGFP-infected 5L cells (Fig. 2B). Furthermore, the data demonstrate that the level of ΔbArnt protein expression does not affect endogenous AhR or pRb protein levels. Coimmunoprecipitation experiments (Fig. 2C) confirm that ΔbArnt protein expression does not interfere with TCDD-dependent AhR-Arnt dimerization or the AhR-pRb interaction at remote binding sites identified previously (Ge and Elferink, 1998; Puga et al., 2000; Elferink et al., 2001; Marlowe et al., 2004).

Flow cytometric analyses on these cells to measure DNA content reveal that the TCDD-inducible G_1 phase growth arrest is completely abolished in cells expressing the ΔbArnt protein (Fig. 3). In contrast, cells infected with the AdGFP control virus retain the TCDD-induced G_1 arrest response. The ability of the ΔbArnt protein to disrupt the AhR-mediated cell cycle arrest is consistent with the Arnt protein functioning as a DNA-binding partner in the AhR-mediated gene expression of p27^{Kip1}. However, despite the evidence for ΔbArnt functioning as a dominant-negative (Fig. 2), it remains formally possible that overexpression of ΔbArnt sequesters the AhR, preventing it from functioning as a corepressor of E2F.

To better discriminate between the proposed models of action, we performed additional experiments using an AhR that was incapable of binding DNA to specifically abolish AhR transcriptional activity through XREs. Previous reports characterizing the AhR-DNA-binding domain identified several lysine and arginine residues necessary for DNA binding

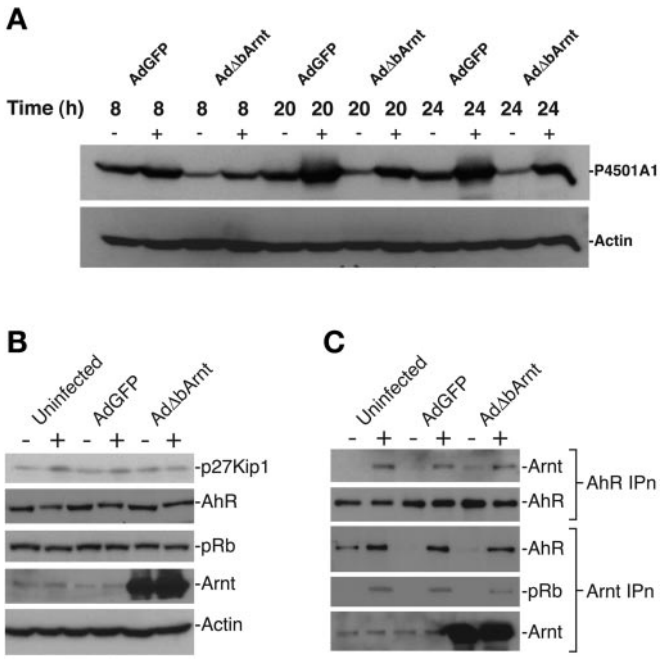

Fig. 2. The ΔbArnt protein suppresses P4501A1 expression in 5L cells. Subconfluent asynchronous cultures of 5L cells were infected with AdΔbArnt or AdGFP at an MOI of 100 to achieve 100% infection. A, cells treated with 6 nM TCDD (+) or Me_2SO (−) 24 h postinfection for the indicated times. Total cell lysates were prepared and analyzed by Western blotting for P4501A1 and actin (loading control). B, uninfected (lanes 1 and 2), AdGFP-infected (lanes 3 and 4), and AdΔbArnt-infected (lanes 5 and 6) cells grown for 24 h before treatment with 6 nM TCDD (+) or Me_2SO (−) for a further 24 h, and total cell lysates analyzed for the indicated proteins. C, cells infected as described in B and treated with 6 nM TCDD (+) or Me_2SO (−) for 1 h before preparation of nuclear extracts. The AhR and Arnt protein were immunoprecipitated from 500 μg of extract as described under *Materials and Methods* and analyzed by Western blotting for the presence of the AhR, Arnt, and pRb protein.

(Fukunaga et al., 1995; Bacsi and Hankinson, 1996; Dong et al., 1996; Swanson and Yang, 1996). Alanine substitutions replacing two arginines at positions 39 (R39A) and 41 (R41A) in the rat AhR encodes a receptor that no longer binds to an XRE (Fig. 4). AhR-negative BP8 rat hepatoma cells were infected for 24 h with the control adenovirus (AdGFP), a virus expressing the wild-type AhR (AdrAhRFL), or a virus expressing the DNA-binding-defective receptor mutant (AdAhR-R39/41A). XRE binding was assayed by the EMSA using the cytosolic AhR after in vitro transformation with 6 nM TCDD. The result confirms that the mutant AhR is unable to form a DNA complex (Fig. 4, lane 4), in contrast to the wild-type AhR that is readily detected as a TCDD-inducible AhR-DNA complex (Fig. 4, lane 6). However, the proximity of the nuclear localization signal with the region involved in DNA binding (Ikuta et al., 1998) limited the choice of residues suitable for site-directed mutagenesis without disrupting receptor nuclear import to Arg39 and Arg41. Indirect immunofluorescence on Me_2SO- and TCDD-treated BP8 cells infected with AdrAhRFL or AdAhR-R39/41A shows that the wild-type and mutant receptors translocate into nuclei to a similar degree, as indicated by the increase in nuclear staining after exposure to TCDD for 1 h (Fig. 5A). Staining for the AhR is specific because uninfected cells (see Fig. 5A) and AdGFP-infected cells (data not shown) are undetectable by immunofluorescence for Alexa Fluor 594. Western blot and coimmunoprecipitation experiments also show that the mutant AhR expressed in BP8 cells translocates into the nucleus in response to ligand stimulation and can interact with the Arnt protein (Fig. 5B). These results indicate that the double alanine substitution did not abolish receptor functional properties beyond the desired loss of DNA binding. As expected, the wild-type AhR regulated both basal and TCDD-induced *CYP1A1* expression, whereas the mutated AhR supports neither basal nor induced *CYP1A1* expression, consistent with the loss of DNA binding. The reason for the presence of AhR protein in nuclear extracts from the uninduced cells infected with AdrAhRFL and to a lesser extent AdAhR-R39/41A may be related to our recent finding that serum-derived growth factors can activate the AhR in these cells (Levine-Fridman et al., 2004). The indirect immunofluorescence data also reveal low-level nuclear staining in Me_2SO-treated cells indicative of basal nuclear AhR (Fig. 5A).

Cell cycle studies performed using BP8 cells infected with the AdrAhRFL or AdAhR-R39/41A virus revealed that expression of either the wild-type or mutant AhR promoted a TCDD-inducible G_1 arrest response, identified as an increase in the number of G_1 phase cells from ~60% in non-TCDD-treated cells (Fig. 6). However, it is noteworthy that the magnitude of the TCDD-induced arrest response is muted (i.e., increased from 60 to 69% of the cells in G_1) in cells expressing the mutant AhR compared with those expressing the wild-type receptor (from 60 to 78% in G_1). BP8 cells infected with the control virus (AdGFP) were nonresponsive to TCDD treatment. These data suggest that at least part of the G_1 arrest response involves a mechanism that does not depend upon direct AhR transcriptional activity but that XRE-dependent transcriptional activity is required for full TCDD-induced arrest.

Complete suppression of the TCDD-inducible arrest response by overexpressing the dominant-negative Arnt protein (Fig. 3) suggests that Arnt either actively participates in both mechanisms or disrupts AhR interactions necessary for cell cycle arrest. To test whether the Arnt protein is absolutely required for the G_1 arrest response, we used an adenovirus-based RNA interference (RNAi) strategy to knockdown Arnt protein expression. A modified adenovirus expression system was engineered to facilitate cloning and expression of siRNA transcripts targeting the rat Arnt transcript. A detailed description of the modified adenovirus system is provided under *Materials and Methods*. In contrast to

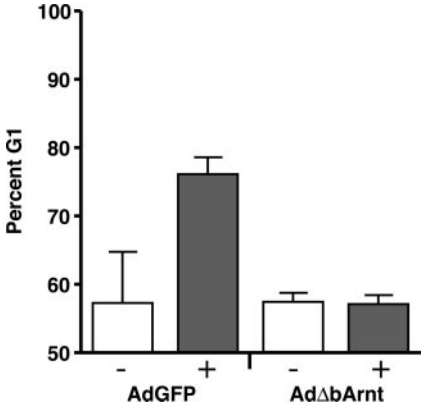

Fig. 3. The ΔbArnt protein suppresses the TCDD-induced G_1 arrest response in 5L cells. Subconfluent asynchronous cultures of 5L cells were infected with AdΔbArnt or AdGFP at an MOI of 100 to achieve 100% infection. After 24 h, cells were treated with 6 nM TCDD (+) or Me_2SO (−) for a further 24 h, fixed in ethanol, and stained with propidium iodide. DNA content was determined on 20×10^3 events using a FACSCalibur cytometer equipped with CellQuest and ModFit software. The percentage of cells in G_1 is indicated and represents the mean ± S.E.M. from at least three independent experiments.

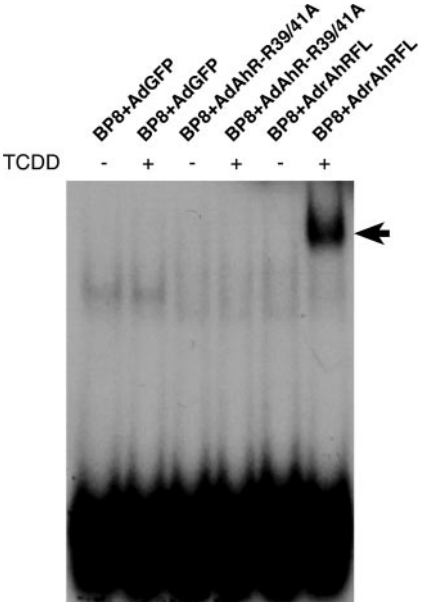

Fig. 4. Electrophoretic mobility shift assay on the wild-type and mutated AhR. Cytosolic extracts were prepared as described previously (Elferink et al., 2001) from asynchronous BP8 cells infected with the AdGFP (control), AdAhR-R39/41A (mutant AhR), or AdrAhRFL (wild-type AhR) adenovirus for 24 h at an MOI of 100. The AhR was transformed in vitro with Me_2SO (−) or 6 nM TCDD (+) for 2 h at 20°C and used in the mobility shift assay with a γ-^{32}P-labeled oligonucleotide probe containing an AhR-binding site (Table 1, sequence 16). The arrow denotes the position of the AhR-DNA complex. The data shown are from a single experiment repeated twice yielding identical results.

BP8 cells, preliminary experiments determined that the siRNA fails to down-regulate Arnt protein expression in 5L cells (data not shown). Why the 5L cells are refractory to the RNAi strategy remains unclear, but this finding precluded us from using siRNA in 5L cells. Infection of BP8 cells with the adenovirus (AdiArnt) completely suppresses Arnt protein expression within 72 h and remains suppressed for at least 120 h (Fig. 7). BP8 cells were coinfected with AdrAhRFL to compensate for the lack of AhR expression. Because the adenovirus AdiArnt coexpresses RFP, it is possible to monitor BP8 cells coinfected with AdrAhRFL and AdiArnt by virtue of GFP and RFP expression, respectively (Fig. 8A). It is worth noting that the excitation and emission spectra for the GFP and RFP are nonoverlapping, thus facilitating accurate identification of cells infected with both viruses. Using an MOI tailored to infect every cell, BP8 cells were initially infected with AdrAhRFL for 24 h to induce AhR expression before infection with AdiArnt (t = 0 h) and maintained for an additional 5 days. At daily intervals, cultures were treated with 6 nM TCDD for 8 h, and lysates were analyzed for Arnt protein, AhR, P4501A1, and actin expression (Fig. 7). The evidence clearly shows that the loss of P4501A1 inducibility matches the decline in Arnt protein expression. Moreover, the complete absence of P4501A1 expression after TCDD treatment confirms the efficacy with which the siRNA specifically down-regulated Arnt protein expression. Specificity for Arnt protein by the siRNA is indicated by the persistent expression of the AhR and actin. Furthermore, prolonged viral infection did not manifest overt cytotoxicity in the cultures (Fig. 8A).

Using this experimental paradigm, BP8 cells were infected with AdrAhRFL for 24 h followed by infection with AdiArnt or AdRFP (a control virus expressing RFP) for 72 h. Cultures were subsequently treated with 6 nM TCDD for a further 24 h, a sufficient period to detect a growth response, and DNA content was measured by flow cytometry. Fluorescence microscopy (Fig. 8A) on the infected cultures confirms that ~100% of the cells are coinfected with the AdrAhRFL (GFP) and the control virus AdRFP (RFP) or the siRNA-expressing virus AdiArnt (RFP). Flow cytometry reveals that suppressing Arnt protein expression completely abrogates the TCDD-induced G_1 arrest response (Fig. 8B). In contrast, BP8 cells

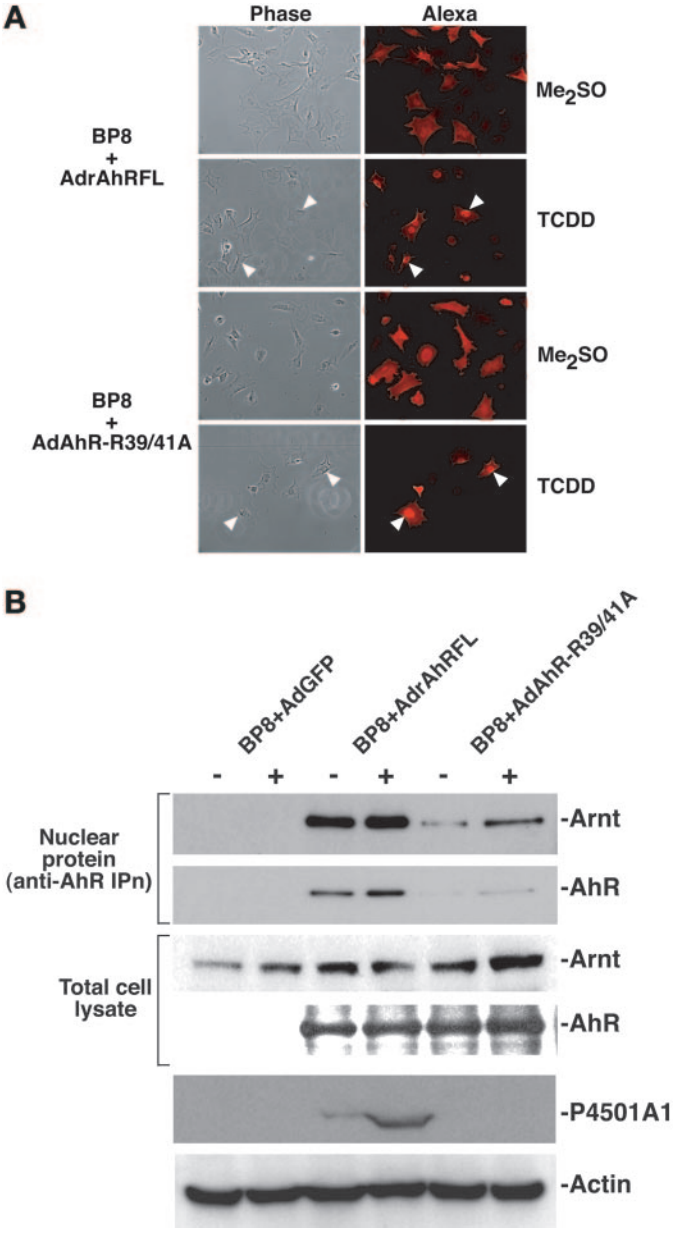

Fig. 5. The AhR DNA-binding mutant translocates into nuclei normally but fails to induce P4501A1. A, BP8 cells plated in chambered slides (3.5×10^4 cells per chamber) infected with AdrAhRFL, AdAhR-R39/41A, or AdGFP (not shown) at an MOI of 100 for 24 h before treatment with 6 nM TCDD for 1 h. Cells were fixed and stained with an anti-AhR antibody and an Alexa Fluor 594-conjugated goat anti-rabbit IgG(H+L) as described under *Materials and Methods*. Cells were visualized by phase contrast (Phase) and indirect fluorescence (Alexa) microscopy using a Zeiss Axiovert 200 microscope equipped with a charge-coupled device camera and AxioVision software. Nuclear AhR staining by immunofluorescence and the corresponding nuclei (Phase) are depicted by the white arrowheads. B, subconfluent cultures of asynchronous BP8 cells infected with the AdGFP (control), AdAhR-R39/41A (mutant AhR), or AdrAhRFL (wild-type AhR) adenovirus for 24 h at an MOI of 100. Cultures were subsequently treated with 6 nM TCDD (+) or Me_2SO (−) for 2 h, and total nuclear protein from 4×10^7 cells were immunoprecipitated with a rabbit anti-AhR antibody for 4 h on ice, followed by precipitation with protein G resin. Immunoprecipitates were analyzed by Western blotting for the AhR and Arnt protein, and total cell lysates were analyzed for the AhR, Arnt, P4501A1, and actin (loading control).

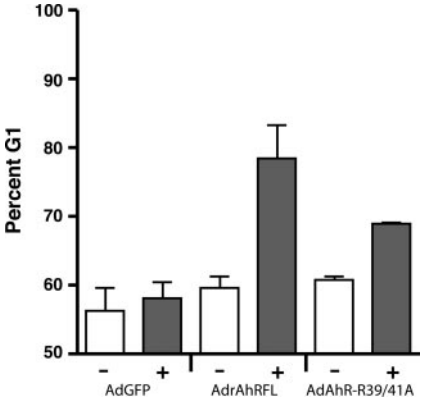

Fig. 6. The DNA-binding-defective AhR protein exhibits a residual capacity to G_1-arrest cells in response to TCDD. Subconfluent cultures of asynchronous BP8 cells were infected with the AdGFP (control), AdAhR-R39/41A (mutant AhR), or AdrAhRFL (wild-type AhR) adenovirus for 24 h at an MOI of 100. Cultures were subsequently treated with 6 nM TCDD (+) or Me_2SO (−) for 24 h, fixed in ethanol, and stained with propidium iodide. DNA content was determined on 20×10^3 events using a FACSCalibur cytometer equipped with CellQuest and ModFit software. The percentage of cells in G_1 is indicated and represents the mean ± S.E.M. from two independent experiments.

coinfected with AdrAhRFL and AdRFP retain the arrest response, consistent with our previously published observations (Elferink et al., 2001). The complete absence of a G_1 arrest response strongly suggests that the Arnt protein is critical to AhR-mediated growth suppression and that it participates in both proposed mechanisms of action.

Discussion

Independent observations made by several groups showed that the AhR suppresses progression through the G_1 phase of the cell cycle (Kolluri et al., 1999; Puga et al., 2000; Elferink et al., 2001). Moreover, the evidence also demonstrated that receptor-mediated growth arrest depended on an interaction with pRb; however, speculation concerning the mechanism of action yielded two very different and seemingly irreconcilable models (depicted in Fig. 1). The fundamental distinction between the two models is predicated on the notion that only coactivation depends on AhR-Arnt heterodimerization and XRE binding, and the selection of AhR and Arnt protein mutations used in this study was designed to specifically disrupt receptor transcriptional activity through the XRE. The findings described in this report suggest that, in fact, both coactivation and corepression contribute to the arrest response.

It is significant that our findings complement those recently published by Marlowe et al. (2004). Marlowe and coworkers used an E2F-driven reporter system to show convincingly that the AhR can function as a corepressor in conjunction with pRb (to deprive p300/CBP access to E2F) and provide a plausible explanation as to why our DNA-binding-defective AhR (AhR-R39/41A) retains some capacity to arrest cells. These researchers also showed that 1) TCDD could G_1-arrest MCF7 and Hepa-1 cells akin to the response in 5L cells (Kolluri et al., 1999; Elferink et al., 2001); 2) $p27^{Kip1}$ is induced by TCDD in Hepa-1 cells as previously reported in 5L cells (Kolluri et al., 1999; Levine-Fridman et al., 2004); 3) pRb can interact with the AhR through more than one domain, consistent with earlier findings (Ge and Elferink, 1998; Puga et al., 2000); and 4) the chromatin immunoprecipitation assay provided evidence for a pRb association with the endogenous *CYP1A1* promoter after TCDD treatment in Hepa-1 cells in addition to p300/CBP as previously reported (Beischlag et al., 2002). This last observation implies that pRb is part of the AhR-Arnt transcriptional complex in vivo and suggests that it may be functioning as a coactivator, consistent with our earlier results (Elferink et al., 2001).

The dominant-negative activity of the ΔbArnt protein suppressing both basal and inducible *CYP1A1* expression in 5L cells (Fig. 2A) and $p27^{Kip1}$ induction (Fig. 2B) is attributed to its ability to heterodimerize with the AhR (Fig. 2C) and form

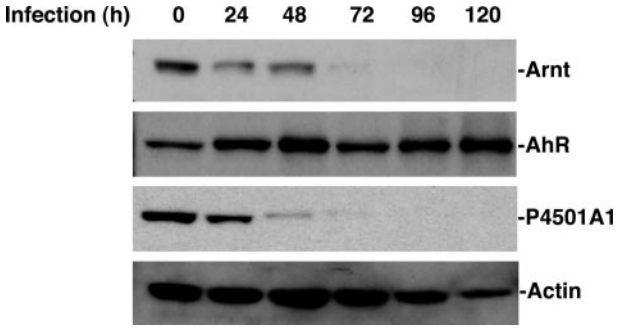

Fig. 7. RNAi-mediated down-regulation of Arnt protein expression efficiently suppresses AhR signaling. Subconfluent cultures of asynchronous BP8 cells were infected with AdrAhRFL at an MOI of 100 for 24 h and subsequently infected with AdiArnt at an MOI of 100 for the indicated period (0–120 h), at which time 6 nM TCDD was added for an additional 8 h before preparation of the total cell lysate for Western blot analysis. Western blotting was performed on SDS-PAGE-fractionated lysates for the Arnt protein, AhR, P4501A1, and actin (loading control).

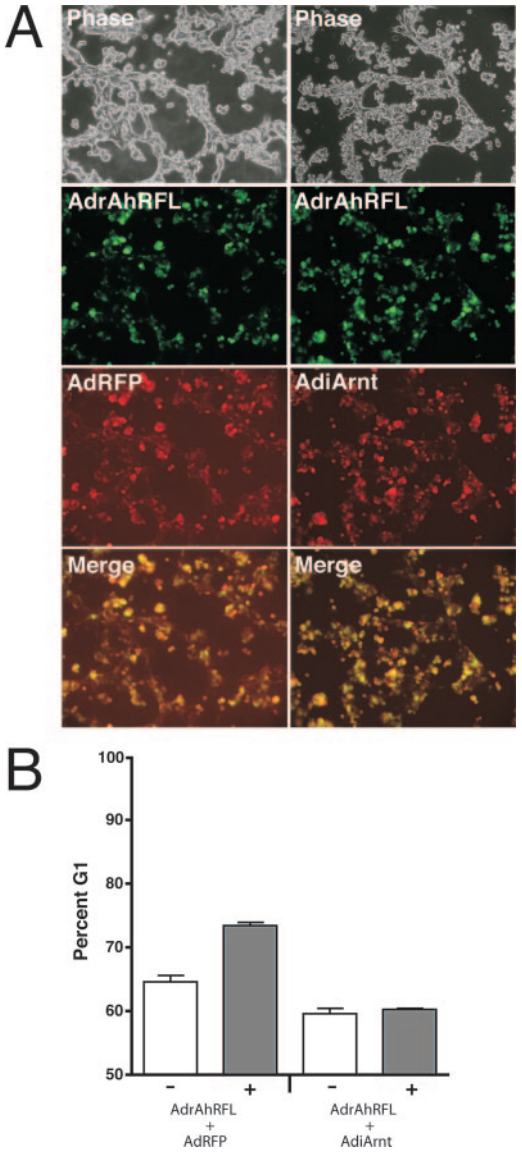

Fig. 8. Down-regulation of Arnt protein expression completely abolishes the TCDD-induced G_1 arrest response. Subconfluent cultures of asynchronous BP8 cells were infected with AdrAhRFL at an MOI of 100 for 24 h before being coinfected with AdRFP (control) or AdiArnt (Arnt protein siRNA) for an additional 48 h. Cultures were subsequently treated with Me_2SO (−) or 6 nM TCDD (+) for a further 24 h. A, phase contrast and fluorescence microscopy of the cultures identified cells infected with AdAhRFL (green) and AdRFP or AdiArnt (red). Coinfected cells are identified by yellow fluorescence in the merged fields. Infections were monitored in live cultures using a Zeiss Axiovert 200 inverted microscope, and images were captured with a charge-coupled device camera using AxioVision software. B, cells collected and fixed in ethanol before staining with propidium iodide and DNA content determined on 20×10^3 events using a FACSCalibur cytometer equipped with CellQuest and ModFit software. The percentage of cells in G_1 is indicated and represents the mean ± S.E.M. from two independent experiments.

a complex unable to bind DNA (Reisz-Porszasz et al., 1994). Hence, the loss of TCDD-inducible G_1 arrest in 5L cells expressing the ΔbArnt protein (Fig. 3) is consistent with the AhR regulating the growth arrest through a coactivation mechanism. However, AhR sequestration by the ΔbArnt protein thereby preventing the receptor from acting as a corepressor of E2F remains a formal possibility. To address this concern, studies were performed using the mutant AhR-R39/41A protein. Because the nuclear localization signal and DNA-binding domains largely overlap (Ikuta et al., 1998; Bunger et al., 2003), we tested the functional consequence of the alanine substitutions at positions 39 and 41 in the rat receptor. The evidence confirms that the mutations abolished AhR DNA binding (Fig. 4) but not nuclear translocation (Fig. 5A) consistent with a previous report (Ikuta et al., 1998), or dimerization with the Arnt protein (Fig. 5B). The reduced AhR protein level detected in the nuclear extracts from BP8 cells infected with AdAhR-R39/41A (Fig. 5B) is attributed to the defect in DNA binding, where the reduced avidity for DNA facilitates AhR loss from the nuclei during the subcellular fractionation as previously noted (Sun et al., 1997). It is worth noting that these point mutations do not affect the AhR-pRb interaction at remote regions of the receptor, namely the LXCXE motif and the C-terminal transactivation domain (Ge and Elferink, 1998; Puga et al., 2000; Elferink et al., 2001; Marlowe et al., 2004). In keeping with the loss of DNA binding, the AhR-R39/41A also fails to induce *CYP1A1* expression, indicating that the mutant receptor is devoid of transcriptional activity. Nevertheless, this protein expressed in BP8 cells exhibits a capacity to at least partially arrest cells in G_1 phase in response to TCDD (Fig. 6). Although the arrest response is weaker than that observed in cells expressing the wild-type receptor, the evidence demonstrates that the AhR can suppress passage through G_1 using a mechanism independent of direct DNA binding to an XRE. Because the receptor's ability to arrest cells in G_1 depends on the interaction with pRb (Puga et al., 2000; Elferink et al., 2001), the data presented suggest that maximal AhR-mediated cell cycle arrest involves both coactivation and corepression. Further studies will determine whether the growth arrest is restricted to induction of $p27^{Kip1}$ and repression of E2F transcription, reflecting coactivation and corepression, respectively. Recent observations, however, suggest that the AhR can also regulate cell growth through an autocrine or paracrine mechanism by modulating growth factor signaling (G. Huang and C. J. Elferink, manuscript in preparation).

Yeast two-hybrid and glutathione *S*-transferase pull-down data previously demonstrated that the AhR-pRb interaction is independent of the Arnt protein (Ge and Elferink, 1998). Furthermore, Marlowe et al. (2004) demonstrated that the Arnt protein is not required for corepression of the E2F-driven reporter. Hence, this represents a major distinction between the models depicted in Fig. 1. To examine the reliance on Arnt protein activity in AhR-mediated cell cycle arrest, Arnt protein expression was down-regulated using RNAi. We generated the adenovirus AdiArnt, engineered to express a siRNA against the Arnt protein mRNA, to suppress expression and, in effect, create a functional knockout phenotype. The evidence shows that the RNAi strategy specifically and completely suppressed the Arnt protein after about 72 h of infection (Fig. 7). An absence of the normally robust *CYP1A1* induction response to TCDD confirms the efficacy of the siRNA knock-down response. We performed these studies in BP8 cells (AhR-negative) coinfected with the AdrAhRFL virus because the 5L cells (AhR-positive)—from which the BP8 cells were derived—are nonresponsive to the RNAi strategy (data not shown). Although surprising, we speculate that the 5L cell line has lost or inactivated one or more of the host protein complexes (e.g., Dicer or the RNA-induced silencing complex) necessary for siRNA processing (Hannon, 2002; Shuey et al., 2002). BP8 cells infected with AdrAhRFL and a control virus (AdRFP) exhibited a TCDD-inducible G_1 arrest response consistent with our previous observations (Elferink et al., 2001). In contrast, coinfection with AdrAhRFL and AdiArnt resulted in complete abolition of the cell cycle arrest response after loss of Arnt protein expression (Fig. 8). Given that Marlowe et al. (2004) showed that the Arnt protein is unnecessary for the corepression of reporter constructs, we speculate that the absolute dependence on Arnt detected in our experimental system reflects a role for Arnt in displacing heat shock protein 90 from the AhR, particularly from the PAS-B domain (Lees and Whitelaw, 1999). Because the LXCXE motif responsible for pRb binding lies within the PAS-B domain, it is conceivable that the Arnt-mediated dissociation of hsp90 from the AhR exposes the PAS-B region and promotes the AhR-pRb interaction necessary for both corepression and coactivation.

In terms of AhR-mediated transcriptional coactivation and corepression, BRG-1 also deserves attention. BRG-1 and related brahma proteins are homologs of the yeast SWI2/SNF2 protein involved in ATP-dependent chromatin remodeling (Fry and Peterson, 2001). BRG-1 is known to bind directly to pRb (Strober et al., 1996) and represses E2F transcription to promote G_1 cell cycle arrest (Trouche et al., 1997; Strobeck et al., 2000), possibly involving recruitment of a histone deacetylase (Brehm et al., 1998). BRG-1 also binds to the AhR; however, in this context, it functions as a coactivator of *CYP1A1* gene expression (Wang and Hankinson, 2002). These researchers mapped the AhR-BRG-1 interaction to the receptor's glutamine-rich region associated with its transactivation domain. Our previous finding that the AhR glutamine-rich region also binds pRb raises some intriguing questions regarding the composition of the quaternary complexes and their functional properties.

In this report, we also describe the modification and use of the adenovirus system to express a siRNA. Using an adenovirus to express siRNA molecules against target transcripts has been recently demonstrated by others, each using distinct strategies to generate viral constructs (Xia et al., 2002; Shen et al., 2003; Zhao et al., 2003). The primary advantage of using the adenoviral system is the ease with which hard-to-transfect mammalian cells can be transduced to express a siRNA, including cells in vivo (Xia et al., 2002). Major attributes of the modifications we introduced include 1) efficient directional cloning of the oligonucleotide encoding the presiRNA, which folds into a short hairpin that is processed into a mature siRNA (Brummelkamp et al., 2002); 2) the CMV promoter transcription start site coincides with the first nucleotide in the siRNA; and 3) coexpression of the GFP or RFP permits identification of coinfected cells. It is noteworthy that the spectral properties of the GFP (excitation at 489 nm; emission at 508 nm) and RFP (excitation at 588 nm; emission at 618 nm) are nonoverlapping, allowing for unambiguous identification of doubly infected cells. In addition,

expression of the siRNA transcript and fluorescent protein (GFP or RFP) are proportional due to the use of distinct but identical CMV promoters; thus, fluorescence intensity in infected cells provides a reliable measure of siRNA expression. We envision that this system offers the versatility to downregulate almost any selected protein. Coinfection with a second virus—expressing the alternate fluorescent protein to identify coinfected cells—provides the opportunity to express an additional siRNA or mutant forms of the suppressed host protein to analyze the impact of targeted mutations on function. If necessary, coinfected cells can be isolated and recovered as a pure population by FACS.

Acknowledgments

We are deeply indebted to Dr. K. Mitchell for discussion and helpful comments during manuscript preparation.

References

Bacsi SG and Hankinson O (1996) Functional characterization of DNA-binding domains of the subunits of the heterodimeric aryl hydrocarbon receptor complex imputing novel and canonical basic helix-loop-helix protein-DNA interactions. *J Biol Chem* **271:**8843–8850.

Bauman JW, Goldsworthy TL, Dunn CS, and Fox TR (1995) Inhibitory effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on rat hepatocyte proliferation induced by 2/3 partial hepatectomy. *Cell Prolif* **28:**437–451.

Beischlag TV, Wang S, Rose DW, Torchia J, Reisz-Porszasz S, Muhammad K, Nelson WE, Probst MR, Rosenfeld MG, and Hankinson O (2002) Recruitment of the NCoA/SRC-1/P160 family of transcriptional coactivators by the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator complex. *Mol Cell Biol* **22:**4319–4333.

Brehm A, Miska EA, McCance DJ, Reid JL, Bannister AJ, and Kouzarides T (1998) Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature (Lond)* **391:**597–601.

Brummelkamp TR, Bernards R, and Agami R (2002) System for stable expression of short interfering RNAs in mammalian cells. *Science (Wash DC)* **296:**550–553.

Bunger MK, Moran SM, Glover E, Thomae TL, Lahvis GP, Lin BC, and Bradfield CA (2003) Resistance to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity and abnormal liver development in mice carrying a mutation in the nuclear localization sequence of the aryl hydrocarbon receptor. *J Biol Chem* **278:**17767–17774.

Carver LA and Bradfield CA (1997) Ligand-dependent interaction of the aryl hydrocarbon receptor with a novel immunophilin homolog in vivo. *J Biol Chem* **272:**11452–11456.

Dong L, Ma Q, and Whitlock JP Jr (1996) DNA binding by the heterodimeric Ah receptor. Relationship to dioxin-induced *CYP1A1* transcription in vivo. *J Biol Chem* **271:**7942–7948.

Eguchi H, Ikuta T, Tachibana T, Yoneda Y, and Kawajiri K (1997) A nuclear localization signal of human aryl hydrocarbon receptor nuclear translocator/hypoxia-inducible factor 1beta is a novel bipartite type recognized by the two components of nuclear pore-targeting complex. *J Biol Chem* **272:**17640–17647.

Elferink CJ, Ge NL, and Levine A (2001) Maximal aryl hydrocarbon receptor activity depends on an interaction with the retinoblastoma protein. *Mol Pharmacol* **59:**664–673.

Elizondo G, Fernandez-Salguero P, Sheikh MS, Kim GY, Fornace AJ, Lee KS, and Gonzalez FJ (2000) Altered cell cycle control at the G_2/M phases in aryl hydrocarbon receptor-null embryo fibroblast. *Mol Pharmacol* **57:**1056–1063.

Fry CJ and Peterson CL (2001) Chromatin remodeling enzymes: who's on first? *Curr Biol* **11:**R185–R197.

Fukunaga BN, Probst MR, Reisz-Porszasz S, and Hankinson O (1995) Identification of functional domains of the aryl hydrocarbon receptor. *J Biol Chem* **270:**29270–29278.

Ge NL and Elferink CJ (1998) A direct interaction between the aryl hydrocarbon receptor and retinoblastoma protein. Linking dioxin signaling to the cell cycle. *J Biol Chem* **273:**22708–22713.

Gonzalez FJ and Fernandez-Salguero P (1998) The aryl hydrocarbon receptor: studies using the AHR-null mice. *Drug Metab Dispos* **26:**1194–1198.

Gu YZ, Hogenesch JB, and Bradfield CA (2000) The PAS superfamily: sensors of environmental and developmental signals. *Annu Rev Pharmacol Toxicol* **40:**519–561.

Hannon GJ (2002) RNA interference. *Nature (Lond)* **418:**244–251.

He T-C, Zhou S, Da Costa LT, Yu J, Kinzler KW, and Vogelstein B (1998) A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci USA* **95:**2509–2514.

Hushka DR and Greenlee WF (1995) 2,3,7,8-Tetrachlorodibenzo-p-dioxin inhibits DNA synthesis in rat primary hepatocytes. *Mutat Res* **333:**89–99.

Ikuta T, Eguchi H, Tachibana T, Yoneda Y, and Kawajiri K (1998) Nuclear localization and export signals of the human aryl hydrocarbon receptor. *J Biol Chem* **273:**2895–2904.

Kazlauskas A, Poellinger L, and Pongratz I (1999) Evidence that the cochaperone p23 regulates ligand responsiveness of the dioxin (aryl hydrocarbon) receptor. *J Biol Chem* **274:**13519–13524.

Kolluri SK, Weiss C, Koff A, and Gottlicher M (1999) $P27^{Kip1}$ Induction and inhibition of proliferation by the intracellular Ah receptor in developing thymus and hepatoma cells. *Genes Dev* **13:**1742–1753.

Kumar MB, Tarpey RW, and Perdew GH (1999) Differential recruitment of coactivator RIP140 by Ah and estrogen receptors. Absence of a role for LXXLL motifs. *J Biol Chem* **274:**22155–22164.

Lahvis GP, Lindell SL, Thomas RS, McCuskey RS, Murphy C, Glover E, Bentz M, Southard J, and Bradfield CA (2000) Portosystemic shunting and persistent fetal vascular structures in aryl hydrocarbon receptor-deficient mice. *Proc Natl Acad Sci USA* **97:**10442–10447.

Lees MJ and Whitelaw ML (1999) Multiple roles of ligand in transforming the dioxin receptor to an active basic helix-loop-helix/PAS transcription factor complex with the nuclear protein Arnt. *Mol Cell Biol* **19:**5811–5822.

Levine-Fridman A, Chen L, and Elferink CJ (2004) Cytochrome P4501A1 promotes G_1 phase cell cycle progression by controlling aryl hydrocarbon receptor activity. *Mol Pharmacol* **65:**461–469.

Lu J and Danielsen M (1998) Differential regulation of androgen and glucocorticoid receptors by retinoblastoma protein. *J Biol Chem* **273:**31528–31533.

Ma Q and Whitlock JP Jr (1996) The aromatic hydrocarbon receptor modulates the Hepa 1c1c7 cell cycle and differentiated state independently of dioxin. *Mol Cell Biol* **16:**2144–2150.

Ma Q and Whitlock JP Jr (1997) A novel cytoplasmic protein that interacts with the Ah receptor, contains tetratricopeptide repeat motifs, and augments the transcriptional response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J Biol Chem* **14:**8878–8884.

Marlowe JL, Knudsen ES, Schwemberger S, and Puga A (2004) Aryl hydrocarbon receptor displaces p300 from E2F-dependent promoters and represses S phase-specific gene expression. *J Biol Chem* **279:**29013–29022.

Meyer BK, Pray-Grant MG, Vanden Heuvel JP, and Perdew GH (1998) Hepatitis B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity. *Mol Pharmacol* **18:**978–988.

Perdew GH (1988) Association of the Ah receptor with the 90-kDa heat shock protein. *J Biol Chem* **263:**13802–13805.

Puga A, Barnes SJ, Dalton TP, Chang C, Knudsen ES, and Maier MA (2000) Aromatic hydrocarbon receptor interaction with the retinoblastoma protein potentiates repression of E2F-dependent transcription and cell cycle arrest. *J Biol Chem* **275:**2943–2950.

Reisz-Porszasz S, Probst MR, Fukunaga BN, and Hankinson O (1994) Identification of functional domains of the aryl hydrocarbon receptor nuclear translocator protein (ARNT). *Mol Cell Biol* **14:**6075–6086.

Shen C, Buck AK, Liu X, Winkler M, and Reske SN (2003) Gene silencing by adenovirus-delivered siRNA. *FEBS Lett* **539:**111–114.

Shen ES, Elferink CJ, and Whitlock JP Jr (1991) Use of gel retardation to analyze protein-DNA interactions upstream of CYPIA1 gene. *Methods Enzymol* **206:**403–409.

Sherr CJ (1996) Cancer cell cycles. *Science (Wash DC)* **274:**1672–1677.

Sherr CJ and Roberts JM (1999) CDK inhibitors: positive and negative regulators of G_1-phase progression. *Genes Dev* **13:**1501–1512.

Shuey DJ, McCallus DE, and Giordano T (2002) RNAi: gene-silencing in therapeutic intervention. *Drug Discov Today* **7:**1040–1046.

Singh P, Coe J, and Hong W (1995) A role for retinoblastoma protein in potentiating transcriptional activation by the glucocorticoid receptor. *Nature (Lond)* **374**:562–565.

Strobeck MW, Knudsen KE, Fribourg AF, DeCristofaro MF, Weissman BE, Imbalzano AN, and Knudsen ES (2000) BRG-1 is required for RB-mediated cell cycle arrest. *Proc Natl Acad Sci USA* **97:**7748–7753.

Strober BE, Dunaief JL, Guha S, and Goff SP (1996) Functional interactions between the hBRM/hBRG1 transcriptional activators and the pRB family of proteins. *Mol Cell Biol* **16:**1576–1583.

Sun W, Zhang J, and Hankinson O (1997) A mutation in the aryl hydrocarbon receptor (AHR) in a cultured mammalian cell line identifies a novel region of the AHR that affects DNA binding. *J Biol Chem.* **272:**31845–31854.

Swanson HI and Yang J (1996) Mapping the protein/DNA contact sites of the Ah receptor and Ah receptor nuclear translocator. *J Biol Chem* **271:**31657–31665.

Tohkin M, Fukuhara M, Elizondo G, Tomita S, and Gonzalez FJ (2000) Aryl hydrocarbon receptor is required for p300-mediated induction of DNA synthesis by adenovirus E1A. *Mol Pharmacol* **58:**845–851.

Trouche D, Le Chalony C, Muchardt C, Yaniv M, and Kouzarides T (1997) RB and hbrm cooperate to repress the activation functions of E2F1. *Proc Natl Acad Sci USA* **94:**11268–11273.

Wang S and Hankinson O (2002) Functional involvement of the Brahma/SWI2-related gene 1 protein in cytochrome P4501A1 transcription mediated by the aryl hydrocarbon receptor complex. *J Biol Chem* **277:**11821–11827.

Weiss S, Kolluri K, Kiefer F, and Gottlicher M (1996) Complementation of Ah receptor deficiency in hepatoma cells: negative feedback regulation and cell cycle control by the Ah receptor. *Exp Cell Res* **226:**154–163.

Xia H, Mao Q, Paulson HL, and Davidson BL (2002) siRNA-mediated gene silencing in vitro and in vivo. *Nat Biotechnol* **20:**1006–1010.

Zhao LJ, Jian H, and Zhu H (2003) Specific gene inhibition by adenovirus-mediated expression of small interfering RNA. *Gene* **316:**137–141.

Address correspondence to: Dr. Cornelis J. Elferink, Assistant Professor, Department of Pharmacology and Toxicology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX. E-mail: coelferi@utmb.edu