A Direct Interaction between the Aryl Hydrocarbon Receptor and Retinoblastoma Protein

LINKING DIOXIN SIGNALING TO THE CELL CYCLE*

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The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor in eukaryotic cells that alters gene expression in response to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). In 5L hepatoma cells, TCDD induces a G1 cell cycle arrest through a mechanism that involves the AhR. The retinoblastoma tumor suppressor protein (pRb) controls cell cycle progression through G1 in addition to promoting differentiation. We examined whether the human AhR or its dimerization partner, the AhR nuclear translocator (Arnt), interacts with pRb as a basis of the TCDD-induced cell cycle arrest. In vivo and in vitro assays reveal a direct interaction between pRb and the AhR but not the AhR nuclear translocator protein. Binding between the AhR and pRb occurs through two distinct regions in the AhR. A high affinity site lies within the N-terminal 364 amino acids of the AhR, whereas a lower affinity binding region colocalizes with the glutamine-rich transactivation domain of the receptor. AhR ligand binding is not required for the pRb interaction per se, although immunoprecipitation experiments in 5L cells reveal that pRb associates preferentially with the liganded AhR, consistent with a requirement for ligand-induced nuclear translocation. These observations provide a mechanistic insight into AhR-mediated cell cycle arrest and a new perspective on TCDD-induced toxicity.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) represents a class of environmental contaminants that include the polychlorinated biphenyls. TCDD triggers a variety of responses in animals including liver toxicity, immune suppression, hyperplasia, and developmental and reproductive toxicity. In humans, TCDD exposure causes chloracne, but concern also extends to TCDD-induced tumor promotion, birth defects, and developmental and reproductive toxicity (1–3). Studies in aryl hydrocarbon receptor (AhR) knockout mice indicate that most TCDD-induced toxic effects are mediated by the AhR (4, 5). The AhR is a ligand-activated transcription factor that acts in concert with the AhR nuclear translocator (Arnt) (6). Target gene expression involves binding of the AhR-Arnt complex to an enhancer sequence called a xenobiotic response element (7). Cloning and characterization of the AhR and Arnt proteins revealed that both contain a basic helix-loop-helix (bHLH) and PAS homology domain (8–10). The basic region confers AhR DNA binding and also contains the nuclear localization signal (3, 11), whereas the HLH motif and PAS domain both mediate protein dimerization (12, 13). Furthermore, the PAS region and AhR ligand-binding domain overlap (10). The C terminus of each protein contains a transactivation domain (TAD). Although both TADs are functionally competent (e.g. in yeast expression systems), in vitro studies indicate that the TAD of AhR predominates, at least in TCDD-induced CYPIA1 gene expression (14, 15).

A new perspective on AhR function and TCDD action comes from two recent reports describing a relationship between AhR activity and the cell cycle (16, 17). Ma and Whitlock (16) examined differences in the growth rates of wild-type (Hepa1) and AhR-defective mouse cell lines and determined that the AhR influences G1 cell cycle progression. AhR-defective cells that contain only 10% of wild-type AhR levels exhibit a prolonged transition through the G1 phase, but when transfected with an AhR expression construct, these cells grew with normal doubling times. Weiss and co-workers (17), using wild-type (5L) and AhR-defective (BP8) rat hepatoma cells, observed a TCDD-dependent G1 arrest only in the 5L cells. Furthermore, expression of the AhR in the BP8 cells ordinarily refractory to TCDD-induced growth arrest reconstituted the inhibitory phenotype, suggesting direct involvement by the AhR in the G1 arrest. Progression through G1 phase of the cell cycle is regulated by pRb, pRb is a 110-kDa nuclear phosphoprotein that undergoes cyclic phosphorylation and dephosphorylation during the cell cycle (18, 19). The hypophosphorylated, active form of pRb triggers cells to arrest in G1, whereas progression beyond the G1 checkpoint and entry into S phase occur following pRb inactivation by cyclin-dependent kinase (Cdk)-mediated hyperphosphorylation (20). Mechanistically, pRb is believed to function as a repressor protein in G1 arrest by binding to the transcription factor E2F, preventing E2F-mediated transcription of genes required for S phase. The adenovirus E1A oncoprotein relieves E2F repression by binding to and sequestering pRb (21). It is noteworthy that E1A also suppresses drug induction of rat CYPIA1 through a mechanism involving the xenobiotic response element (22). It is conceivable that E1A disrupts a functional interaction between the AhR-Arnt complex and pRb necessary for AhR-mediated gene expression. Similarly, the TCDD-inducible G1 cell cycle arrest in 5L cells may involve an interaction between pRb and the AhR-Arnt complex.

These observations prompted us to examine whether the
AhR or Arnt protein is capable of interacting with pRB. Immunoprecipitation results detected an interaction between the AhR-Arnt complex and pRB. Using the yeast two-hybrid system and the GST fusion protein pull-down assay, we show a direct interaction between pRB and the AhR. In contrast, the Arnt protein does not interact with pRB. The pRB-Ahr interaction involves primarily a sequence(s) located within the N-terminal 364 amino acids of the AhR, although a C-terminal 83-amino acid region encompassing the glutamine-rich domain is also involved. The data also suggest that the pRB-Ahr interaction per se is not contingent upon ligand binding. Collectively, the data demonstrate that the AhR and pRB directly interact in vivo and in vitro.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases and other DNA modifying enzymes (T4 DNA ligase, calf intestinal alkaline phosphatase) were purchased from Life Technologies Inc. and New England Biolabs (Beverly, MA). Reduced glutathione and glutathione-agarose were from Sigma. The MATCHMAKER LexA two-hybrid system used and yeast culture media were from CLONTECH. The Toq and KlenTaq DNA polymerases were obtained from Qiagen and Sigma, respectively. The pRB coding (GS-245) was from Pierce. TCD2 was from the National Cancer Institute Chemical Carcinogen Reference Standard Repository. Western-Star and Galacton-Star kits were purchased from Tropix (Bedford, MA). Radioactive compounds were acquired from Amersham Pharmacia Biotech. The TNT-coupled transcription and translation system was from Promega (Madison, WI). Protein G-coupled Sepharose resin and custom synthesized oligonucleotides were from Life Technologies Inc.

**Oligonucleotides (5'-3')**—Sequences of the oligonucleotides used are presented below. Bold type denotes restriction sites used in cloning, and underlining depicts template sequences. The primers used in the yeast two-hybrid screen are: hAhR-F, CCATCGGGCGCCGCTTATGATAACGACGGAGCCGAC; hAhR-R, GCATGCGCTACTGATACG; pGEXAhR537–774. An Arnt-encoding cDNA was generated by PCR using plasmid pBM5NeoM1-1 and primers GST-hArnt510-F and GST-hArnt510-R, and modified by directional cloning into EcoRI/HindIII-cut vector pGEX-KG vector generating pGEXArnt510–780. GST fusion proteins were expressed in Escherichia coli following induction by 1 mM isopropyl-thio-β-D-galactopyranoside for 3 h. Fusion proteins were recovered from cells resuspended in NETN buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 8.0, 1% Nonidet P-40) containing protease inhibitors, following two passages through the French press and clarified by centrifugation. The clarified supernatants were purified using glutathione-agarose beads.

**In Vitro Binding Assay**—A cDNA encoding full-length human pRB was generated by PCR amplification of p2206TRB using the primers pCR3Rb-F and pCR3RbR and cloned into pCR3.1 (Invitrogen) by TA cloning. In *vitro* transcription and translation of pRB was performed in the TNT T7-coupled rabbit reticulocyte lysate system in the presence of [35S]methionine according to the protocol provided by the manufacturer. Protein samples were carried out on ice for 3 h and the bound proteins were fractionated by SDS-PAGE and visualized on a GS-525 MolecularImager (Bio-Rad).

**Cell Culture**—Wild-type rat hepatoma 5L cells and the AhR-defective BP8 variants were grown in 100-mm plates as monolayers in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in 5% CO₂ atmosphere at 37 °C.

**Immunoprecipitation and Western Blots**—Subconfluent cultures of 5L cells were treated with 5 µM TCDD or 0.05% (v/v) vehicle (Me₃SO) for 1 h and then lysed on ice for 30 min by addition of 1 ml plate lysate buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 10 µM glutathione, 1 mM diethiothreitol, 0.1% Tween 20, 10% glycerol, 10 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM NaF, 1 mM phenylmethylsulfonfluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 1 µg/ml pepstatin A). The cell debris was removed by centrifugation at 12,000 × *g* for 30 min at 4 °C. Immunoprecipitations were performed on total cell lysate from 3 × 10⁵ cells using the GS-245 anti-pRB monoclonal antibody (5 µg/ml) on ice for 4 h followed by binding to protein G-coupled beads. Beads were washed four times with lysate buffer, and the bound proteins were fractionated by 7.5% SDS-PAGE, transferred to PVDF membrane, and blocked with 4% Blotto in Tris-buffered saline containing 0.1% Tween 20. Filters were probed with the anti-pRB antibody and antibodies against mouse AhR and Arnt (Ref. 26; 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 MolecularImager.
INTERACTION BETWEEN THE AH R AND pRB

RESULTS

The TCDD-inducible G1 arrest in 5L cells may bespeak an interaction between pRB and the AhR-Arnt complex and is supported by immunoprecipitation experiments (Fig. 1). Immunoprecipitates from control or TCDD-treated 5L cell lysates using an antibody against human pRB were analyzed by Western blotting for the presence of AhR, Arnt protein, and pRB. A TCDD-inducible coprecipitation of the AhR and Arnt protein is revealed by the enrichment of these proteins without a corresponding increase in pRB levels (compare lanes 2 and 3). Digital imaging and quantitation of the bands reveal that AhR and Arnt levels are increased 2.5- and 2.2-fold, respectively, after normalization for pRB. Hence, the data suggest that the AhR-Arnt complex does indeed interact with pRB upon TCDD activation of the AhR. Furthermore, the result also reveals that the AhR-Arnt complex is capable of interacting with the hypophosphorylated, active form of pRB, because the inactive hyperphosphorylated form of pRB detectable in the 5L cell lysate (lane 1, pRB) is not a component of the immunoprecipitates. Precipitation of pRB, AhR, or Arnt was not observed with nonimmune IgG (data not shown).

To examine whether the AhR or Arnt protein directly contacts pRB, we used the yeast two-hybrid assay. The assay uses two reporter genes (LEU2 and lacZ) under the control of multiple LexA operators, detectable as growth of the leucine auxotrophs and blue colony formation on leucine-deficient X-gal plates. Fusion constructs linking the full-length human AhR or Arnt protein to the DNA-binding domain of the LexA repressor induced both reporters in the absence of the plasmid pB42ADpRB (Fig. 2, A, AhRF1, and G, ArntFL), indicating that the TADs contained in the full-length AhR and Arnt are both transcriptionally competent. This agrees with past observations (14). For the purpose of the yeast two-hybrid assay, however, this domain needed to be removed. Because the AhR TAD is complex, comprising several regions each capable of transcription (27), the AhR C terminus was progressively deleted in a series of LexA fusion constructs to remove the entire TAD (AhR1–672, 1–589, and 1–528). In contrast, the TAD of Arnt is confined primarily to the last 34 amino acids (27). The Arnt1–694 construct removes the TAD as well as the glutamine-rich region. When transformed into cells in the absence of pB42ADpRB, none of the AhR and Arnt truncated constructs induced β-galactosidase expression nor grew on leucine-deficient plates (Fig. 2, −pRB). Cotransformation with pB42ADpRB stimulated β-galactosidase expression and growth on selective medium in cells transformed with the truncated AhR proteins (Fig. 2, +pRB). Restoration of reporter expression by the pLexA bait and pB42AD prey constructs is interpreted as evidence for a direct pRB-AhR interaction. Specificity for this interaction is suggested by the failure of pRB to interact with Arnt1–694.

The AhR-pRB interaction and the impact of ligand binding were examined quantitatively by the yeast two-hybrid assay using liquid cultures (Fig. 3). Background β-galactosidase activity in cells (i.e. transformed with the reporter plasmid p8op-lacZ) was not increased in cells cotransformed with the pB42ADpRB construct, indicating that pRB alone is incapable of transcriptional activation. The full-length AhR in the absence of pRB triggered a 200-fold increase in β-galactosidase activity (Fig. 3, compare columns 1 and 5). Cotransformation of pRB fails to induce AhRF1-driven β-galactosidase activity further (compare columns 5 and 7), suggesting that the pRB interaction with AhRF1 is probably masked by the potent TAD of the receptor. In contrast, the truncated AhR constructs were almost devoid of inherent AhR transcription activity inducing at most a 2–3-fold increase in reporter expression (Fig. 3, compare column 1 with columns 9 and 11). Interactions between pRB and the truncated AhR proteins stimulated a 10–100-fold increase in β-galactosidase activity, being most pronounced with the AhR1–672 construct (compare columns 9 and 11).
Fig. 3. Quantitative analysis of the AhR-pRb interaction. The presence or absence of the indicated plasmid constructs is indicated by + and − symbols, respectively. Vehicle (Me₃SO) and βNF (1 μM)-treated liquid cultures are similarly identified by + and − symbols. Cells containing the LacZ reporter (p8op-LacZ) were cotransformed with plasmids expressing the full-length AhR (pYAhRFL, columns 5–8; pYAhR1–672, columns 9–12; pYAhR1–589, columns 13–16; pYAhR1–528, columns 17–20) and Arnt (pYArntFL, columns 21 and 22; pYArnt1–694, columns 23 and 24) in the absence (−) and presence (+) of pRb (pB42ADpRb). Liquid cultures were grown to log phase and assayed for β-galactosidase expression as described under “Experimental Procedures.” The data represent the means of three to five independent triplicate assays ± S.E.

Fig. 4. In vitro binding between the AhR and pRb. The scheme depicts the GST (stippled), bHLH (black), PAS (shaded), and glutamine-rich (hatched) domains in the GST fusion proteins used in the pull-down assays. The location of the LXCXE motif in the AhR is also shown. In vitro transcribed and translated 35S-radiolabeled full-length human pRb (Input) was used in binding assays with bacterially expressed GST fusion proteins encoding AhR amino acids 1–364 and 537–774 and Arnt residues 510–780 as described under “Experimental Procedures.” Bound pRb protein was fractionated by SDS-PAGE and visualized by autoradiography. Relative pRb binding is presented below the figure normalized against binding to naive beads.
motif. The second site lies between residues 537 and 774. In fact, we can narrow this down further to an 83-amino acid glutamine-rich region between residues 589 and 672, because deleting this region resulted in a ~20-fold decrease in β-galactosidase reporter expression (Fig. 3).

**DISCUSSION**

This paper presents evidence for a direct interaction between the AhR and pRb and seems to involve at least two distinct regions within the AhR. The GST-AhR pull-down data place one site in the N terminus of the AhR encompassing the first 364 amino acids and a second site within residues 537–774. The ~20-fold decrease in β-galactosidase activity in the two-hybrid assay following removal of residues 589–672 suggests that the latter site lies within this 83-amino acid region. The N-terminal region contains both a bHLH and LXXCE motif, whereas the C-terminal sequence harbors a glutamine-rich TAD. Studies on the high mobility group-box transcription factor, HBP1, revealed that its LXXCE motif and activation domain functioned as both high and low affinity pRb-binding sites, respectively (28). This resembles the ~4-fold enhanced binding of pRb to GST-hAhR (aa1–364) containing a LXXCE motif relative to GST-hAhR (aa537–774) harboring the TAD (Fig. 4). Because the MyoD-pRb interaction involves the bHLH domain in MyoD (29), we are currently investigating whether the AhR-pRb interaction occurs through the bHLH or LXXCE motif. However, the very existence of the LXXCE motif in the AhR and the absence of pRb binding by the bHLH domain in Arnt suggests that the AhR-pRb interaction occurs through the LXXCE motif.

We hypothesize that a mechanism involving the AhR-pRb interaction is responsible for the TCDD-inducible G1 arrest in 5L cells. A role for the AhR in cell cycle arrest is supported by several observations. For example, benz[a]pyrene induces an AhR-dependent G1 arrest in murine Swiss 3T3 cells (30). In MCF7 human breast epithelial cells, TCDD causes a significant decrease in cell number when compared with the untreated counterparts over a 72-h period (31). Whether this is due to a G1 arrest is currently unknown, but MCF7 cells exposed to indole-3-carbinol at concentrations 4-fold above the Kd for binding to the AhR arrest in G1 phase concomitant with Cdk6 down-regulation (32, 33). TCDD induces the Cdk inhibitors p15INK4a, p21Waf1, p27Kip1, as well as pRb in a dose-dependent manner in mice (34), consistent with conditions inducing G1 arrest. Also, ras activation in NIH3T3 cells up-regulates cyclin D1 levels resulting in pRb hyperphosphorylation (35). This is noteworthy because activated ras down-regulates AhR function in MCF10A cells (36), suggesting that ras may indirectly suppress AhR activity by affecting pRb phosphorylation. This implies that AhR function may rely on an active pRb and predicts that AhR activity is cell cycle-dependent. Consistent with this idea, nodocazole-treated Hepa1 cells arrested in G2/M no longer induce CYP1A1 expression following TCDD exposure (37).

From a functional standpoint, pRb is generally viewed as a transcriptional repressor (18, 19). pRb binds to the E2F activation domain actively suppressing E2F-regulated transcription of S phase-specific genes involved in the G2 to S phase transition. This involves pRb-mediated recruitment of the histone deacetylase, HDAC1, believed to modify chromatin into a transcriptionally inactive form (38). Whether AhR-mediated G1 arrest requires pRb transcriptional repression or activation is currently unclear. E1A suppression of CYP1A1 induction is consistent with the latter, because E1A functionally inactivates pRb by sequestration (21, 28). Interpreting the E1A effect on CYP1A1 expression is complicated, however, by the recent observation that the coactivator p300/CBP interacts with the TAD in Arnt (22). E1A also binds p300/CBP, raising the prospect that E1A inhibits Arnt transactivation by interfering with p300/CBP function. Given that induction of CYP1A1 requires the AhR TAD (15), coupled with our results that pRb interacts with the AhR TAD, we speculate that E1A inhibits CYP1A1 induction primarily by disrupting the AhR-pRb interaction. This scenario impugns that in the context of CYP1A1 expression, pRb functions as a transcriptional activator rather than a repressor. Positive regulation by pRb is known in a few cases, most notably in glucocorticoid receptor-mediated transcriptional activation (39). In glucocorticoid receptor-mediated transcription, pRb acts in concert with hBrm, a homolog of the Saccharomyces cerevisae SWI2/SNF2 protein, which functions in nucleosome disruption (40). Because nucleosome disruption also occurs during AhR-mediated induction of CYP1A1 (15), the prospect that a similar mechanism is involved must be considered.

Protein-DNA cross-linking studies (41) and purification (42) of the rat liver AhR DNA-binding complex detected a 110-kDa protein comprising part of the AhR-DNA complex that is distinct from Arnt. Given that hypophosphorylated pRb is 110 kDa, we speculate this species may in fact be pRb, suggesting that pRb is an integral component of the AhR complex. DNA binding experiments using purified, baculovirus-expressed AhR and Arnt revealed that AhR DNA binding could be reconstituted only following addition of “a heat-sensitive factor(s) present in soluble extracts from a variety of cell types,” including mammary gland, and plant sources (43). The broad distribution of pRb or functional homologs (44, 45) raises the possibility that these varied cell extracts promoted AhR-Arnt DNA binding by virtue of an pRb activity in the extracts. Because DNA binding by the AhR and Arnt alone may occur under some in vitro conditions, however (46), the precise role for pRb in AhR DNA binding requires further study. Attempts to demonstrate the presence of pRb in the DNA-bound AhR complex from rat liver using anti-pRb antibodies failed to supershift or disrupt the complex in a gel mobility shift assay (data not shown). This may reflect occlusion of the epitope during DNA binding. E box-binding complexes containing MyoD similarly failed to supershift with antibodies against pRb, even though MyoD and pRb bind to one another, and E box binding by MyoD-containing heterodimers is stabilized by pRb (29).

From a toxicological perspective, the AhR-pRb interaction and TCDD-induced G1 arrest in 5L cells appear at odds with the notion that TCDD is a tumor promoter. Tumor formation is observed in animals treated with relatively high doses of TCDD, yet epidemiological evidence from a population in Seveso, Italy, exposed to low dioxin levels correlates with a significant reduction in total tumors (47). Hence, a thorough examination of the role played by the AhR in regulating cell cycle progression should clarify some of the confusion surrounding the involvement of TCDD in tumor promotion. In contrast, the toxic effects of TCDD on tissue differentiation are reconcilable with the role pRb plays in development and differentiation (18, 19). It is conceivable that persistent TCDD signaling through the AhR may disrupt normal pRb-mediated differentiation processes. Hence, the realization that the AhR and pRb interact is pivotal in our efforts aimed at deciphering the mechanism of TCDD toxicity.

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