# ACTIVATION OF THE ARYL HYDROCARBON RECEPTOR BY DIOXIN INDUCES CELL CYCLE ARREST

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## Introduction

One of the most puzzling aspects of the biological impact of the halogenated aromatic hydrocarbons (HAHs) is that they elicit a variety of toxic, teratogenic, and carcinogenic responses in exposed animals and in humans. Recent long-term epidemiologic studies with TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), the prototypic HAH, have shown a strong link between exposure to this agent and certain types of cancers and cardiovascular disease in humans [1-3]. In rodents, if administered during embryogenesis, TCDD is teratogenic, causing craniofacial anomalies such as cleft palate, and hydronephrosis [4,5], whereas in the adult it causes disturbances of lipid metabolism and immunotoxic [6], reproductive and endocrine effects [7], some of which appear to be present also in exposed humans [8].

At the cellular level the effects of TCDD are just as diverse, and often contradictory, inducing proliferation [9] as well as terminal differentiation [10-12] of human keratinocytes. Immature thymocytes from rats and mice treated with TCDD *in vivo*, but not *in vitro*, show increased apoptosis[13] [14,15] while in rat hepatocytes TCDD has been reported to induce apoptosis [16], to inhibit uv-induced apoptosis [17], and to increase [16] and to decrease [18,19] proliferation rates.

Most, if not all, effects of dioxins are mediated by a cytosolic receptor known as the Ah (for aromatic hydrocarbon) receptor (AHR) [20,21], which has been cloned from several vertebrates, including fish, mice, rats and humans [22-25]. The mouse Ah receptor is an 805 amino acid-long ligand-activated transcription factor that forms a transcriptionally active heterodimer with the aromatic hydrocarbon nuclear translocator (ARNT) [26]. In the cytosol, the unliganded AHR is found in a complex with two HSP90 molecules and at least one p45 protein, recently identified as an immunophilin [27,28]. Ligand binding disrupts this complex and causes the nuclear translocation of the AHR and the activation of a transcriptionally competent AHR/ARNT heterodimeric complex. This complex binds to CACGC DNA motifs (AhREs; also termed DREs and XREs) in the regulatory regions of the *CYP1A1, CYP1B1* and *CYP1A2* cytochromes P450 genes and of several genes coding for phase II detoxification enzymes (see ref. 29 for a review).

Ligand activation of the Ah receptor may cause cell cycle arrest, cell proliferation, differentiation, or apoptosis, depending on cell type and lineage. This property of the AHR suggests that the Ah receptor has activities other than induction of phase I detoxification genes and that these activities involve the perturbation of one or more cell cycle regulatory components. Our observations, and those of others [30-32] strongly support the notion that the Ah receptor forms protein complexes with the retinoblastoma protein (RB) and that these complexes function as efficient inhibitors of cell cycle progression, suggesting that one of the functions of the Ah receptor is to serve as an environmental sensor that signals cell cycle arrest in the presence of its toxic environmental ligands.

## Methods

All methods for plasmid construction, transfection assays, Western immunobloting, cell cycle analysis and growth conditions of cell lines have been described previously [31,33,34] and will not be repeated here for the sake of brevity.

**Table 1.** Alignment of LXCXE motifs in AHR and in other RB-binding proteins.

| | | | |
|---|---|---|---|
| Human AHR | 325-HAADM | **LYCAE** | SHIRM |
| Mouse AHR | 320-HAADI | **LHCAE** | SHIRM |
| Rat AHR | 334-HAADM | **LHCAE** | SHIRM |
| Rabbit AHR | 333-HAADM | **LYCAE** | SHIRM |
| Fish AHR | 323-HAADM | **LYCAE** | NHVRM |
| *C. elegans* | 326-HAKHR | **LLCQE** | KEGSV |
| Hum. Cyclin D1 | 1- MEHQ | **LLCCE** | VETIR |
| Hum. Cyclin D2 | 1- ME | **LLCHE** | VDPVR |
| Hum. Cyclin D3 | 1- ME | **LLCCE** | GTRHA |
| Hum. TF elf-1 | 46-NSYAG | **LACVE** | EPNDM |
| Hum. RBB1 | 98-IGPET | **LVCHE** | VDLDD |
| Hum. RBB2 | 267-SLEPN | **LFCDE** | EIPIK |
| Adeno5 E1A | 117-PEVID | **LTCHE** | AGFPP |
| Polyoma BK Tag | 55-FDEQD | **LHCDE** | ELEPS |
| Polyoma BK tag | 88-LCPDT | **LYCKE** | WPICS |
| SV40 Tag | 98-FNEEN | **LFCSE** | EMPSS |
| HPV-9 E7 | 20-QPTAD | **LHCYE** | ELTEE |

**Results and Discussion**

*AHR activation leads to RB-dependent inhibition of cell cycle progression*

In the process of studying the mechanisms of constitutive AHR activation independent of exogenous ligands, we noticed that variant AHR-less mouse hepatoma cells that expressed AHR ectopically were more likely to enter apoptosis than control cells. These results, together with reports of TCDD-dependent [35,36] and -independent [36] Ah receptor effects on cell cycle progression, prompted us to examine possible mechanisms that would explain the apparent association of the Ah receptor with the cell cycle. Amino acid sequence comparisons revealed that, near the carboxyl terminus of the PAS domain, all six Ah receptor proteins sequenced up to that time, including the putative AHR homolog in *C. elegans*, had a conserved LXCXE domain, in common with all known RB-binding proteins (Table 1). Outside this domain, sequence conservation is high within protein groups, and low between groups. In addition, the domains of AHR, polyoma large T-antigen and small t-antigen and HPV E7 differ by only one amino acid. These observations suggested the possibility that AHR, like the D-cyclins and the transforming oncoproteins, could bind to RB and be involved in cell cycle regulation. This possibility was confirmed experimentally. We used the yeast two-hybrid system to determine that AHR and RB bound to each other and we used *in vitro* pull-down experiments with truncated AHR peptides to show that at least two separate AHR domains, one of which included the LXCXE motif, formed independent complexes with hypophosphorylated RB [31], in agreement with results from other investigators [30]. Co-immunoprecipitation of whole cell lysates from human breast carcinoma MCF-7 cells, which express both proteins endogenously, revealed that AHR associates with RB *in vivo* and that the association required ligand-dependent nuclear translocation of the Ah receptor, since RB is always localized to the nucleus. In addition, several controls demonstrated that the observed interaction occurs *in vivo* and is not merely a consequence of spurious protein associations following lysis. Interestingly, ARNT, the AHR nuclear translocator and transcriptional heterodimerization partner, was not required for, nor was it a part of the AHR/RB complex. Ectopic expression of AHR and RB in human osteosarcoma SAOS-2 cells, which lack endogenous expression of both proteins, showed that AHR synergizes with RB to repress E2F-dependent transcription and to induce cell cycle arrest. Furthermore, AHR partly blocked T-antigen-mediated reversal of RB-dependent transcriptional repression. These results uncover a potential function for the AHR in cell cycle regulation and suggest that this function may be that of serving as an environmental sensor that signals cell cycle arrest when cells are exposed to certain environmental toxicants.

The data briefly presented here point at a mechanism that implicates the Ah receptor in bringing about cell cycle arrest. It may be the failure to respond to one such "AHR checkpoint", perhaps in combination with the specific genetic make-up of exposed cells or animals, that causes TCDD to be a powerful tumor promoter in mice [37] and a rodent carcinogen [38]. Both AHR and RB are responsive to environmental signals. There are more than 400 environmental toxicants and endogenous compounds that are known AHR ligands [39]. Many of these ligands are oxygenated by the cytochrome P450 monooxygenases induced by AHR/ARNT-dependent transactivation and converted into highly oxidized metabolites, many of which, such as the benzo[a]pyrene diol-epoxides, are known DNA-damaging agents which elicit cell cycle checkpoints [40,41]. Consistent with a checkpoint role for AHR, homozygous deletion of the mouse *Ahr* gene does not lead to uncontrolled cell proliferation, but changes the cellular response to environmental insult [42,43], suggesting that the role of AHR in the regulation of cell cycle progression is subtle and not critical for survival. A similar phenotype is observed in fibroblasts from RB-deficient mice, in which cell cycle progression is not inherently mis-regulated [44], but responsiveness to environmental signals is compromised, with failure to arrest in response to DNA damage or TGF-ß [44-46]. Like AHR, RB is also responsive to a plethora of environmental signals [47] that inhibit cell cycle progression by bringing about the dephosphorylation of RB, thereby activating RB as a transcriptional repressor and a cell cycle inhibitory molecule [47,48]. The finding that AHR cooperates with RB via a direct interaction suggests an additional mechanism through which environmental signals can function to activate RB. One of the critical functions of the liganded Ah receptor appears to be to act as an environmental sensor that, in the presence of DNA damaging environmental toxicants, binds to RB and signals cell cycle arrest.

**Acknowledgments**

The work described here was supported by USPHS NIEHS grants ES06273 and P30 ES06096.

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