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# A cycloheximide-sensitive factor regulates TCDD-induced degradation of the aryl hydrocarbon receptor

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**Abstract**

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), a prototype of environmental halogenated aromatic hydrocarbons, induces a rapid reduction in steady state aryl hydrocarbon receptor (AhR). Here, we analyzed the biochemical pathway and function of the downregulation. Our results reveal that TCDD downregulates the AhR protein by shortening the halflife of AhR. The TCDD-induced degradation of AhR is inhibited by MG132, a potent inhibitor of the 26S proteasome, indicating the ubiquitin-26S proteasome mediated proteolysis as a mechanism for the degradation of AhR. Furthermore, inhibition of protein synthesis by cycloheximide blocks the degradation of AhR by TCDD, suggesting a labile factor in controlling the stability of ligand-activated AhR (hence, designated as AhR degradation promoting factor, or ADPF). Analyses of nuclear AhR demonstrated that cycloheximide increases nuclear AhR protein and functional AhR/Arnt DNA-binding complex, resulting in superinduction of *CYP1A1*. Lastly, genetic analyses by using AhR- or Arnt-defective variant cells demonstrate that superinduction by cycloheximide requires the transcription activation (TA) domain of AhR, implicating the TA domain in the control of AhR turnover by ADPF. These findings provide new insights into the mechanism by which TCDD-activated AhR is regulated in nucleus through the 26S proteasome protein degradation pathway. 

*Keywords:* Ah receptor; 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; Degradation; Superinduction; 26S proteasome

*Abbreviations:* AhR, aryl hydrocarbon receptor; Arnt, AhR nuclear translocator; AIP, Ah receptor-interacting protein; hsp, heat shock protein; HIF1α, hypoxia inducible factor 1α; bHLH, basic helix–loop helix; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DMSO, dimethyl sulphoxide; CHX, cycloheximide; TA, transcription activation.

## 1. Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated, basic helix–loop helix PAS (bHLHPAS) transcription factor (Poland and Knutson, 1982; Hankinson, 1995; Whitlock, 1999). Mouse genetic studies implicate AhR in most of the biological responses to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), which is the prototype for a class of structurally related halogenated aromatic hydrocarbons (HAHs), including polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls (Poland and Glover, 1980; Fernandez-Salguero et al., 1996; Mimura et al., 1997). These HAH chemicals are mostly by-products of industrial processes involving chlorine chemistry and combustion of fuels. Many such chemicals are also widespread and persistent environmental contaminants. TCDD is the most potent among the chemicals; animals exposed to TCDD exhibit a wide range of toxic and adaptive responses, including a wasting syndrome, tumor promotion in skin and liver, cleft palate, chloracne, immune and endocrine dysfunctions, and induction of a number of drug metabolizing enzymes (Luster et al., 1979; Poland and Knutson, 1982; Safe, 1986; Okey et al., 1994; Hankinson, 1995;

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Whitlock, 1999). Humans exposed to TCDD exhibit certain skin lesions such as chloracne. The possibility that TCDD exposure causes certain neuro- and psychopathological alterations (Oliver, 1975; Klawans, 1987), some forms of cancers and diabetic conditions (Fingerhut et al., 1991; Calvert et al., 1999), and reproductive lesions is a particular concern of human health.

Studies on the induction of *CYP1A1* gene expression by TCDD provided major mechanistic understanding of the action and regulation of AhR (Whitlock et al., 1996). In uninduced cells, AhR is located in the cytoplasm, complexed with hsp90 (Perdew, 1988) and AIP, which is an immunophillin type of chaperon protein (Carver and Bradfield, 1997; Ma and Whitlock, 1997; Meyer et al., 1998). Binding with an agonist triggers the dissociation of AhR from the associated proteins and translocation of AhR into nucleus, where AhR heterodimerizes with Arnt, which is another bHLHPAS transcription factor (Hankinson, 1995). The AhR/Arnt dimer binds to a specific nucleotide sequence termed DRE (dioxin responsive element) in the enhancer region of the *CYP1A1* gene. The transactivation domains of the AhR are essential for the subsequent transcriptional events, including alterations in chromatin structure, binding of general transcription factors to the promoter, and induction of transcription of the gene (Ma et al., 1995; Ko et al., 1996; Ko et al., 1997).

Several cellular mechanisms have been recognized for regulation of AhR activity during the induction of *CYP1A1*. For example, prolonged treatment (from several hours to overnight) with TCDD downregulates the steady state level of the AhR protein in total cell extract (Ma and Baldwin, 2000) and the AhR/Arnt/DNA interactions in the nucleus of a rat hepatocyte-derived cell line (Reick et al., 1994). The functional relevance and the mechanism of such down regulation of the AhR protein remain to be studied. In another instance, treatment of cells with TCDD plus cycloheximide, a potent inhibitor of protein synthesis, superinduces the transcription of *CYP1A1*. Early studies established that the superinduction involves an increase in the rate of transcription of the gene, requires functional DREs, but does not change several measurable properties of the TCDD-receptor complex such as the sedimentation velocity of the complex (Israel et al., 1985; Lusska et al., 1992). The superinduction is accompanied by an inhibition of the synthesis of total protein in cell, implicating a "labile", inhibitory factor in AhR function. However, the nature and the mechanism of action of the putative "labile" factor remain unknown.

To understand the molecular mechanism of TCDD-induced down regulation of AhR, we analyzed the biochemical pathway and function of AhR degradation. We found that TCDD induces turnover of AhR through the 26S proteasome pathway. Cycloheximide, an inhibitor of protein synthesis, blocks TCDD-induced AhR degradation. Furthermore, blocking of AhR degradation by cycloheximide increases the amount and function of AhR, resulting in superinduction of *CYP1A1*. Lastly, we show that the superinduction by cycloheximide requires the transactivation domains of AhR. Our findings provide a novel mechanism of regulating agonist-activated, nuclear AhR through a cycloheximide-sensitive, "labile" protein factor by controlling the stability of nuclear AhR.

## 2. Experimental procedures

### 2.1. Materials

AmpliTaq DNA polymerase was from Perkin Elmer (Foster City, CA). Restriction endonucleases and other DNA-modifying enzymes were from New England Biolabs (Beverly, MA). Radioactive compounds were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Cell culture materials were from Life Technologies, Inc. (Grand Island, NY). Cycloheximide (CHX), dimethyl sulphoxide (DMSO), aprotinin, leupeptin, and PMSF were from Sigma (St. Louis, MO). MG132 was from Calbiochem–Novabiochem Corp. (San Diego, CA). TCDD was purchased from AccuStandard (New Haven, CT). Reagents for immunoblotting and Northern blotting are as described below.

### 2.2. Cell culture and treatment

The mouse hepa1c1c7, Ah receptor-defective (AhR-D), and Arnt-defective (Arnt-D) variant cells were obtained from ATCC (Rockville, MD); the reconstituted cells of the AhR- or Arnt-D variants were gifts from Dr. J.P. Whitlock Jr., and associates (Stanford University) and were described elsewhere (Ko et al., 1997). The cells were grown as monolayer in α-minimal essential medium (αMEM), containing 10% fetal bovine serum and 5% $CO_2$ at 37 °C, as described previously (Miller et al., 1983). The cells were treated with TCDD or other agents as described in figure legends; DMSO was used as the solvent control for TCDD.

### 2.3. Preparation of nuclear extract and total cell lysate

Nuclear extracts were prepared according to published procedures (Denison et al., 1988). Briefly, wild type hepa1c1c7 cells, after treatment, were washed in a hypotonic buffer and homogenized in a Dounce homogenizer; the nuclei were obtained by differential centrifugation; nuclear extracts were prepared by incubation of the nuclei with a high salt buffer, followed by centrifugation at 100,000*g* for 1 h. For preparation of total cell lysate, cells were grown to near confluency in a

60 mm dish, washed twice with phosphate buffered saline, and scraped into 300 μl of a reporter lysis buffer (Promega, Madison, WI). The cells were disrupted by brief sonication; total cell lysate was obtained by centrifugation at 13,000*g* for 10 min in a refrigerated microcentrifuge.

### 2.4. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was carried out using nuclear extract from hepa1c1c7 cells, as described previously (Ausubel et al., 1998), except that 6% polyacrylamide gels were used. The DNA probe contains the DNA recognition sequence for the AhR/Arnt heteromer designated as DRE D (Lusska et al., 1993). The probe was labeled with [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase (New England Biolabs, Beverley, MA). The nuclear extracts were incubated with poly (dIdC) for 15 min at room temperature. The $^{32}$P-labeled probe was then added and incubated for another 15 min at room temperature, followed by nondenaturing gel electrophoresis; the AhR/Arnt/DRE complexes were visualized by autoradiography.

### 2.5. Immunoblot analysis

For immunoblotting, total cell lysate or nuclear extract of 5 μg were fractionated on SDS-polyacrylamide gels, and transferred to nitrocellulose membranes according to established procedures (Ausubel et al., 1998). The blots were blocked with 5% dry milk, 0.1% Tween 20 in phosphate buffered saline for 1 h with shaking. Blots were then incubated with a polyclonal antibody against AhR (Ma and Whitlock, 1996) for 1 h, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for an additional 1 h. Signals were visualized by chemiluminescence using an ECL kit (Amersham, Piscataway, NJ). To ensure equal loading of the samples, the same blots were reprobed with a monoclonal anti-mouse actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by incubation with alkaline phosphatase-conjugated secondary antibodies (Promega) and color visualization with the Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate system (Promega). For quantitation of the blotting results, the visualized results were scanned and analyzed using the ImageQuaNT (version 4.2) program (Molecular Dynamics, San Jose, CA). All data were corrected for loading variations by comparing the amount of actin of each sample analyzed.

### 2.6. RNA analysis

For Northern blotting of *CYP1A1*, a cDNA fragment (~700 bp) encoding the 5′-untranslated region of the mouse *CYP1A1* messenger RNA was used to generate a riboprobe for *CYP1A1*. To prepare an actin probe, a cDNA fragment of mouse actin was generated by RT-PCR with primers specific for mouse actin (Stratagene, La Jolla, CA), subcloned into pCRII (Invitrogen, Carlsbad, CA), and used as a template for riboprobe synthesis. Riboprobes were synthesized in the presence of DIG-UTP using a DIG RNA-labeling kit (Roche Molecular Biochemicals, Indianapolis, IL). Total RNA was isolated from cells using a Qiagen total RNA isolation kit (Qiagen, Valencia, CA). RNA samples of 5 μg each were electrophoresed in a 1% agarose-formaldehyde gel and transferred to a Nytran membrane. After cross-linking, the membranes were hybridized with the DIG-labeled riboprobes at 68 °C overnight; signals were visualized by chemiluminescence using a DIG RNA detection kit with CDP star as a substrate (Roche Molecular Biochemicals). For all samples analyzed, parallel blots were assayed at the same time for both *CYP1A1* and actin mRNAs.

### 2.7. Pulse-chase labeling

Cells grown to near confluence were incubated in methionine-free medium with 10% dialyzed FBS (Life Technologies, Inc.) for 1 h and incubated for another hour in fresh methionine-free medium with 10% dialyzed FBS plus $^{35}$S-methionine (100 μCi per ml, Amersham). The cells were then incubated in αMEM with 10% FBS and treated with DMSO, cycloheximide (10 μg/ml), TCDD (1 nM), or TCDD plus cycloheximide for various time periods. The cells were scraped into RIPA buffer (1% Ipegal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 100 μM PMSF, and 10 μg/ml aprotinin in phosphate buffered saline). The $^{35}$S-labeled AhR was precipitated with the anti-AhR antibodies, fractionated by SDS-PAGE (10%), and visualized by fluorography.

### 2.8. Immunoprecipitation

AhR was precipitated with anti-AhR antibodies according to a standard method (Ausubel et al., 1998). Briefly, cells grown in six well plates were scraped into RIPA buffer. Cell extracts were prepared by centrifugation at 13,000*g* for 10 min, followed by preclearing by incubation with normal rabbit IgG (Santa Cruz Biotechnology, Inc.) and protein A-agarose (Life Technologies, Inc.) for 30 min at 4 °C. The extracts were then incubated with the anti-AhR antibodies (Dong et al., 1996; Ma and Whitlock, 1996) for 1 h and with protein A-agarose for an additional hour. The precipitated agarose beads were washed three times with the RIPA buffer and resuspended in a loading buffer for analysis by SDS-PAGE.

## 3. Results

### 3.1. TCDD induces turnover of AhR through the 26S proteasome pathway

Treatment of mouse hepa1c1c7 cells with TCDD (1 nM, 5 h) results in a marked reduction in the steady state level of the AhR protein, whereas the level of the Arnt protein was not affected (Fig. 1). Thus, the TCDD-induced protein downregulation exhibits a high specificity toward AhR. To examine the mechanism of AhR downregulation, pulse-chase experiments were performed to measure the halflife ($t_{1/2}$) of AhR. The unliganded AhR has a $t_{1/2}$ of ~28 h (Ma and Baldwin, 2000); while TCDD shortens the $t_{1/2}$ of AhR to ~3 h (Fig. 5). The pathway that mediates AhR degradation was analyzed by testing inhibitors of the 26S proteasome and various proteases. Cotreatment of the cells with TCDD and MG132, a potent inhibitor of the 26S proteasome, totally blocks TCDD-induced reduction of AhR (Fig. 2); whereas, inhibitors of calpains, lysosomal proteases, serine-, or serine/cysteine proteases, do not show inhibitory activity (data not shown (Ma and Baldwin, 2000)). These results indicate that TCDD induces AhR turnover through the 26S proteasome mediated proteolysis.

### 3.2. Cycloheximide blocks TCDD-induced turnover of the AhR protein

Cycloheximide, a potent inhibitor of protein synthesis, superinduces the transcription of *CYP1A1* in the presence of an agonist of AhR. As shown in Fig. 3, cycloheximide alone does not affect *CYP1A1* gene expression (lane 3), whereas cotreatment of hepa1c1c7 cells with TCDD (1 nM) and cycloheximide (10 μg/ml) for 5 h increases the induction of *CYP1A1* by TCDD (compare lanes 4 and 2). These results indicate that the superinduction requires activation of AhR by an agonist, suggesting that AhR or a component of the AhR signaling pathway serves as a primary target of cycloheximide. These findings raised the question of whether cycloheximide modulates the agonist-induced degradation of AhR as a mechanism of superinduction. Therefore, we analyzed the effect of cycloheximide on the protein level of AhR during the superinduction by immunoblotting. As shown in Fig. 4, treatment of the wild type cells with TCDD (1 nM, 4 h) downregulates the steady state AhR to less than 20% of the wild type.

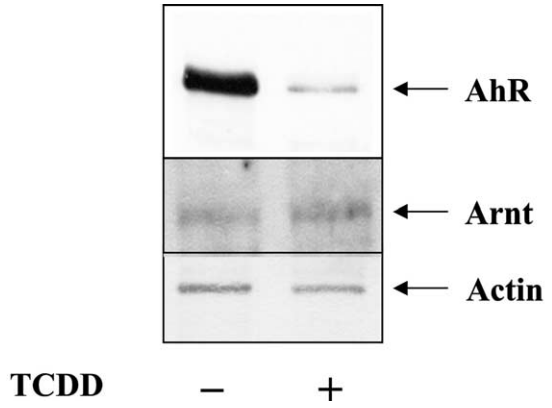


Fig. 1. TCDD-induced downregulation of AhR. Hepa1c1c7 cells were grown in 60 mm dishes and treated with TCDD (1 nM) or DMSO for 5 h. Total cell lysate was prepared and analyzed by immunoblotting (5 μg/lane) as described under experimental procedures. The blot was first probed for AhR or Arnt proteins, visualized by chemiluminiscence, then reprobed for the actin protein, and visualized by color development.

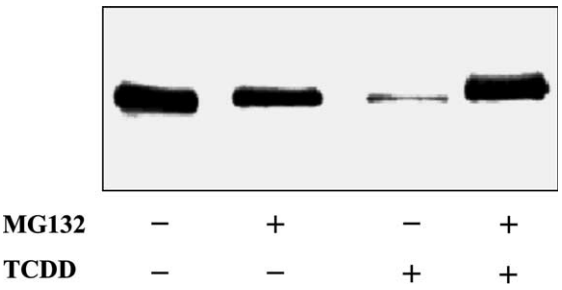


Fig. 2. Inhibition of TCDD-induced degradation of AhR by MG132. Cells were grown in 60 mm dishes and treated with TCDD (1 nM), MG132 (25 μM), or both for 5 h. Total cell lysate was prepared and analyzed by immunoblotting (5 μg/lane) for AhR as described for Fig. 1.

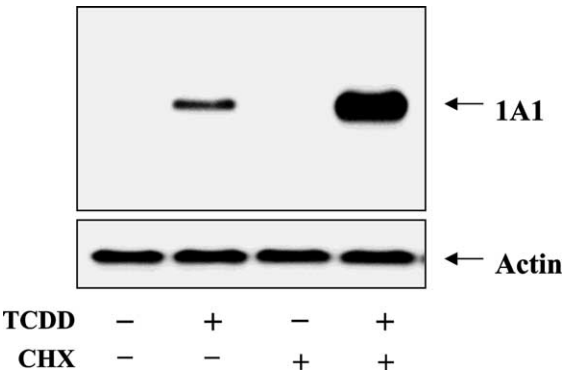


Fig. 3. Superinduction of *CYP1A1* by cycloheximide. Cells were grown to near confluency in a 100 mm dish, and were treated with TCDD (1 nM), CHX (10 μg/ml), or both for 5 h. Total RNA was prepared, fractionated on agarose-formaldehyde gels, blotted to Nytran membranes, and probed with DIG-labeled riboprobes for *CYP1A1* (upper panel) and actin (lower panel) as described under Section 2. Each lane contains 5 μg of RNA.

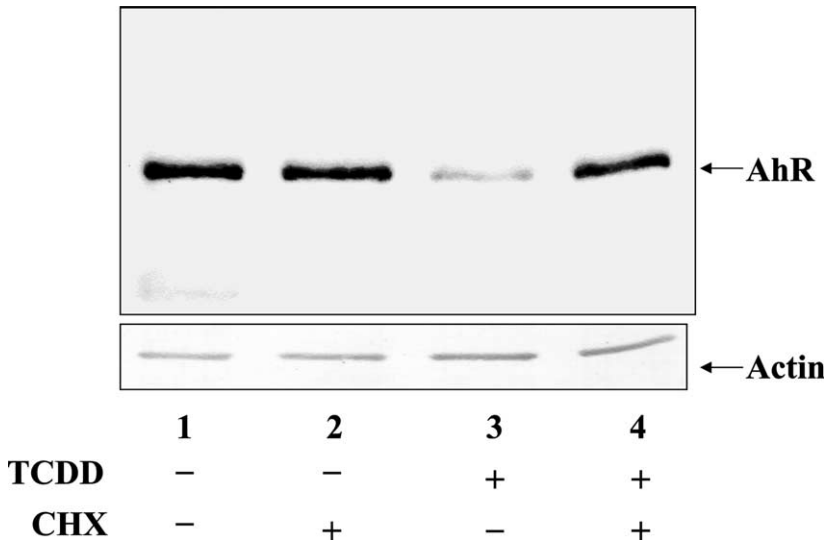


Fig. 4. Blocking of TCDD-induced downregulation of AhR by cycloheximide. Cells were grown in 60 mm dishes and treated with TCDD (1 nM), CHX (10 μg/ml), or both for 4 h. Total cell lysate was prepared and analyzed by immunoblotting (5 μg/lane) for AhR and Actin as described for Fig. 1.

Cycloheximide alone (10 μg/ml) does not affect the protein level of AhR (lane 2). However, the level of the AhR protein in cells treated with TCDD plus cycloheximide (TCDD, 1 nM; CHX, 10 μg/ml; 4 h) is nearly the same as the controls (compare lane 4 with 1 and 2). Thus, cycloheximide completely blocks the reduction of the steady state AhR protein by TCDD.

We next tested if cycloheximide inhibits the TCDD-induced turnover of AhR by measuring the halflife ($t_{1/2}$) of AhR. Pulse-chase labeling experiments reveal that AhR in DMSO treated cells is relatively stable with a $t_{1/2}$ of 28 h (Ma and Baldwin, 2000) and that cycloheximide alone does not affect the $t_{1/2}$ of AhR (data not shown). TCDD shortens the $t_{1/2}$ of AhR to ~3 h (Fig. 5). However, the $t_{1/2}$ value of AhR in cells treated with TCDD plus cycloheximide is nearly the same as that in DMSO treated cells (i.e. ~28 h). Therefore, cycloheximide fully inhibits the TCDD-induced turnover of the AhR protein. Since cycloheximide inhibits over 90% of total protein synthesis in cells, our results implicate a cycloheximide-sensitive, labile or inducible factor in promoting the TCDD-induced degradation of AhR;

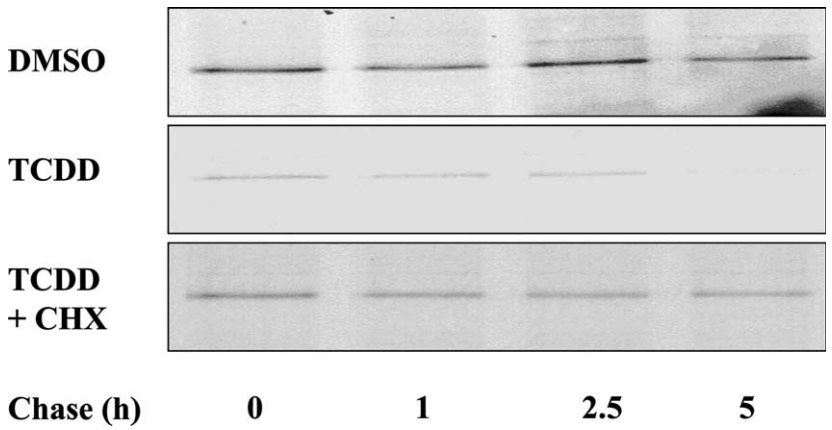


Fig. 5. Pulse-chase labeling of AhR. Hepa1c1c7 cells were labeled with $^{35}$S-methionine and were treated with DMSO, TCDD (1 nM), or TCDD + CHX (10 μg/ml). The AhR protein was immunoprecipitated with a polyclonal anti-AhR antibody, fractionated by SDS-PAGE, and visualized by fluorography, as described under Section 2. The hours indicate the time period of treatment after pulse labeling.

hence, we designated the factor as AhR degradation promoting factor, or ADPF.

### 3.3. Cycloheximide enhances the function of nuclear AhR

Since cycloheximide blocks the degradation of AhR following activation by agonist, we envision that cycloheximide enhances AhR function in the nucleus by increasing the amount and function of agonist-activated, nuclear AhR, which mediates the superinduction. To test this possibility, we first analyzed the AhR protein level in nuclear extract. As shown in Fig. 6, cycloheximide treatment increases the amount of AhR protein in nuclear extract of cells treated with TCDD plus cycloheximide dose-dependently; the increase is observable at 100 ng/ml of cycloheximide and is maximal at 10 μg/ml. Next, we examined the nuclear AhR/Arnt complex by EMSA, which measures the amount and activity of the AhR/Arnt complex in nuclear extract. The EMSA analyses revealed a dose-dependent increase in the interaction of the AhR/Arnt heteromer with the DRE sequences (Fig. 7). The increase in the band shift by EMSA is observable at 10 ng/ml of cycloheximide. Taken together, these results reveal that cycloheximide increases the accumulation of both the AhR protein and the functional AhR/Arnt complex in the nucleus, resulting in superinduction of AhR regulated genes. These findings suggest that the agonist induced degradation of AhR serves as a mechanism by which the function of nuclear AhR is controlled through proteolysis of the receptor.

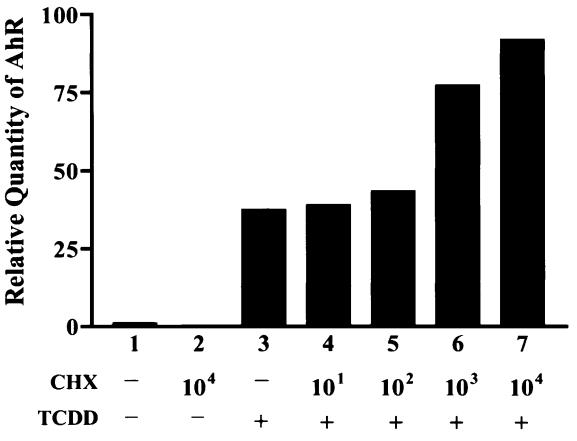


Fig. 6. Immunoblotting of nuclear AhR. Cells grown in 100 mm dishes were treated with TCDD (1 nM), or TCDD plus CHX at indicated concentrations (in ng/ml) for 4 h, and nuclear extracts were prepared as described under Section 2. The nuclear extracts (5 μg/lane) were analyzed by immunoblotting for the AhR protein. The results from immunoblotting were quantified by densitometry and analyzed using the Image-QuaNT software.

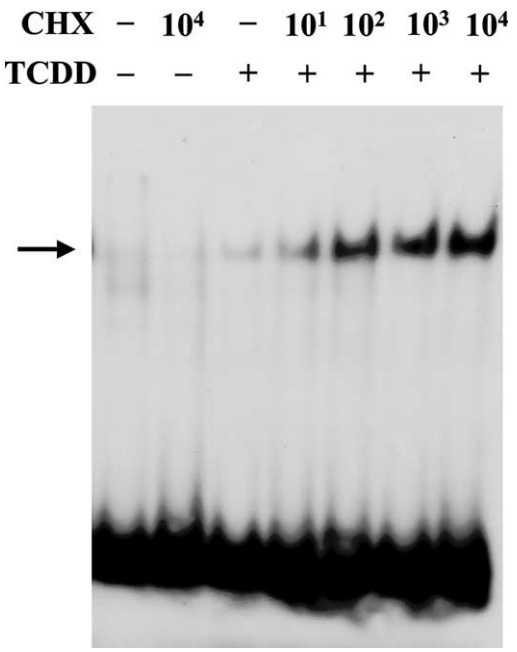


Fig. 7. Electrophoretic mobility shift assay. Nuclear extracts were prepared from cells treated with TCDD (1 nM), or TCDD plus CHX at indicated concentrations (in ng/ml) for 4 h. EMSA was performed using nuclear extract preparations and a $^{32}$P-labeled DNA probe containing a functional DRE sequence as described under Section 2. The arrow indicates the AhR/Arnt/DRE complex. Shown at the bottom of the film are the $^{32}$P-labeled, free DRE probes.

### 3.4. Superinduction by cycloheximide requires the transcription activation domain of AhR

The AhR protein consists of several modular structures each associated with different functions, such as DNA-binding and transcription activation (TA). To analyze the mechanism of action of ADPF, we examined the role of functional domains of AhR in cycloheximide action by using AhR- or Arnt-D variants reconstituted with AhR, Arnt, or their deletion mutants. Cotreatment of the wild type cells with TCDD (1 nM) and cycloheximide (10 μg/ml) for 5 h results in superinduction of *CYP1A1* mRNA (Fig. 8(A) and (B)). The induction and superinduction were largely reduced, but measurable, in AhR-D variant cells (AhR-D, Fig. 8(A), compare lanes 1–4 with lanes 5–8). However, both the induction and superinduction were not observed in Arnt-D cells (Arnt-D; Fig. 8(B), compare lanes 1–4 with lanes 5–8). Reconstitution of the variant cells with the full length AhR or Arnt completely restores both the induction and superinduction (Fig. 8(A) and (B), lanes 9–12); thus, the superinduction requires the presence of both the AhR and Arnt proteins.

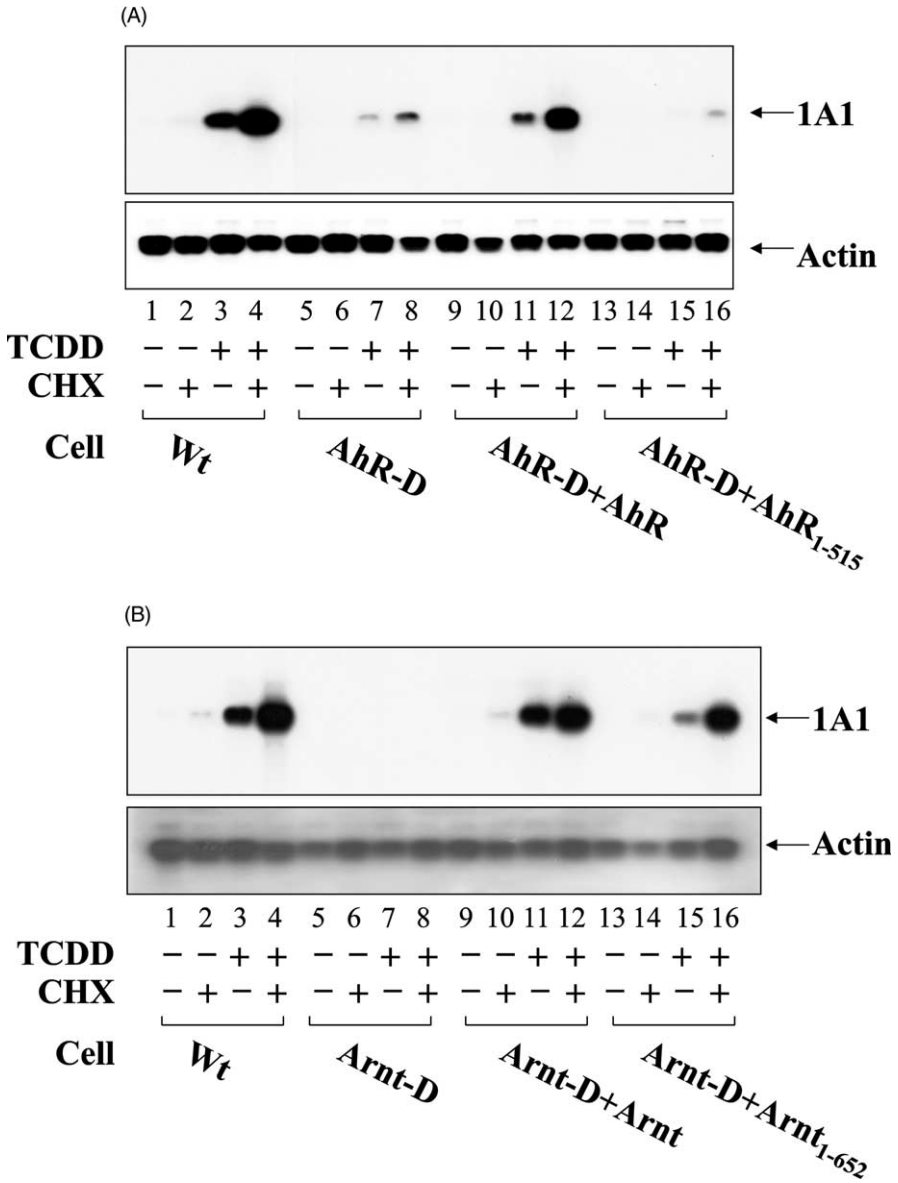


Fig. 8. Superinduction of *CYP1A1* in AhR-defective, Arnt-defective and reconstituted cells. Cells were grown in 100 mm dishes, and were treated as described for Fig. 3. Total RNA of 5 μg each was analyzed for both the *CYP1A1* and actin mRNA expression using DIG-labeled probes. (A) Northern blotting of superinduction in AhR-defective variant and its reconstituted cells and (B) northern blotting of superinduction in Arnt-defective variant and its reconstituted cells.

AhR and Arnt contain, at their carboxyl halves, modular structures termed TA domains. The TA domains of AhR are regulated through interaction with an inhibitory module of AhR (Ma et al., 1995), and are required for mediating the induction of *CYP1A1* in the intact cell; whereas, the TA domains of Arnt are dispensable in the induction process (Ko et al., 1996). As shown in Fig. 8(A), the AhR mutant that lacks its TA domains (AhR-D + $AhR_{1\text{-}515}$) was incapable of restoring the induction and superinduction, implicating its requirement for both of the processes (Fig. 8(A), lanes 13–16). On the contrary, reconstitution of the Arnt-D variant cells with an Arnt mutant that lacks its TA domains (Arnt-D + $Arnt_{1\text{-}652}$) retained both the induction and the superinduction (Fig. 8(B), lanes 13–16). These analyses established that the superinduction of *CYP1A1* by cycloheximide is mediated through AhR/Arnt via the TA domains of AhR. In a previous study, we found that

the AhR mutant (AhR-D + $AhR_{1-515}$) is resistant to TCDD-induced protein degradation. Taken together, these results implicate the TA domains of AhR in ADPF-mediated regulation of AhR degradation. Cloning of ADPF will provide insights into the molecular interplay between ADPF and the TA domains of AhR.

## 4. Discussion

Protein degradation through the ubiquitin-proteasome pathway has been demonstrated in the signal transduction of a number of biologically important proteins; these include short-lived factors, such as p53, c-Myc, and c-Jun, and stable proteins that undergo signal-induced degradation, such as IκBα and the estrogen receptor α. In either scenario, proteasomal degradation of the proteins involves ubiquitination of the transcription factors or their associated proteins for targeting to the proteasome (for review, see reference Hershko and Ciechanover (1998)). In this study, we analyzed the regulation of the Ah receptor by TCDD via protein degradation. Our data reveal that the unliganded, cytoplasmic AhR protein is stable, with a $t_{1/2}$ of 28 h. The $t_{1/2}$ of TCDD-activated AhR, however, is shortened to only 3 h. Thus, TCDD induces a rapid turnover of AhR. The degradation of AhR by TCDD is blocked by inhibition of the 26S proteasome, implicating the proteasome pathway in the degradation of AhR. In a separate study, we have demonstrated that TCDD induces ubiquitination of the AhR protein (Ma and Baldwin, 2000). Thus, TCDD increases the turnover of nuclear AhR by activating the ubiquitin-26S proteasome pathway. Others have obtained a similar conclusion by analyzing steady state AhR using immunoblotting (Pollenz, 1996).

The induction of *CYP1A1* transcription by TCDD through AhR-mediated signal transduction constitutes a model response for analyzing the mechanism of action of TCDD at the level of gene regulation (Whitlock et al., 1996). Cycloheximide, an inhibitor of protein synthesis, superinduces the transcription of the *CYP1A1* gene in the presence of TCDD; superinduction of the gene by cycloheximide implicates a cellular mechanism by which the TCDD-elicited responses can be modulated after activation of the receptor by agonist. The mechanism of action of cycloheximide in superinduction is unclear at present. In this report, we demonstrate that the Ah receptor protein is a primary target of cycloheximide in the superinduction. We show that cycloheximide fully inhibits the degradation of AhR by TCDD; the half-life of AhR in cells treated with TCDD plus cycloheximide is nearly the same as that of DMSO-treated cells. Thus, our data, for the first time, clearly establish that cycloheximide enhances the stability of the agonist-activated AhR protein by inhibiting the turnover of the AhR protein following activation by agonist.

Since cycloheximide is a potent inhibitor of protein synthesis, it is conceivable that the mechanism by which cycloheximide inhibits AhR degradation involves a trans-factor, which acts as a negative regulator of AhR function by promoting the degradation of agonist-activated AhR in the nucleus. Here, we designate the trans-factor as AhR degradation promoting factor, or ADPF. Inhibition of the synthesis of ADPF by cycloheximide releases the negative control of AhR by increasing the stability of the AhR, resulting in superinduction. Two possible mechanisms can explain the sensitivity of ADPF to inhibition of protein synthesis. First, the cycloheximide-sensitive ADPF is a TCDD-inducible protein. The negative regulation of AhR involves a TCDD-induced, autoregulatory mechanism; blockage of the induction of ADPF by inhibiting protein synthesis by cycloheximide or puromycin disrupts the autoregulation. Alternatively, ADPF is a short-lived, labile protein, and therefore is sensitive to inhibitors of protein synthesis. These two possibilities are not mutually exclusive. The observation that pretreatment of cells with TCDD (for over 10 h) before treatment with cycloheximide also superinduces the *CYP1A1* gene (Lusska et al., 1992) supports the second possibility that the cycloheximide-sensitive ADPF is a labile protein; whether it is inducible by TCDD remains to be examined. Regulation of protein stability by labile or inducible factors has been observed for other transcription factors. For example, the tumor suppressor protein p53 is induced by ionizing irradiation as an adaptive response to DNA-breaking damage. The activity of p53 is regulated by degradation of the protein through the ubiquitin-proteasome pathway shortly after the induction of p53 (Agawal et al., 1998). The proteasomal degradation of p53 is controlled by an oncoprotein Mdm2; Mdm2 possesses the ubiquitin-ligase (E3) activity and promotes the degradation of p53 by catalyzing ubiquitination of p53 (Haupt et al., 1997; Honda et al., 1997). The Mdm2 protein is induced by p53 and is degraded by ubiquitin-proteasomal proteolysis.

How does the ADPF regulate the degradation of AhR? One possible mechanism involves destabilization of the nuclear AhR protein. For example, ADPF can alter the conformation of the activated receptor through protein–protein interaction with the receptor, promote modifications of the receptor such as phosphorylation/dephosphorylation, or dissociate the receptor from protective mechanisms that shield AhR from proteolysis in the nucleus; these changes render the receptor susceptible to the ubiquitin-proteasomal degradation mechanism. Alternatively, ADPF can be a component of the proteolytic system; it serves as a recognition factor that channels the nuclear AhR to proteolysis. By analogy with the findings for the regulation of the degra-

dation of p53 by Mdm2, it is possible that ADPF directly controls ubiquitination of the agonist-activated AhR in nucleus. This notion is supported by the observation that cycloheximide inhibits ubiquitination of AhR. [1] Cloning and functional analysis of the ADPF protein will provide direct evidence to distinguish these possibilities. Understanding the mechanism of the TCDD-induced AhR degradation will reveal new aspects of the signal transduction and regulation of AhR in the nucleus.

Controlling cellular protein levels by ubiquitin-mediated proteolysis has been implicated in the regulation of a number of transcription factors (Hershko and Ciechanover, 1998). However, the functional relevance and the mechanism of such regulation are largely unclear at the present. Our demonstration of the link between the superinduction of *CYP1A1* and inhibition of the agonist-induced degradation of AhR by cycloheximide underscores the importance of controlling the AhR protein level through proteolysis in AhR function. It will be intriguing to examine if such a connection between AhR degradation and function could be observed in more complex, AhR-mediated functions or responses, such as in the toxicity of TCDD. The bHLH/PAS proteins comprise a growing family of transcription factors that participate in a number of important biological functions, such as circadian rhythm control (Sassone-Corsi, 1997), the hypoxic response (Semenza, 1998), embryonic development (Crews, 1998), and the response to xenobiotics (Whitlock et al., 1996). The bHLH/PAS proteins exhibit large similarities in their structure and signaling mechanisms, and overlap in certain functions. Regulation of the bHLH/PAS factors following activation by a specific signal remains to be analyzed. Therefore, the signal transduction and regulation of AhR can serve as a useful model for other bHLH/PAS proteins. Since the superinduction of *CYP1A1* involves a complex mechanism of regulation of the AhR degradation, elucidating the mechanism of the superinduction will provide new insights into the mechanism by which the bHLH/PAS proteins are regulated through ubiquitin-proteasome mediated proteolysis in the cell.

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[1] Unpublished results.

## References

Agawal, M.L., Taylor, W.R., Chernov, M.V., Chernova, O.B., Stark, G.R., 1998. The p53 network. J. Biol. Chem. 273, 1–4.

Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., Struhl, K., 1998. Current Protocols in Molecular Biology. Wiley, New York.

Calvert, G.M., Sweeney, M.H., Deddens, J., Wall, D.K., 1999. Evaluation of diabetes mellitus, serum glucose, and thyroid function among United States workers exposed to 2,3,7,8-tetracholorodibenzo-*p*-dioxin. Occup. Environ. Med. 56, 270–276.

Carver, L.A., Bradfield, C.A., 1997. Ligand-dependent interaction of the Aryl hydrocarbon receptor with a novel immunophilin homolog in vivio. J. Biol. Chem. 272, 11452–11456.

Crews, S., 1998. Control of cell lineage-specific development and transcription by bHLH-PAS proteins. Genes Dev. 12, 607–620.

Denison, M.S., Fisher, J.M., Whitlock Jr., J.P, 1988. Inducible, receptor-dependent protein-DNA interactions at a dioxin-responsive transcriptional enhancer. Proc. Natl. Acad. Sci. USA 85, 2528–2532.

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

Fernandez-Salguero, P.M., Hilbert, D.M., Rudikoff, S., Ward, J.M., Gonzalez, F., 1996. Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced toxicity. Toxicol. Appl. Pharmacol. 140, 173–179.

Fingerhut, M.A., Halerpin, W.E., Marlow, D.A., Piacitelli, L.A., Honchar, P.A., Sweeney, M.H., Greife, A.L., Dill, P.A., Steenland, K., Suruda, A.J., 1991. Cancer motality in workers exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. N. Engl. J. Med. 324, 212–218.

Hankinson, O., 1995. The aryl hydrocarbon receptor complex. Annu. Rev. Pharmacol. Toxicol. 35, 307–340.

Haupt, Y., Maya, R., Kazaz, A., Oren, M., 1997. Mdm2 promotes the rapid degradation of p53. Nature 387, 296–299.

Hershko, A., Ciechanover, A., 1998. The ubiquitin system. Annu. Rev. Biochem. 67, 425–479.

Honda, R., Tanaka, H., Yasuda, H., 1997. Oncoprotein Mdm2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett. 420, 25–27.

Israel, D., Estolano, M.G., Galeazzi, D.R., Whitlock Jr., J.P, 1985. Superinduction of cytochrome P1-450 gene transcription by inhibition of protein synthesis in wild type and variant mouse hepatoma cells. J. Biol. Chem. 260, 5648–5653.

Klawans, H.L., 1987. Acta Neurol. Scand. 2, 255–261.

Ko, H.P., Okino, S.T., Ma, Q., Whitlock Jr., J.P, 1996. Dioxin-induced CYP1A1 transcription in vivo: the aromatic hydrocarbon receptor mediates transactivation, enhancer-promoter communication, and changes in chromatin structure. Mol. Cell Biol. 16, 430–436.

Ko, H.P., Okino, S.T., Ma, Q., Whitlock Jr., J.P, 1997. Transactivation domains facilitate promoter occupancy for

the dioxin-inducible CYP1A1 gene in vivo. Mol. Cell Biol. 17, 3497–3507.

Lusska, A., Shen, E., Whitlock Jr., J.P, 1993. Protein-DNA interactions at a dioxin-responsive enhancer. Analysis of six bona fide DNA-binding sites for the liganded Ah receptor. J. Biol. Chem. 268, 6575–6580.

Lusska, A., Wu, L., Whitlock Jr., J.P, 1992. Superinduction of CYP1A1 transcription by cycloheximide. J. Biol. Chem. 267, 15146–15151.

Luster, M., Faith, R., Clark, G., 1979. Laboratory studies on the immune effects of halogenated aromatics. Ann. N. Y. Acad. Sci. 31, 473–486.

Ma, Q., Baldwin, K.T., 2000. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced degradation of aryl hydrocarbon receptor (AhR) by the ubiquitin-proteasome pathway. Role of the transcription activation and DNA-binding of AhR. J. Biol. Chem. 275, 8432–8438.

Ma, Q., Dong, L., Whitlock Jr., J.P, 1995. Transcriptional activation by the mouse Ah receptor. Interplay between multiple stimulatory and inhibitory functions. J. Biol. Chem. 270, 12697–12703.

Ma, Q., Whitlock Jr., J.P, 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., Whitlock Jr., J.P, 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. 272, 8878–8884.

Meyer, B.K., Pray-Grant, M.G., Heuvel, J.P., Perdew, G.H., 1998. Hepatitisis B virus X-associated protein 2 a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity. Mol. Cell. Biol. 18, 978–988.

Miller, A.G., Israel, D.L., Whitlock Jr., J.P, 1983. Biochemical and genetic analysis of variant mouse hepatoma cells defective in the induction of benzo(a)pyrene-metabolizing enzyme activity. J. Biol. Chem. 258, 3523–3527.

Mimura, J., Yamashita, K., Nakamura, K., Morita, M., Takagi, T.N., Nakao, K., Ema, M., Sogawa, K., Yasuda, M., Katsuki, M., Fujii-Kuriyama, Y., 1997. Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. Genes Cells 2, 645–654.

Okey, A.B., Riddick, D.S., Harper, P.A., 1994. The Ah receptor: mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds. Toxicol. Lett. 70, 1–22.

Oliver, R., 1975. Br. J. Indust. Med. 32, 49–53.

Perdew, G.H., 1988. Association of the Ah receptor with the hsp90-kDa heat shock protein. J. Biol. Chem. 263, 13802–13805.

Poland, A., Glover, E., 1980. 2,3,7,8,-Tetrachlorodibenzo-*p*-dioxin: segregation of toxocity with the Ah locus. Mol. Pharmacol. 17, 86–94.

Poland, A., Knutson, J.C., 1982. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. Annu. Rev. Pharmacol. Toxicol. 22, 517–554.

Pollenz, R.S., 1996. The aryl-hydrocarbon receptor, but not the aryl-hydrocarbon receptor nuclear translocator protein, is rapidly depleted in hepatic and nonhepatic culture cells exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Mol. Pharmacol. 49, 391–398.

Reick, M., Robertson, R.W., Pasco, D.S., Fagan, J.B., 1994. Down-regulation of nuclear aryl hydrocarbon receptor DNA-binding and transactivation functions: requirement for a labile or inducible factor. Mol. Cell. Biol. 14, 5653–5660.

Safe, S.H., 1986. Comparative toxicity and mechanism of action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. Annu. Rev. Pharmacol. Toxicol. 26, 371–399.

Sassone-Corsi, P., 1997. PERpetuating the PASt. Nature 389, 443–444.

Semenza, G.L., 1998. Hypoxia-induced factor 1: master regulator of O2 homeostasis. Curr. Opinion in Genetics and Development 8, 588–594.

Whitlock Jr., J.P, 1999. Induction of cytochrome P4501A1. Annu. Rev. Pharmacol. Toxicol. 39, 103–125.

Whitlock Jr., J.P., Okino, S.T., Dong, L., Ko, H.P., Clarke-Katzenberg, R., Ma, Q., Li, H., 1996. Cytochromes P450 5: induction of cytochrome P4501A1: a model for analyzing mammalian gene transcription. FASEB J. 10, 809–818.