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Superinduction of *CYP1A1* Gene Expression

REGULATION OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN-INDUCED DEGRADATION OF Ah RECEPTOR BY CYCLOHEXIMIDE*

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Cycloheximide superinduces the transcription of *CYP1A1* in the presence of an agonist for the Ah receptor (AhR). To investigate the molecular target for "superinduction," we analyzed the agonist-induced degradation of AhR. Whereas 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a potent agonist of AhR, induces a rapid reduction of the AhR protein, cycloheximide blocks the down-regulation of steady state AhR. Analyses of the turnover of AhR reveal that cycloheximide blocks the shortening of the half-life of AhR by TCDD. Blocking of the TCDD-induced AhR degradation requires inhibition of protein synthesis, because (*a*) cycloheximide inhibits protein synthesis at the concentration at which it causes superinduction and inhibition of AhR degradation; and (*b*) puromycin, an inhibitor of protein synthesis by mimicking aminoacyl-tRNA, also blocks the TCDD-induced AhR degradation. The blocking of the TCDD-induced AhR degradation correlates with the superinduction of *CYP1A1* gene expression in a time- and dose-dependent manner. Furthermore, cycloheximide is shown to increase the accumulation of the TCDD-activated AhR and the functional AhR·Arnt complex in nucleus. Collectively, our results reveal a mechanism of superinduction by cycloheximide by enhancing the stability of agonist-activated AhR. The finding that inhibition of protein synthesis blocks the TCDD-induced AhR turnover implicates a cycloheximide-sensitive, labile factor (designated as AhR degradation promoting factor, or ADPF) in controlling the removal of agonist-activated AhR in nucleus.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD,[1] dioxin) represents the prototype for a class of structurally related halogenated aromatic hydrocarbons, including polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls (1, 2). These man-made compounds 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 drug metabolizing enzymes (2–6). The health effect of TCDD on human beings remains a matter of debate. Humans exposed to TCDD exhibit certain skin lesions such as chloracne; the possibility that TCDD exposure causes certain neuro- and psychopathological alterations (7, 8), some forms of cancers and diabetic conditions (9, 10), and reproductive lesions is a particular concern of public health.

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor with a basic helix-loop-helix PAS (bHLH/PAS) modular structure (2, 3, 11, 12). Mouse genetic studies implicate AhR in most of the biological responses to TCDD, presumably by affecting the expression of target genes (13–15). Recent observations also imply that AhR plays certain roles in embryonic development and liver and immune functions in mice (16, 17), and modulate the growth, differentiation, and apoptotic processes in certain cell lines and mouse liver (18–22); these activities or functions of AhR were observed in the absence of known exogenous agonists, implicating a mechanism(s) of activating AhR under physiological conditions *in vivo*. Because of the broad range and the complexity of the biological responses that AhR contributes to, it is conceivable that the signal transduction of AhR involves a complex process during which AhR is regulated through different cellular mechanisms in a tissue-, species-, and developmental stage-dependent manner.

The TCDD-inducible *CYP1A1* gene encodes cytochrome P4501A1, a major inducible form of microsomal P450 in mammalian species; P4501A1 oxygenates polycyclic aromatic hydrocarbons, such as the carcinogen benzo(*a*)pyrine (23), as the initial step in the metabolism of the chemicals to water soluble metabolites for excretion from body. Studies on the induction of *CYP1A1* gene expression by TCDD provided major mechanistic understanding of the mechanism of action and regulation of AhR (2, 3). In uninduced cells, AhR is localized in the cytoplasm, complexed with hsp90 (24) and AIP, an immunophillin-type chaperon protein (25–27). Binding with an agonist triggers the dissociation of AhR from the associated proteins and translocation into nucleus, where AhR dimerizes with Arnt, another bHLH/PAS transcription factor (28). The AhR/Arnt dimer binds to a specific nucleotide sequence termed DRE (dioxin responsive element) in the enhancer region of the *CYP1A1* gene (29); the transcription activation domains of 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 (30–32).

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[1] The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; Arnt, AhR nuclear translocator; Me_2SO, dimethyl sulfoxide; LPS, lipopolysaccharide; CHX, cycloheximide; TA, transcription activation; bHLH, basic helix loop helix; ADPF, AhR degradation promoting factor; EMSA, electrophoretic mobility shift assay; DIG, digitonin; PAGE, polyacrylamide gel electrophoresis.

Several cellular mechanisms have been recognized for the regulation of the AhR activity during the induction of *CYP1A1*. For example, cycloheximide enhances the induction of *CYP1A1* gene expression by TCDD, a phenomenon termed "superinduction." 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 (33, 34). Since cycloheximide is known to inhibit protein synthesis, it is assumed that a labile, inhibitory protein factor regulates the AhR activity. However, the nature and the mechanism of action of the putative "labile" factor remain unknown. In another scenario, treatment with TCDD shortens the half-life of the AhR protein from 28 to 3 h (35). The TCDD-induced turnover of AhR is mediated through the 26 S proteasome, involves ubiquitination of AhR, and requires the transcription activation domain of AhR (35). Moreover, inhibition of the 26 S proteasome by proteasome inhibitors increases the induction of *CYP1A1* by TCDD; these findings implicate the agonist-induced AhR degradation in the regulation of AhR function.

To identify the molecular target of cycloheximide, we analyzed the TCDD-induced AhR turnover in the superinduction. We show here that cycloheximide blocks TCDD-induced degradation of AhR. Inhibition of the TCDD-induced AhR degradation requires inhibition of protein synthesis and correlates with the superinduction in a time- and dose-dependent manner. Furthermore, cycloheximide is shown to increase the accumulation of the AhR and the functional AhR·Arnt complex in nucleus. In addition, we show that inhibition of the 26 S proteasome superinduces *CYP1A1* expression in a similar fashion to cycloheximide. To our knowledge, this report is the first study demonstrating that cycloheximide blocks the agonist-induced degradation of the AhR protein. Our findings provide a novel mechanism of superinduction of *CYP1A1* in which a cycloheximide-sensitive, labile protein factor (designated as AhR degradation promoting factor, or ADPF) negatively regulates the stability of agonist-activated, nuclear AhR.

EXPERIMENTAL PROCEDURES

Materials—AmpliTaq 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), puromycin, dimethyl sulfoxide (Me_2SO), lipopolysaccharide (LPS), aprotinin, leupeptin, and phenylmethylsulfonyl fluoride were from Sigma. Lactacycstin and MG132 were 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.

Cell Culture and Treatment—The mouse hepa1c1c7 cells were gifts from Dr. J. P. Whitlock, Jr. (Stanford University). The cells were grown as monolayer in α-minimal essential medium, containing 10% fetal bovine serum and 5% CO_2 at 37 °C, as described previously (36). The cells were treated with TCDD or other agents as described in figure legends; Me_2SO was used as the solvent control for TCDD.

Preparation of Nuclear Extract and Total Cell Lysate—Nuclear extracts were prepared according to published procedures (29). 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.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was carried out using nuclear extract from hepa1c1c7 cells, as described previously (29), except that 6% polyacrylamide gels were used. The DNA probe contains the DNA recognition sequence for the AhR/Arnt heteromer designated as DRE D (37). The probe was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England Biolabs). The nuclear extracts were incubated with poly(dI-dC) 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.

Immunoblot Analysis—For immunoblotting, total cell lysate or nuclear extract of 5 μg was fractionated on SDS-polyacrylamide gels, and transferred to nitrocellulose membranes according to established procedures (38). 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 (25) 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 Pharmacia Biotech). 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 by 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.

RNA Analysis—For Northern blotting of *CYP1A1*, a cDNA fragment (~700 base pairs) 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 reverse transcriptase-polymerase chain reaction 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. Quantitation of the blotting results were performed by using the ImageQuaNT program as described above. All data were corrected for loading variations by comparing the amount of actin of each sample analyzed.

Pulse-Chase Labeling—Cells grown to near confluence were incubated in methionine-free medium with 10% dialyzed fetal bovine serum (Life Technologies, Inc.) for 1 h and incubated for another hour in fresh methionine-free medium with 10% dialyzed fetal bovine serum plus [^{35}S]methionine (100 μCi/ml, Amersham Pharmacia Biotech). The cells were then incubated in α-minimal essential medium with 10% fetal bovine serum and treated with Me_2SO, 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 phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin in phosphate-buffered saline). The ^{35}S-labled AhR was precipitated with the anti-AhR antibodies, fractionated by SDS-PAGE (10%), and visualized by fluorography.

Immunoprecipitation—AhR was precipitated with anti-AhR antibodies according to a standard method (39). Briefly, cells grown in 6-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 (20, 40) for 1 h and with protein A-agarose for an additional hour. The precipitated agarose beads were washed 3 times with the RIPA buffer and resuspended in a loading buffer for analysis by SDS-PAGE.

Immunofluorescence Staining and Confocal Microscopy—Immunofluorescent staining of cells with anti-AhR IgG was performed according to standard procedures (39). Briefly, cells grown on coverslips were washed with 1 × phosphate-buffered saline, fixed in 3.7% formaldehyde for 10 min, and permeabilized with methanol at −20 °C for 6 min. The cells were then blocked in 1% bovine serum albumin for 30 min with shaking, and blotted with an affinity-purified polyclonal anti-mouse

AhR IgG (Biomol, Plymouth Meetings, PA) in 1% bovine serum albumin for 1 h, followed by incubation with a fluorescein-conjugated anti-rabbit IgG (Chemicon International Inc., Temecula, CA) for an additional 1 h in the dark. The glass coverslips were mounted onto slides with Prolong (Molecular Probes, Eugene, OR), an anti-fade mounting medium. Fluorescence was visualized using a Sarastro 2000 laser scanning confocal microscope fitted with an argon-ion laser (Molecular Dynamics, Inc., Sunnyvale, CA) and an Optiphot-2 microscope (Nikon, Inc., Melville, PA). Confocal images were recorded through a ×60 lens objective using a 488-nm laser line.

RESULTS

Cycloheximide Blocks TCDD-induced Turnover of the AhR Protein—Cycloheximide superinduces the transcriptional gene expression of *CYP1A1* in the presence of an agonist of AhR. As shown in Fig. 1*A*, cycloheximide alone does not affect *CYP1A1* gene expression (*lane 3*), whereas co-treatment of hepa1c1c7 cells with TCDD (1 nM) and cycloheximide (10 μg/ml) for 5 h increases the induction of *CYP1A1* by TCDD by 6-fold (compare *lanes 2* and *4*). 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. In a recent study on the turnover of the AhR protein, we showed that TCDD shortens the half-life of AhR from 28 to 3 h through ubiquitin-proteasome mediated proteolysis (35). Furthermore, inhibition of the 26 S proteasome by using proteasome inhibitors enhances the induction of *CYP1A1* by TCDD. 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. 1, *B* and *C*, treatment of the cells with TCDD (1 nM, 4 h) down-regulates the steady state AhR to less than 20% of the control. 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.

Since TCDD down-regulates the AhR protein by increasing its turnover, we next tested if cycloheximide inhibits the TCDD-induced degradation of AhR by measuring the half-life ($t_{1/2}$) of AhR. Pulse-chase labeling experiments reveal that AhR in Me_2SO-treated cells is relatively stable with a $t_{1/2}$ of 28 h (Fig. 2, *A* and *B*, Ref. 35); cycloheximide alone does not affect the $t_{1/2}$ of AhR. TCDD shortens the $t_{1/2}$ of AhR to ~3 h. However, the $t_{1/2}$ value of AhR in cells treated with TCDD plus cycloheximide is comparable with that in Me_2SO-treated cells (*i.e.* ~28 h). Therefore, cycloheximide fully inhibits the TCDD-induced turnover of the AhR protein.

Blocking of the AhR Turnover by Cycloheximide Requires Inhibition of Protein Synthesis—Cycloheximide inhibits protein synthesis by blocking the peptidyl synthetase activity of eukaryotic ribosomes. To explore the mechanism of inhibition of TCDD-induced AhR degradation by cycloheximide, we analyzed the role of protein synthesis in AhR degradation. IκBα, a regulatory subunit of the NFκB transcription factor, is known to undergo a rapid, signal-induced degradation during the activation of NFκB, followed by recovery of the protein through protein synthesis (41). Therefore, we first used the signal-induced degradation and synthesis of IkBα as a control to test if cycloheximide inhibits protein synthesis under the experimental condition for superinduction. As shown in Fig. 3, treatment with LPS (5 μg/ml), an activator of NFκB, results in a rapid reduction of IκBα (*lanes 1–5*); the reduction is followed by recovery of the protein level through protein synthesis (*lane 6*). However, co-treatment with LPS and CHX (10 μg/ml) blocks

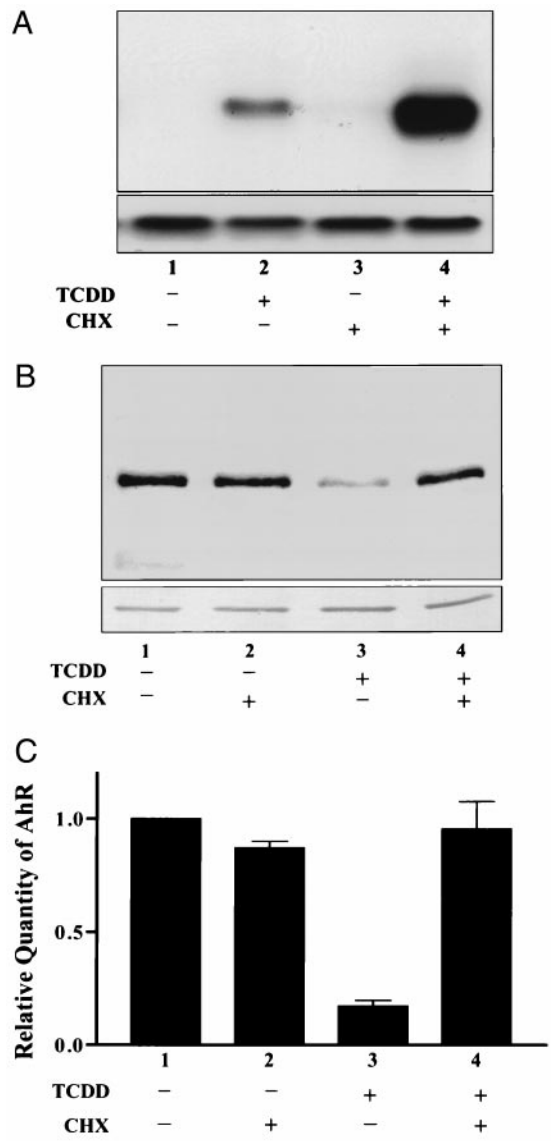

FIG. 1. **Superinduction of *CYP1A1* and inhibition of TCDD-induced down-regulation of AhR by cycloheximide.** *A,* Northern blotting. Hepa1c1c7 cells were grown to near confluency in a 100-mm dish, and were treated with TCDD (1 nM), CHX (10 μg/ml), or both for 5 h. Total RNA was prepared, fractionated 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 "Experimental Procedures." Each lane contains 5 μg of RNA. *B,* immunoblotting. 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) as described under "Experimental Procedures." The blot was first probed for AhR protein, visualized by chemiluminescence, then reprobed for the actin protein, and visualized by color development. *C,* quantitation of the immunoblotting results. Data represent three separate experiments. The results were quantitated by using the ImageQuaNT software (Molecular Dynamics) and were corrected for loading variations by comparing the amount of actin in each sample. Statistical analysis was performed using the GraphPad PRISM program (GraphPad Software, Inc.) and standard deviation was used to represent variations.

the recovery of IκBα (compare *lane 12* with *lane 6*). These results reveal that cycloheximide inhibits the synthesis of labile proteins, such as IκBα, at a concentration at which it superinduces *CYP1A1* and inhibits the TCDD induced AhR degradation. Others have reported inhibition of total protein synthesis by cycloheximide at a similar concentration (34). These findings implicate inhibition of protein synthesis in the action of cycloheximide on AhR degradation. These results also

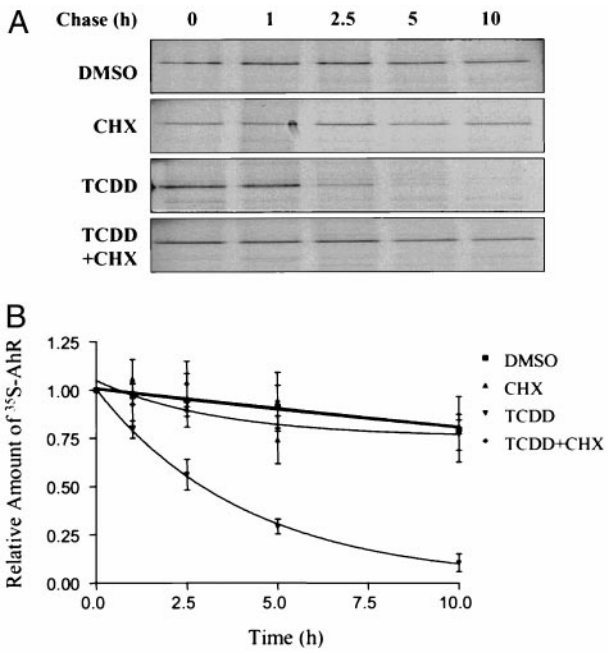

FIG. 2. **Blocking the TCDD-induced turnover of AhR by cycloheximide.** *A,* pulse-chase labeling of AhR. Hepa1c1c7 cells were labeled with [^{35}S]methionine and were treated with Me_2SO (*DMSO*), CHX (10 μg/ml), TCDD (1 nM), or CHX + TCDD as indicated. The AhR protein was immunoprecipitated with a polyclonal anti-AhR antibody, fractionated by SDS-PAGE, and visualized by fluorography, as described under "Experimental Procedures." The hours indicate the time period of treatment after pulse labeling. *B,* $t_{1/2}$ of AhR. The results from pulse-chase experiments were quantified by densitometry and analyzed using the ImageQuaNT software. The $t_{1/2}$ of AhR was calculated and plotted using the Prism program. Data represent means and standard deviation from three separate experiments.

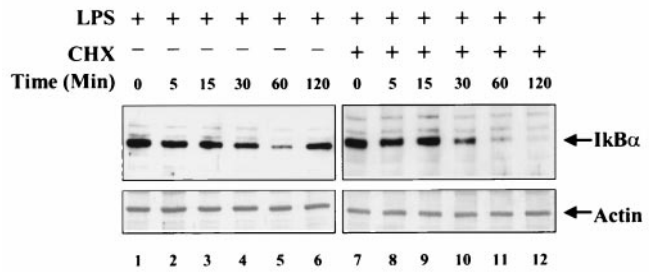

FIG. 3. **Cycloheximide blocks the synthesis of IκBα after LPS stimulation.** Wild type cells were seeded in 6-well plates at a density of 5×10^4 per well and allowed to grow to near confluency. The cells were incubated in α-minimal essential medium, 0.5% serum, 5% CO_2 for ~18 h for synchronization; the cells were then incubated in the normal α-minimal essential medium with 10% serum and 5% CO_2. One hour later, the cells were treated with LPS (5 μg/ml) or LPS plus CHX (10 μg/ml, added to the cells 1 h before treatment with LPS). The cells were harvested for immunoblotting at indicated time points for analyses of IκBα and actin; 5 μg of protein was used for each lane.

imply that inhibition of protein degradation by cycloheximide exhibits certain specificity toward target proteins, because cycloheximide does not inhibit the LPS-induced degradation of IκBα.

We next examined if inhibition of protein synthesis is sufficient for blocking of AhR degradation. Puromycin, an analog of aminoacyl-tRNA, inhibits protein synthesis and superinduces *CYP1A1* with a similar potency to cycloheximide (33). Therefore, we tested if puromycin blocks AhR degradation by TCDD. As shown in Fig. 4, while puromycin alone does not affect the AhR protein level, co-treatment of cells with TCDD and puromycin fully inhibits the degradation of AhR by TCDD. Since puromycin inhibits protein synthesis through a different mechanism from that of cycloheximide, these results indicate that inhibition of protein synthesis is sufficient for inhibition of TCDD-induced AhR turnover. Together, these data support the mechanism of inhibition of AhR degradation through inhibition of protein synthesis. These results implicate a cycloheximide-sensitive, labile, or inducible factor in promoting the TCDD-induced degradation of AhR; hence, we designated the factor as ADPF. Cloning of ADPF will provide new insights into the interplay between inhibition of protein synthesis and inhibition of protein degradation.

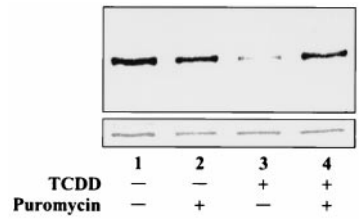

FIG. 4. **Inhibition of TCDD-induced AhR degradation by puromycin.** Cells were grown in 60-mm dishes and treated with TCDD (1 nM), puromycin (30 μg/ml), or both for 5 h. Total cell lysate was prepared and analyzed by immunoblotting (5 μg/lane). The blot was first probed for AhR protein, visualized by chemiluminescence, then reprobed for the actin protein, and visualized by color development as described for Fig. 1.

Time and Dose Curves of Inhibition of AhR Degradation and Superinduction by Cycloheximide—The finding that cycloheximide blocks TCDD-induced AhR degradation at the concentration of superinduction suggests a mechanism of superinduction by which cycloheximide enhances the stability of agonist-activated AhR through inhibition of AhR degradation. To test this notion, we examined the time and dose curves of the inhibition of AhR degradation and the superinduction of *CYP1A1* by cycloheximide. Treatment of cells with TCDD causes a time-dependent reduction of the level of the AhR protein (Fig. 5, *A* and *B, lanes 6–10*), which approaches the maximum reduction at 5 h. Cycloheximide blocks the TCDD-induced reduction (*lanes 11–15*) and increases the AhR protein to levels comparable to those treated with cycloheximide alone (*lanes 1–5*). Analyses of the induction of *CYP1A1* under similar conditions reveal that TCDD induces *CYP1A1* time dependently with the maximum induction at 5 h. Cycloheximide enhances the maximal induction (Fig. 5, *C* and *D*, compare *lanes 9* and *10* with *lanes 13–15*). Furthermore, cycloheximide treatment shortens the time to reach the maximal induction (*i.e.* 2.5 h), indicating that inhibition of AhR degradation at early time points markedly increases the rate of the induction. The block of the AhR degradation by cycloheximide is dose-dependent; the effect is observable at a concentration of 100 ng/ml cycloheximide (Fig. 6, *A* and *B, lane 8*) and maximal at 10 μg/ml (*lane 9*), at which the AhR level is comparable to those of the control samples. Cycloheximide treatment causes dose-dependent superinduction of *CYP1A1*; the superinduction is observed at 100 ng/ml cycloheximide and reaches the maximal level at 10 μg/ml (Fig. 6, *C* and *D*). Thus, blocking the TCDD-induced degradation of AhR by cycloheximide correlates with the superinduction of the *CYP1A1* gene in a time- and dose-dependent manner; these findings support the mechanism of superinduction by cycloheximide by inhibiting the TCDD-induced down-regulation of AhR.

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 nucleus by increasing the amount and function of agonist-activated, nuclear AhR, which mediates the superinduction. To examine this possibility, we first analyzed the AhR protein level in nuclear extract. As shown in Fig. 7, cycloheximide treatment increases the amount of AhR protein in

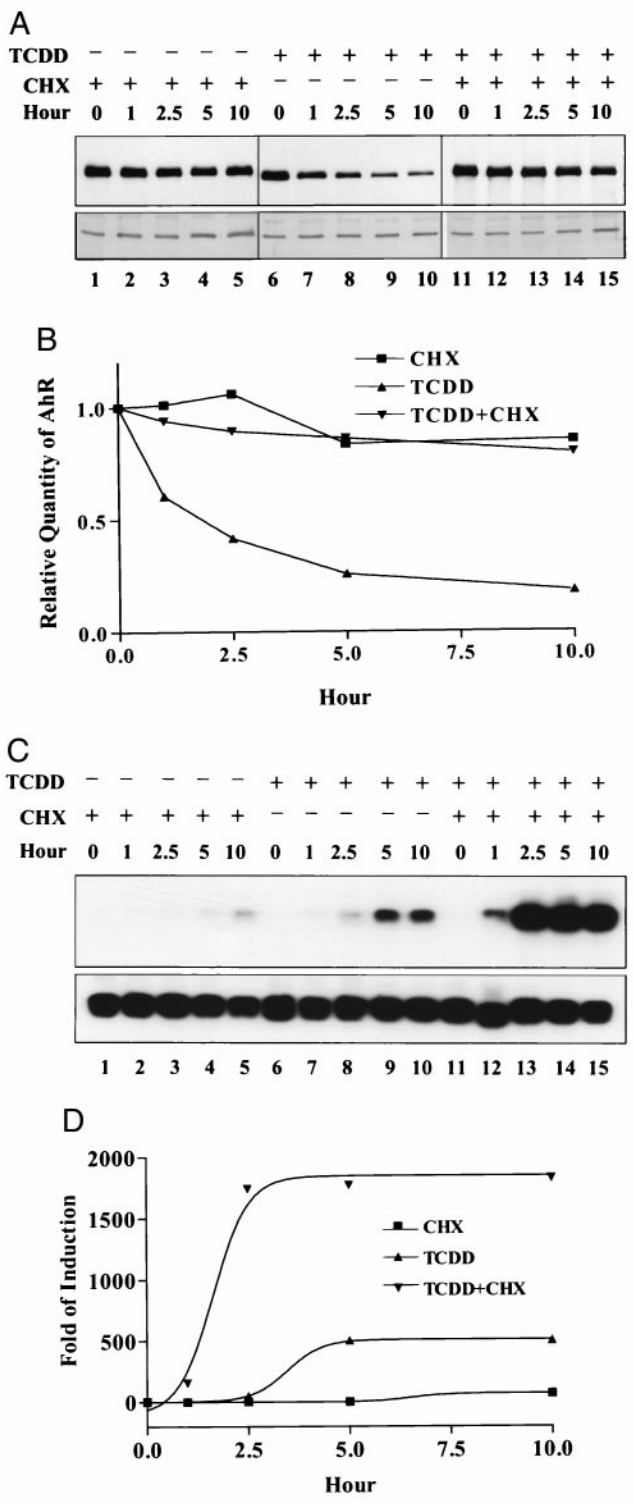

FIG. 5. **Time curves of inhibition of AhR degradation and superinduction of *CYP1A1* by cycloheximide.** Cells were grown to near confluency, treated with TCDD (1 nM), CHX (10 μg/ml), or TCDD plus CHX, and harvested for analyses of proteins and RNAs at indicated time points. *A,* immunoblotting of the AhR and actin proteins. Each lane contains 5 μg of proteins. *B,* quantitation of the immunoblotting results by using the ImageQuaNT program. *C,* Northern blotting of the *CYP1A1* and actin mRNAs. Each lane contains 5 μg of total RNA. *D,* quantitation of the Northern blotting results by using the ImageQuaNT program.

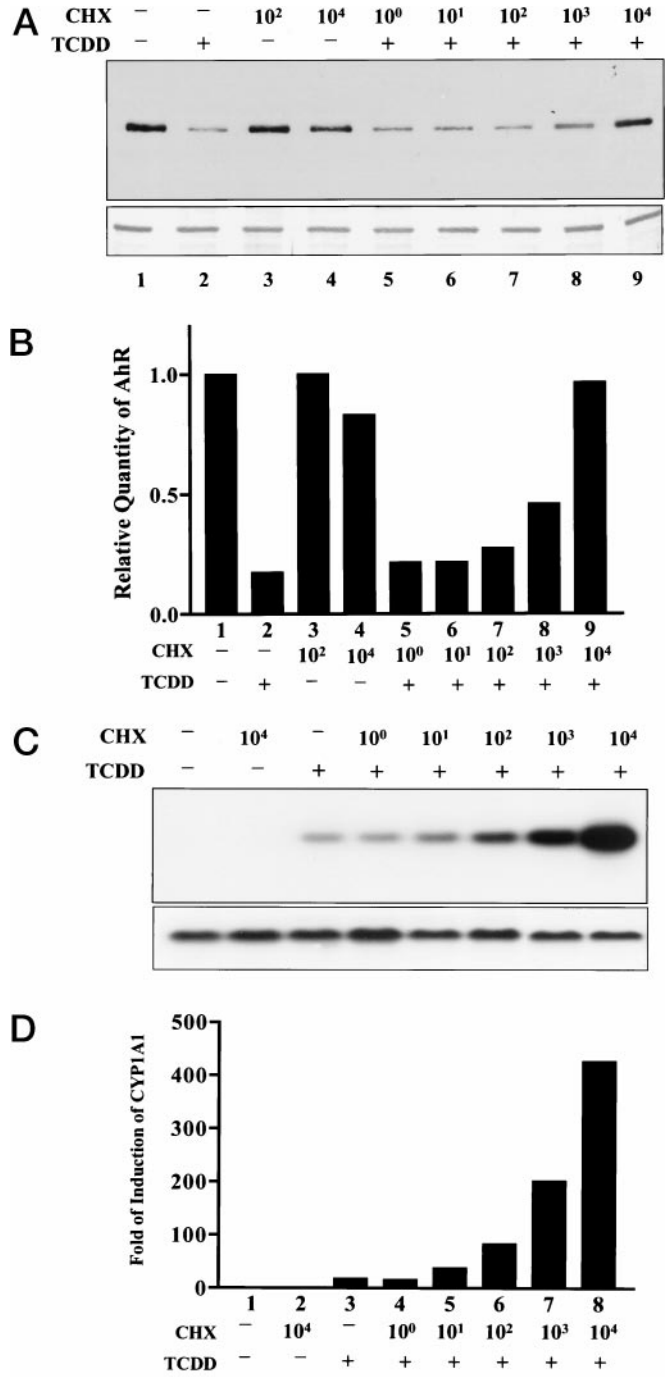

FIG. 6. **Dose curves of inhibition of AhR degradation and superinduction of *CYP1A1* by cycloheximide.** Cells were grown to near confluency, treated with TCDD (1 nM), or TCDD plus CHX at the indicated concentrations (in ng/ml) for 4 h, and were harvested for analyses of proteins and RNAs. *A,* immunoblotting of the AhR and actin proteins. Each lane contains 5 μg of protein. *B,* quantitation of the immunoblotting results by using the ImageQuaNT program. *C,* Northern blotting of the *CYP1A1* and actin mRNAs. Each lane contains 5 μg of RNA. *D,* quantitation of the Northern blotting results by using the ImageQuaNT program.

nuclear extract of cells treated with TCDD plus cycloheximide dose-dependently; the increase is observable at 100 ng/ml cycloheximide and is maximal at 10 μg/ml. It has been shown that TCDD induces translocation of AhR from cytoplasm into nucleus, followed by reduction of the nuclear AhR through degradation (42). Therefore, we next examined if cycloheximide affect the distribution and the protein level of AhR in cytoplasm and nucleus by immunofluorescent confocal microscopy. Fig. 8 shows that the AhR staining is mostly in the cytoplasm in Me_2SO-treated cells. Treatment with TCDD (1 nM) for 6 h reduces the cytoplasmic and total staining of AhR; whereas

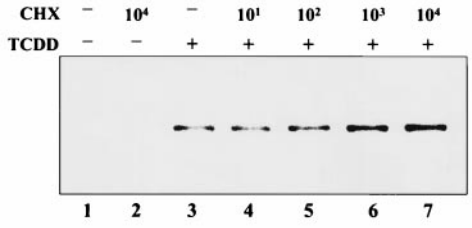

FIG. 7. **Immunoblotting of nuclear AhR.** Cells grown in 100-mm dishes were treated with TCDD (1 nM), or TCDD plus CHX at indicated concentrations (in ng/ml) for 4 h, and nuclear extracts were prepared as described under "Experimental Procedures." The nuclear extracts (5 μg/lane) were analyzed by immunoblotting for the AhR protein.

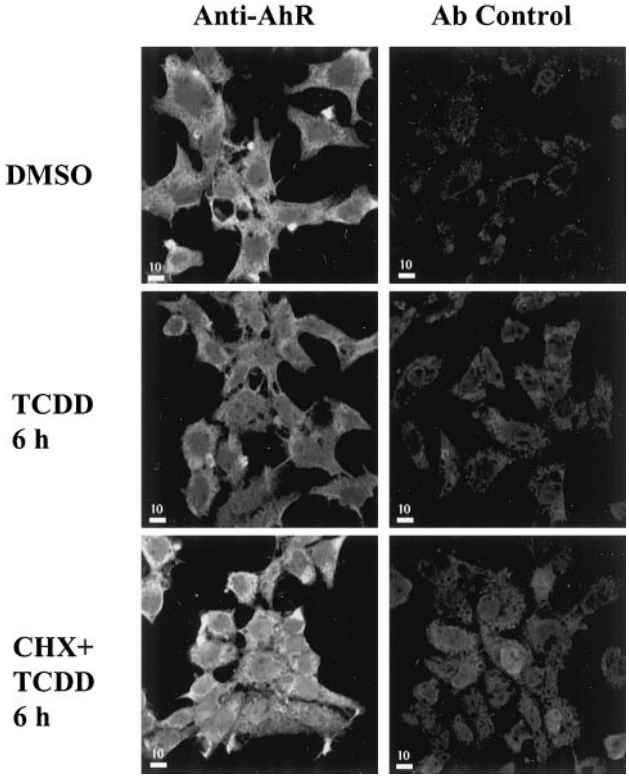

FIG. 8. **Immunofluorescent confocal microscopy.** Hepa1c1c7 cells were grown on coverslides, treated with Me_2SO, TCDD (1 nM), CHX (10 μg/ml), or TCDD plus CHX as indicated. The cells were fixed for immunofluorescent staining of AhR, and were examined under confocal microscopy as described under "Experimental Procedures." Cells stained with the fluorescein-conjugated anti-rabbit IgG were used as background control (Ab control). *DMSO*, Me_2SO.

co-treatment of the cells with TCDD and cycloheximide (10 μg/ml, 6 h) increases both nuclear and total staining of AhR. Lastly, 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 reveal a dose-dependent increase in the interaction of the AhR/Arnt heteromer with the DRE sequences (Fig. 9). The increase in the band shift by EMSA is observable at 10 ng/ml cycloheximide. Taken together, these results reveal that cycloheximide increases the accumulation of both the AhR protein and the functional AhR·Arnt complex in nucleus.

Since lactacystin and MG132, which inhibit TCDD-induced AhR degradation by inhibiting the 26 S proteasome, enhance the induction of *CYP1A1* by TCDD (35), we compared the superinduction of *CYP1A1* by cycloheximide and by the proteasome inhibitors. As shown in Fig. 10, lactacystin and MG132 increase the induction of *CYP1A1* by TCDD by ~4-fold, whereas cycloheximide superinduces *CYP1A1* by ~6-fold.

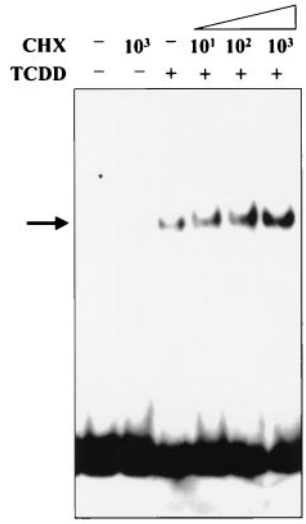

FIG. 9. **Electrophoretic mobility shift assay.** Nuclear extracts were prepared from cells treated with TCDD (1 nM), or TCDD plus CHX at the indicated concentrations for 4 h. EMSA was performed using the nuclear extract preparations and a ^{32}P-labeled DNA probe containing a functional DRE sequence as described under "Experimental Procedures." The *arrow* indicates the AhR·Arnt·DRE complex. Shown at the *bottom* of the film are the ^{32}P-labeled, free DRE probes.

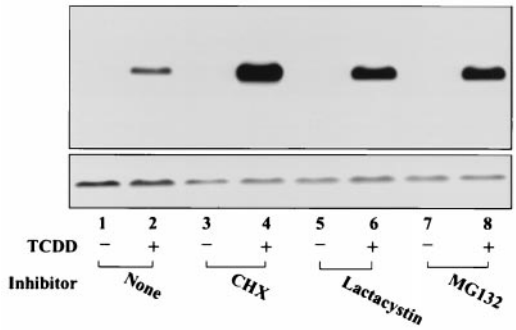

FIG. 10. **Comparison of superinduction of *CYP1A1* by cycloheximide and proteasome inhibitors.** Total RNA was prepared from cells treated with TCDD, CHX, lactacystin, or MG132 for 5 h as indicated. RNA samples (5 μg/lane) were analyzed by Northern blotting for *CYP1A1* and actin as described in the legend to Fig. 1.

These results further support the notion that increasing the stability of AhR by inhibiting its degradation superinduces *CYP1A1*. These data also imply that, while both cycloheximide and the proteasome inhibitors fully inhibit AhR degradation, the mechanism and the functional impact of the inhibition by the chemicals are distinct, because cycloheximide superinduces *CYP1A1* to a larger extent than the proteasome inhibitors.

DISCUSSION

Inhibition of the Turnover of Agonist-activated AhR as a Mechanism of Superinduction—The induction of *CYP1A1* transcription by TCDD through the AhR-mediated signal transduction constitutes a model response for analyzing the mechanism of action of TCDD at the level of gene regulation (43). Cycloheximide, an inhibitor of protein synthesis, superinduces the transcription of the *CYP1A1* gene in the presence of TCDD; the 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 the 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 have previously shown that TCDD markedly increases the turnover of the AhR protein; activation of AhR by TCDD shortens the half-life of AhR from 28 to 3 h (35). The TCDD-induced AhR degradation is mediated by the 26 S proteasome and involves ubiquitination of the AhR protein. Here, 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 Me_2SO- or cycloheximide only-treated cells (Fig. 2). 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.

Several lines of evidence support the hypothesis that enhanced stability of the activated AhR accounts for the superinduction of *CYP1A1*. First, inhibition of the TCDD-induced degradation of AhR by cycloheximide correlates with the superinduction of *CYP1A1* gene expression in a time- and dose-dependent manner (Figs. 5 and 6). The observation that cycloheximide not only enhances the maximal induction of *CYP1A1* by TCDD, but also shortens the time required to reach the maximal induction (from 5 to 2.5 h) suggests that inhibition of the degradation of the activated AhR at early time points markedly increases the rate of the induction. Second, analyses of the nuclear AhR reveal that cycloheximide increases the accumulation of the activated AhR and the AhR·Arnt complex in nucleus (Figs. 7–9). Third, puromycin, which superinduces *CYP1A1*, inhibits TCDD-induced AhR degradation similarly to cycloheximide (Fig. 4). Last, inhibition of the 26 S proteasome, which mediates the TCDD-induced AhR degradation, also enhances the induction of *CYP1A1* by TCDD (Fig. 10 and Ref. 35). Collectively, these findings established that the mechanism of superinduction involves enhancing the stability of nuclear AhR by cycloheximide through inhibition of AhR degradation. However, this conclusion does not exclude the possibility that cycloheximide regulates other aspects of the AhR protein or downstream events of AhR signaling in gene regulation (for more discussion, see below).

Regulation of the Stability of Agonist-activated AhR by a Cycloheximide-sensitive, Labile Factor ADPF—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 nucleus. Here, we designate the trans-factor as ADPF. Inhibition of the synthesis of ADPF by cycloheximide releases the negative control of AhR by increasing the stability of the AhR, resulting in a superinduction. This hypothesis is supported by the following findings: 1) cycloheximide inhibits the synthesis of labile factors, such as IkBα (Fig. 3), or total protein (34), at the concentration at which it causes superinduction and inhibition of AhR degradation; and 2) puromycin, which inhibits protein synthesis through a different mechanism from that of cycloheximide, blocks AhR degradation similarly to cycloheximide (Fig. 4), implying that inhibition of protein synthesis is sufficient for inhibition of AhR degradation. 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; block of the induction of ADPF by inhibiting protein synthesis by cycloheximide or puromycin disrupts the autoregulation. Alternatively, ADPF is a short-lived, labile protein, 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 (34) 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 (44). 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 (45–47). The Mdm2 protein is induced by p53 and is degraded through the 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 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 degradation 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.[2] 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 nucleus.

We have shown that both cycloheximide and the proteasome inhibitors, lactacystin and MG132, completely inhibit AhR degradation; however, cycloheximide enhances the induction of *CYP1A1* to a larger extent than the proteasome inhibitors (*i.e.* 6-fold *versus* 4-fold). These findings suggest that, in addition to controlling the stability of AhR, the ADPF protein may regulate other aspects of AhR function. In a previous study, we demonstrated that the C-terminal portion of AhR, which consists of the transcription activation (TA) domains of AhR, is required for the ubiqutin-proteasomal degradation of AhR (35). It is possible that ADPF inhibits the activity of the TA domain of activated AhR, while promoting ubiquitination of the TA domain. Similar mechanisms have been implicated in the regulation of p53 by Mdm2; the Mdm2 protein binds to the TA domain of p53, inhibits the its TA activity in control of gene regulation, and catalyzes ubiquitination of the domain (47, 48). Further analyses are needed to prove the regulation of the AhR TA activity by ADPF. Since the TA domain of AhR mediates the enhancer-promoter communication in the induction of *CYP1A1* (31), a process involving modulation of chromatin structure of the gene, ADPF may also interfere with the interactions among AhR/Arnt, DNA, and chromatin.

Controlling protein levels in cell by the ubiquitin-mediated proteolysis has been implicated in the regulation of a number of transcription factors (for review, see Ref. 49). 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

[2] Q. Ma, and K. T. Baldwin, unpublished results.

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 (50), hypoxic response (51, 52), embryonic development (53), and response to xenobiotics (43). The bHLH/PAS proteins exhibit large similarities in the structure and signaling mechanism, and overlaps 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 cell.

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Superinduction of *CYP1A1* Gene Expression: REGULATION OF 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN-INDUCED DEGRADATION OF Ah RECEPTOR BY CYCLOHEXIMIDE

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