THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 275, No. 12, Issue of March 24, pp. 8432–8438, 2000
Printed in U.S.A.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced Degradation of Aryl Hydrocarbon Receptor (AhR) by the Ubiquitin-Proteasome Pathway

ROLE OF THE TRANSCRIPTION ACTIVATON AND DNA BINDING OF AhR*

(Received for publication, December 20, 1999, and in revised form, January 11, 2000)

Qiang Ma‡ and Kimberly T. Baldwin

From the Molecular Toxicology Laboratory, Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, West Virginia 26505

Activation of the aryl hydrocarbon receptor (AhR) by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a potent agonist of AhR, induces a marked reduction in steady state AhR. To analyze the mechanism of regulation of ligand-activated AhR, we examined the biochemical pathway and function of the down-regulation of the receptor by TCDD. Pulse-chase experiments reveal that TCDD shortens the half-life ($t_{1/2}$) of AhR from 28 to 3 h in mouse hepatoma cells. Inhibitors of the 26 S proteasome, lactacystin and MG132, block the TCDD-induced turnover of AhR. The TCDD-induced degradation of AhR involves ubiquitination of the AhR protein, because (*a*) TCDD induces formation of high molecular weight, ubiquitinated AhR and (*b*) degradation of AhR is inhibited in ts20 cells, which bear a temperature-sensitive mutation in the ubiquitin-activating enzyme E1, at a nonpermissive temperature. Inhibition of proteasomal degradation of AhR increases the amount of the nuclear AhR·Arnt complex and "superinduces" the expression of endogenous *CYP1A1* gene by TCDD, indicating that the proteasomal degradation of AhR serves as a mechanism for controlling the activity of the activated receptor. We also show that deletion of the transcription activation domain of AhR abolishes the degradation, whereas a mutation in the DNA-binding region of AhR or Arnt reduces the degradation; these data implicate the transcription activation domain and DNA binding in AhR degradation. Our findings provide new insights into the regulation of TCDD-activated AhR through ubiquitin-mediated protein degradation.

The aryl hydrocarbon receptor (AhR)[1] is a ligand-activated transcription factor with a basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) domain structure (1–3). Genetic and biochemical studies reveal that AhR mediates most of the biological responses to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The biological effects of TCDD include adaptive responses, such as the induction of drug-metabolizing enzymes, or toxic effects, such as tumor promotion, wasting syndrome, and toxicity to the skin, immune, developmental, or endocrine systems (4–9). The health effects of TCDD and related chemicals on the human population remain a matter of debate. The endogenous ligand(s) for AhR has not been identified; however, genetic evidence implicates AhR in mouse embryonic development, liver, and immune functions, as well as cell growth and differentiation (10–15).

The mechanism of action of AhR involves a multi-step, ligand-induced signal transduction process. Binding of a ligand to AhR in liver cells triggers the dissociation of AhR with associated proteins, including heat shock protein 90 and an immunophilin-type chaperon, AhR-interacting protein (AIP) (16–18). The activated AhR translocates into the nucleus, dimerizes with Arnt, another bHLH/PAS transcription factor, and activates the transcription of target genes by binding to specific enhancer sequences in the regulatory region of the genes; transcription of a target gene involves disruption of the chromatin structures associated with the gene (for review see Ref. 1).

Signal-activated transcription factors are often regulated in cells after activation so that the transcriptional response can be controlled to meet the need of cellular homeostasis. Degradation of a protein through the ubiquitin-proteasome pathway has been shown to be involved in the regulation of many cellular proteins, including transcription factors, such as p53, NF-κB, IκBα, c-Jun, c-Myc, and estrogen receptor α (19–22). Proteasomal degradation of a protein involves covalent attachment of ubiquitin, a 76-residue peptide molecule, to the target protein. Repeated rounds of ubiquitination result in a highly ubiquitinated target protein that is rapidly degraded by the 26 S proteasome. Alternatively, ubiquitination can occur on an associated protein, which assists recognition of the target protein by the proteasome system. Ubiquitination of a protein is catalyzed by a multi enzyme system(s) that includes the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and the ubiquitin ligase (E3), on a specific structural element(s) of the protein called a "degron." Selective degron recognition and subsequent ubiquitination of lysine residues by specific E2 and E3 is central to the specificity and regulation of ubiquitin-mediated proteolysis.

Several observations suggest that AhR is regulated after activation by an agonist. For example, treatment of cells with TCDD causes a time-dependent reduction in the DNA binding activity of the nuclear AhR·Arnt complex (23) and down-regulation in the steady state level of AhR in cultured cells (24). The mechanism of this down-regulation remains unclear. Recent

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: CDC/NIOSH/HELD/TMBB, Mailstop 3014, 1095 Willowdale Rd., Morgantown, WV 26505. Fax: 304-285-5708; E-mail: qam1@cdc.gov.

[1] The abbreviations used are: AhR, aryl hydrocarbon receptor; Arnt, Ah receptor nuclear translocator; bHLH, basic helix-loop-helix; DRE, dioxin-responsive element; TA, transcription activation; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PMSF, phenylmethylsulfonyl fluoride; MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; PD150606, 3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; CMV, cytomegalovirus; PAGE, polyacrylamide gel electrophoresis; DIG, digoxigenin; EMSA, electrophoretic mobility shift assay.

evidence obtained by using inhibitors of the 26 S proteasome (MG132) and nuclear export (leptomycin B) suggests that the proteasome and nuclear export are involved in the reduction of steady state AhR by TCDD (25). However, direct evidence of AhR degradation by TCDD (*i.e.* changes in the half-life of AhR) is lacking, and whether TCDD induces ubiquitination of AhR has not been addressed. In this study, we analyzed the biochemical pathway and functional implication of AhR degradation by TCDD. Our results reveal that activation of AhR by TCDD induces a marked shortening of the half-life ($t_{1/2}$) of the receptor through degradation of the receptor by the 26 S proteasome. The proteasomal degradation of AhR involves ubiquitination of AhR. Inhibition of the 26 S proteasome enhances the induction of *CYP1A1* by TCDD, implicating the ubiquitin-proteasome pathway in controlling the activity of ligand-activated AhR. Furthermore, we show that AhR degradation requires the transcription activation (TA) domain and DNA binding activity of AhR. Our findings provide new insights into the mechanism by which AhR is regulated through the ubiquitin-proteasome pathway after activation by a ligand.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—The mouse hepa1c1c7 cells, AhR-defective variant (AhR-D), and Arnt-defective variant (Arnt-D) cells were provided by Dr. J. P. Whitlock, Jr. (Stanford University). The AhR-D cells expressing AhR, AhR_{1-515}, or AhRR39A and the Arnt-D cells expressing Arnt, $Arnt_{1-652}$, or ArntR87A were originally from the laboratory of Dr. J. P. Whitlock, Jr. Generation of these cell lines by using the MFG retroviral expression system and characterization of the cell lines for AhR or Arnt function were described previously (26–28). The cells were grown as a monolayer in α-minimal essential medium, containing 10% fetal bovine serum, and 5% CO_2, as described elsewhere (29). E36 and ts20 cells (provided by Dr. R. R. Kopito, Stanford University) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 5% CO_2, at the indicated temperatures. Cells were treated with TCDD (AccuStandard, New Haven, CT) or other reagents as described in the figure legends. Me_2SO (0.1%) was used as the vehicle control for TCDD. Lactacystin, MG132, proteasome inhibitor I, calpastatin, and PD150606 were from Calbiochem-Novabiochem (San Diego, CA). Chloroquine, aprotinin, leupeptin, and PMSF were from Sigma. Treatment involving protease inhibitors was for 5 h or less to avoid toxicity to the cells; over 90% of the cells were viable under the experimental conditions.

Stable Transfection and Retroviral Gene Expression—Transfection of pRc/CMV (Invitrogen, Carlsbad, CA) or pAhR/CMV (30) into E36 and ts20 cells was performed with the LipofectAMINE PLUS reagent (Life Technologies, Inc.), followed by selection with G418 (400 μg/ml) for 10 days. Stable transfectants from the same transfection were pooled for further analysis. AhR_{1-421} was expressed by using the Retro-X System (CLONTECH, Palo Alto, CA), according to protocols from CLONTECH. Briefly, AhR cDNA encoding residues 1–421 was subcloned into the pLNCX vector to generate $pAhR_{1-421}$/LNCX, which was transfected into the RetroPack PT67 packaging cells by using the calcium method. 48 h after transfection, the supernatant was collected and used to infect the AhR-D cells. The cells expressing AhR_{1-421} were enriched by selection in the presence of G418 (400 μg/ml) for 10 days.

Immunoblot Analysis—Total cell extracts were fractionated on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and blotted with antibodies according to established procedures (31). For immunoblotting of AhR, an affinity purified rabbit polyclonal antibody against the mouse AhR was used (14). Horseradish peroxidase-conjugated anti-rabbit IgG (Promega, Madison, WI) was used as the secondary antibody. The blots were visualized by chemiluminescence using the ECL kit (Amersham Pharmacia Biotech). The same blots were reprobed with a monoclonal anti-actin IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by incubation with alkaline phosphatase-conjugated anti-mouse IgG (Promega) and color visualization using the 5-bromo-4-chloro-3-indolyl phosphate toluidinium/nitroblue tetrazolium substrate system (Promega). The amount of actin detected in the blots was used as an internal control to ensure equal loading of the samples. For immunodetection of ubiquitinated AhR, the AhR protein was immunoprecipitated with the anti-AhR antibodies as described below. The immunoprecipitates were fractionated by SDS-PAGE at 70 volts and blotted onto nitrocellulose membranes by overnight transfer at 200 mA. The blots were denatured in a buffer (6 M guanidine-HCl, 20 mM Tris-HCl, pH 7.5, 5 mM β-mercaptoethanol, 1 mM PMSF) for 1 h, blocked with 5% albumin in phosphate-buffered saline, incubated with a mouse monoclonal IgG against ubiquitin (Zymed Laboratories Inc., South San Francisco, CA) overnight, and detected with horseradish peroxidase-conjugated anti-mouse IgG (Promega) and the ECL kit.

Immunoprecipitation—AhR was precipitated with the anti-AhR antibodies according to a standard method (31). Briefly, cells grown in 6-well plates 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). Cell extracts were prepared by centrifugation at 13,000 $\times$ *g* for 10 min, followed by preclearing by incubation with a normal rabbit IgG (Santa Cruz Biotechnology, Inc.) and protein A-agarose (Life Technologies, Inc.) for 30 min at 4 °C. The extracts were incubated with the anti-AhR antibodies (14, 28) for 1 h and then with protein A-agarose for an additional hour. The precipitated agarose beads were washed three times with RIPA buffer and resuspended in a loading buffer for analysis by SDS-PAGE.

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 were labeled in a fresh supplemented, methionine-free medium plus [^{35}S]methionine (100 μCi/ml, Amersham Pharmacia Biotech) for 1 h. The cells were then incubated in supplemented α-minimal essential medium, treated with TCDD or Me_2SO for various time periods, and scraped into RIPA buffer. The ^{35}S-labeled AhR was precipitated with the anti-AhR antibodies, fractionated by SDS-PAGE (10%), and visualized by fluorography.

Northern Blot—Total RNA was isolated from cells by using a Qiagen total RNA isolation kit (Qiagen, Valencia, CA). RNA samples of 5 μg each were fractionated in a 1% agarose-formaldehyde gel and transferred to a Nytran membrane. The blot was probed with a DIG-labeled riboprobe prepared with the DIG-labeling kit (Roche Molecular Biochemicals), which recognizes a 700-base pair fragment in the 5′-untranslated region of the mouse *CYP1A1* mRNA. Signals were visualized by chemiluminescence using a DIG RNA detection kit with CDP star as a substrate (Roche Molecular Biochemicals). Parallel blots of the same samples were probed with a DIG-labeled actin probe to ensure equal loading.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was carried out by using nuclear extracts prepared from hepa1c1c7 cells, as described previously (32), except that 6% polyacrylamide gels were used. The DNA probe contains the DNA recognition sequence for the AhR·Arnt heteromer, designated DRE D (33). The probe was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). 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 complex was visualized by autoradiography.

RESULTS

TCDD Shortens the Half-life of AhR through the 26 S Proteasome Pathway—TCDD, a potent agonist of the Ah receptor, both activates AhR and induces a marked reduction in the steady state level of the receptor. As shown in Fig. 1*A*, treatment of hepa1c1c7 cells with TCDD (1 nM, 5 h) results in reduction of steady state AhR to less than 10% of the control, whereas the level of the Arnt protein is not affected. To analyze the biochemical pathway of TCDD-induced down-regulation of the AhR protein, we measured the half-life of unliganded and TCDD-activated AhR by using pulse-chase experiments to test whether the down-regulation is due to an increase in the turnover of the receptor. After pulse-labeling of the mouse hepa1c1c7 cells with [^{35}S]methionine, the AhR protein was immunoprecipitated with a polyclonal antibody against AhR, resolved in SDS-PAGE, and visualized by fluorography. As shown in Fig. 1 (*B* and *C*), the AhR of untreated cells is relatively stable with a half-life ($t_{1/2}$) of about 28 h. Treatment with 1 nM TCDD reduces the $t_{1/2}$ to 3 h. The time course for the reduction of the pulse-labeled AhR by TCDD is similar to that of the steady state level of AhR (data not shown). Thus, TCDD treatment markedly increases the turnover of AhR, accounting for the down-regulation of steady state AhR by TCDD.

Signal-induced degradation of cellular proteins is often me-

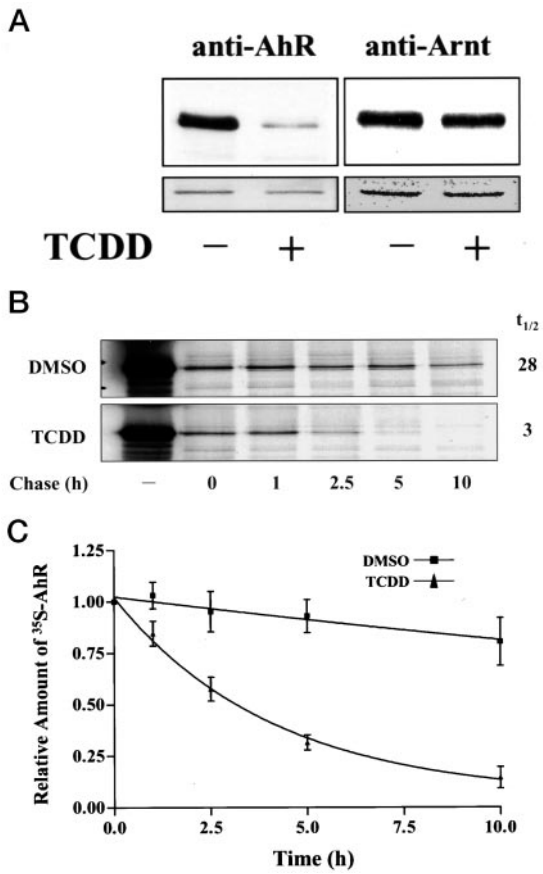

FIG. 1. **TCDD-induced degradation of AhR.** *A*, Down-regulation of steady state AhR. Hepa1c1c7 cells were treated with TCDD (1 nM) for 5 h. Total cell extracts (5 μg each) were prepared, and the blot was analyzed with an anti-AhR antibody or anti-Arnt antibody and visualized using the ECL kit as described under "Experimental Procedures" (*upper panels*). The same blot was reprobed with a monoclonal anti-actin antibody and detected with color development (*lower panels*). *B*, pulse-chase labeling of AhR. Hepa1c1c7 cells were labeled with [^{35}S]methionine, and the AhR protein was immunoprecipitated with a polyclonal anti-AhR antibody, fractionated by SDS-PAGE, and visualized by fluorography. The first lane was loaded with ^{35}S-labeled, *in vitro* transcribed and translated mouse AhR (30) as a marker for AhR. The hours indicate the time period of treatment after pulse labeling. *DMSO*, dimethyl sulfoxide. *C*, $t_{½}$ of AhR. The results from pulse-chase experiments were quantified by densitometry and analyzed by using ImageQuaNT software (Molecular Dynamics). The $t_{½}$ of AhR was calculated and plotted using the GraphPad PRISM program (GraphPad Software, Inc.). Data represent means and standard deviation from four separate experiments.

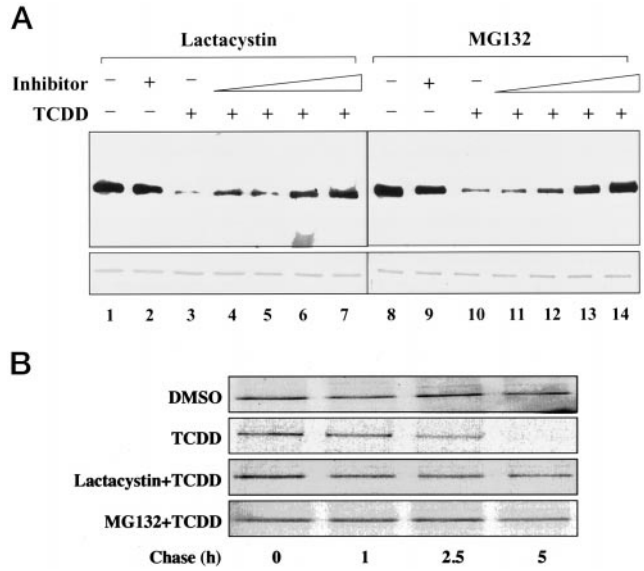

FIG. 2. **Inhibition of AhR degradation by proteasome inhibitors.** *A*, dose dependence of inhibition. Cells were co-treated with TCDD and various amounts of lactacystin or MG132 for 5 h. AhR (*upper panel*) and actin (*lower panel*) of each sample were analyzed by immunoblot as described above. *Lane 2*, 20 μM lactacystin; *lanes 4–7*, 0.1, 1.0, 10, and 20 μM lactacystin, respectively. *Lane 9*, 25 μM MG132; *lanes 11–14*, 0.125, 1.25, 12.5, and 25 μM MG132, respectively. *B*, pulse-chase experiment. Cells were labeled with [^{35}S]methionine and were treated with Me_2SO, TCDD (1 nM), or TCDD plus lactacystin (20 μM), or MG132 (25 μM). The cells were harvested at the indicated time points. The ^{35}S-labeled AhR was precipitated with an anti-AhR antibody and analyzed by SDS-PAGE and fluorography as described for Fig. 1. *DMSO*, dimethyl sulfoxide.

diated through a specific proteolytic system(s), such as the 26 S proteasome (19) or calpains (34). To identify the proteolytic activity for the TCDD-induced degradation of AhR, we tested a panel of inhibitors that specifically inhibit the 26 S proteasome, calpains, lysosomal enzymes, and other proteases. Immunoblot analyses reveal that co-treatment of the cells with TCDD (1 nM) and lactacystin, an irreversible, potent and specific proteasome inhibitor (19), or MG132, a reversible, potent but less specific proteasome inhibitor (35), inhibits the TCDD-induced reduction of AhR in a dose-dependent manner; maximal inhibition occurs at a concentration of 20 μM lactacystin or 25 μM MG132 (Fig. 2*A*). Others have observed a similar effect with MG132 via immunoblotting of AhR (25). To directly test whether the 26 S proteasome is required in the TCDD-induced AhR degradation, pulse-chase experiments were performed to analyze the effect of the proteasomal inhibitors on the turnover of pulse-labeled AhR. As shown in Fig. 2*B*, when co-treated with TCDD, both lactacystin and MG132 block the degradation of pulse-labeled Ah receptor by TCDD (compare treatments of Me_2SO, TCDD, and TCDD plus lactacystin or MG132). These results provide a direct proof that the TCDD-induced AhR degradation requires the 26 S proteasome.

It has been shown *in vitro* that AhR in the cytoplasmic preparations of mouse liver and hepatoma cells is rapidly degraded through a Ca^{2+}-dependent, calpain II-like protease process (36). To analyze the role of calpains and other proteases in TCDD-induced degradation of AhR in intact cells, we examined the effect of inhibitors of calpains and other cellular proteases on AhR degradation. Fig. 3*A* shows that co-treatment with TCDD and calpastatin or PD150606, specific inhibitors of calpains (37, 38), does not inhibit the degradation of AhR by TCDD. Inhibitors of lysosomal proteases (chloroquine), serine proteases (PMSF and aprotinin), and serine/cysteine proteases (leupeptin) do not exhibit inhibitory activity toward the TCDD-induced AhR degradation (Fig. 3*B*). Collectively, these results indicate that the 26 S proteasome mediates the TCDD-induced turnover of AhR in intact cells.

TCDD-induced AhR Degradation Involves Ubiquitination of AhR—Degradation of a specific protein by the 26 S proteasome is often preceeded by ubiquitination of the protein through a multi-component, ubiquitin enzyme system. This modification serves as a marker, which targets the protein to the proteasome for degradation (21, 22). Proteasomal degradation of proteins not ubiquitinated may require a secondary, ubiquitinated protein that functions to assist in directing the target proteins to the 26 S proteasome for degradation. To analyze the mechanism of the proteasomal degradation of AhR, we examined whether TCDD induces ubiquitination of AhR. As shown in Fig. 4*A*, TCDD treatment induces accumulation of high molecular weight, ubiquitinated proteins, that are recognized by a monoclonal antibody specific for ubiquitin, as compared with the Me_2SO control. To test whether AhR is ubiquitinated in response to TCDD, AhR from control and TCDD-treated cells

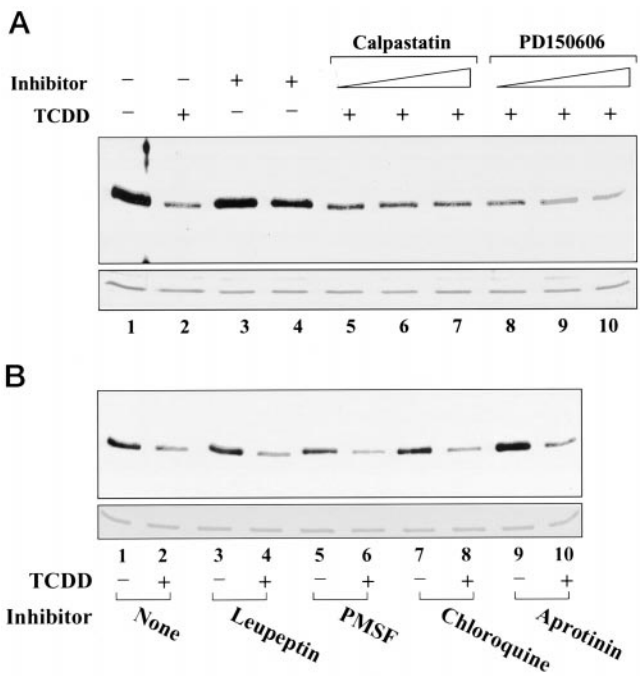

FIG. 3. **Effect of protease inhibitors on AhR degradation.** *A*, calpain inhibitors. Cells were treated with TCDD (1 nM) plus calpastatin or PD150606 for 5 h. AhR and actin were analyzed by immunoblotting as described for Fig. 1. *Lanes 3* and *4* were controls for calpastatin and PD150606, respectively. *Lane 3*, 2.5 μg/ml calpastatin; *lanes 5–7*, 0.05, 0.5, and 2.5 μg/ml calpastatin, respectively. *Lane 4*, 25 μM PD150606; *lanes 8–10*, 0.5, 5.0, and 25 μM PD150606, respectively. *B*, inhibitors of other proteases. Cells were treated with leupeptin (10 μg/ml), PMSF (100 μM), chloroquine (100 μM), or aprotinin (10 μg/ml) with or without TCDD (1 nM) for 5 h. AhR and actin were analyzed by immunoblotting as described above.

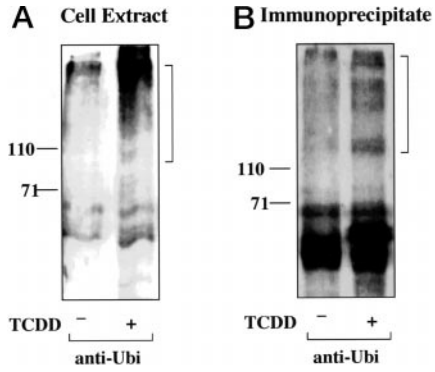

FIG. 4. **Immunoblotting of ubiquitinated AhR.** Cells were treated with TCDD (1 nM) for 4 h. *A*, total cell extracts were prepared and analyzed by SDS-PAGE and immunoblotted with a mouse monoclonal antibody specific for ubiquitin, as described under "Experimental Procedures," except that the cell extracts were prepared in phosphate buffered saline containing 10 mM *N*-ethylmaleimide. *B*, total cell extracts were prepared in RIPA buffer. AhR was precipitated with an anti-AhR antibody and immunoblotted with the anti-ubiquitin antibody, as described under "Experimental Procedures," except that MG132 (25 μM) was added to RIPA buffer to inhibit proteasome activity. The bracket indicates ubiquitinated AhR. The calculated molecular mass of the native mouse AhR is 95 kDa.

was immunoprecipitated with an anti-AhR antibody and blot analyzed with the anti-ubiquitin antibody. Fig. 4*B* shows that TCDD induces the formation of high molecular weight forms of AhR that are immunoprecipitated with the anti-AhR antibody and recognized by the anti-ubiquitin antibody. In a separate experiment, AhR was immunoprecipitated and blotted with the anti-AhR antibody; the data reveal that TCDD treatment results in both accumulation of high molecular weight AhR and

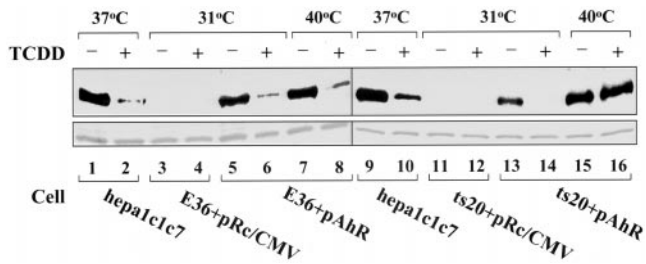

FIG. 5. **Degradation of AhR in E1 mutant cells.** AhR was expressed in E36 and ts20 cells by stable transfection. The plasmid pRc/CMV was used as a vector control (*lanes 3*, *4*, *11*, and *12*); hepa1c1c7 cells were used as a positive control for TCDD treatment (*lanes 1*, *2*, *9*, and *10*). The cells were treated with TCDD (1 nM) or Me_2SO for 5 h at the indicated temperatures in incubators with 5% CO_2. For cells treated at 40 °C (*lanes 7*, *8*, *15*, and *16*), the cells were incubated at 40 °C for 2 h prior to being treated with TCDD.

reduction of the native, nonubiquitinated AhR (data not shown). Together, these findings indicate that TCDD induces ubiquitination of the AhR protein.

Next, we examined whether ubiquitination of AhR is required for AhR degradation by TCDD. Because ubiquitination of a target protein requires activation of the ubiquitin molecule by E1, cells defective in E1 are compromised in protein ubiquitination and proteasomal degradation (39, 40). Therefore, we analyzed the degradation of AhR in cells bearing a temperature-sensitive mutation in E1. Mouse AhR was expressed in wild type (E36) and temperature-sensitive mutant cells (ts20) by stable transfection. E36 and ts20 cells transfected with vector only express low levels of AhR (Fig. 5, *lanes 3*, *4*, *11*, and *12*). At 31 °C, the cells transfected with the pAhR/CMV plasmid express the AhR protein to a level lower than that of the mouse hepa1c1c7 cells. Interestingly, the level of AhR in untreated ts20 cells at 40 °C (nonpermissive temperature for E1 function in ts20) is 3-fold higher than that at 31 °C (permissive temperature for E1) (compare *lanes 13* and *15*), suggesting that E1 negatively controls the AhR level in untreated cells. Treatment of E36 cells with TCDD induces degradation of AhR at both 31 and 40 °C. However, TCDD induces degradation of AhR in ts20 cells only at 31 °C but not at 40 °C. These findings implicate E1 in the degradation of AhR in both untreated and TCDD-treated cells. Thus, both biochemical and genetic studies indicate that the degradation of AhR by TCDD involves ubiquitination of the AhR protein.

Inhibition of the Proteasomal Degradation of AhR "Superinduces" CYP1A1 Gene Expression—The fact that TCDD induces both activation and degradation of the Ah receptor raises the question of whether the TCDD-induced degradation of AhR serves as a means of controlling the activity of ligand-activated AhR in the nucleus. To test this possibility, we examined the effect of proteasome inhibitors on the induction of endogenous *CYP1A1* gene expression by TCDD, a well characterized transcriptional response mediated by AhR. As shown in Fig. 6*A*, TCDD induces *CYP1A1* gene expression, whereas co-treatment with TCDD (1 nM) and lactacystin (20 μM) or MG132 (25 μM) for 5 h enhances the induction to 4.0- or 3.5-fold higher (compare *lanes 4* and *8* with *lane 2*). Proteasome inhibitor I, a weaker proteasome inhibitor than lactacystin and MG132, also enhances the induction of *CYP1A1*, when added at a concentration of 25 μM (~3.0-fold). PMSF, an inhibitor of serine proteases, does not affect the induction (compare *lanes 2* and *10*). Therefore, inhibition of proteasomal degradation of AhR by TCDD superinduces *CYP1A1* gene expression. We next examined, via EMSA, whether inhibition of AhR degradation increases the formation of the functional AhR·Arnt complex in the nucleus, thereby serving as a mechanism for the superin-

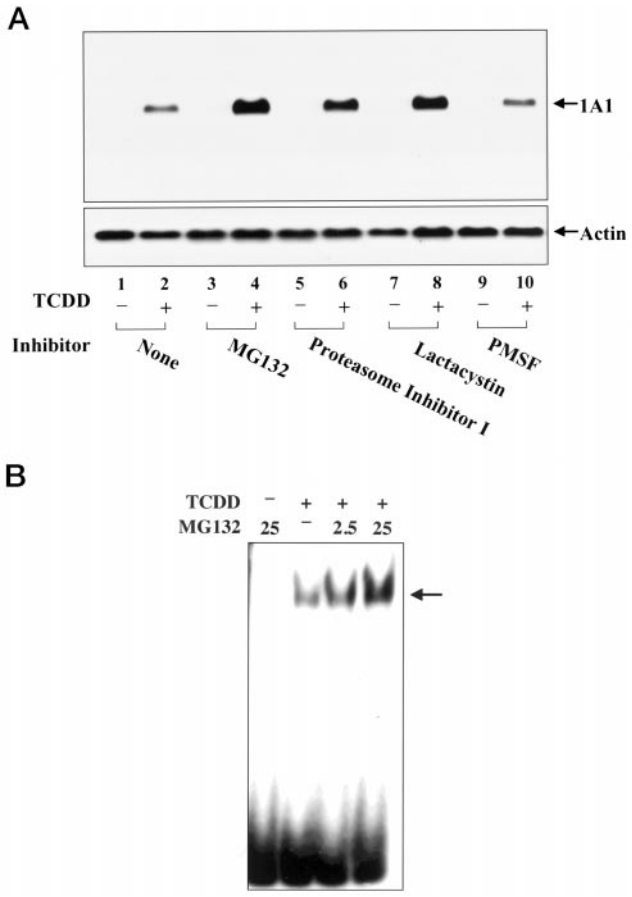

FIG. 6. **Inhibition of the 26 S proteasome enhances nuclear AhR function.** *A*, superinduction of *CYP1A1*. Cells were co-treated with TCDD and MG132 (25 μM), proteasome inhibitor I (25 μM), lactacystin (20 μM), or PMSF (100 μM) as indicated, for 5 h. Total RNA was prepared and analyzed for the messenger RNAs of *CYP1A1* and actin as described under "Experimental Procedures." *B*, EMSA. Cells were treated with TCDD (1 nM) or TCDD plus MG132 (2.5 or 25 μM) for 5 h as indicated. Nuclear extract was prepared and EMSA was performed using 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.

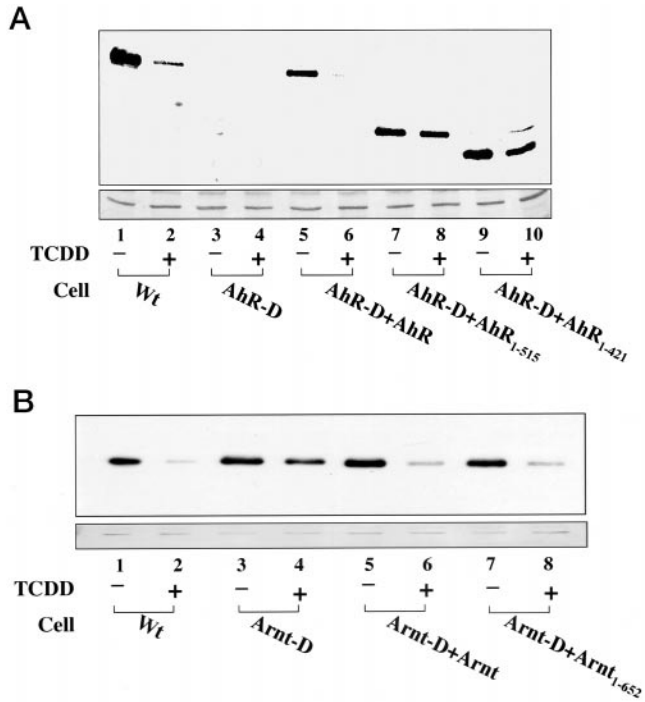

FIG. 7. **Deletion analysis for AhR degradation.** The AhR, AhR$_{1-515}$, or AhR$_{1-421}$ proteins were expressed in AhR-D variant (*A*) and the Arnt or Arnt$_{1-652}$ expressed in Arnt-D variant cells (*B*) by using retroviral expression as described under "Experimental Procedures." The cells were treated with TCDD (1 nM) for 5 h; total cell extracts were analyzed by SDS-PAGE and immunoblotted with an anti-AhR antibody against the N-terminal portion of mouse AhR (BioMol, Plymouth Meeting, PA). The same blot was reprobed with an anti-actin antibody as described in the legend for Fig. 1. *Wt*, wild type.

duction of CYP1A1 expression. Fig. 6*B* shows that MG132 enhances the TCDD-induced gel mobility shift, which reflects the formation of the AhR·Arnt·DRE complex, dose-dependently, indicating that inhibition of the 26 S proteasome increases the amount of functional AhR in the nucleus. Taken together, these data suggest that the ubiquitin-proteasomal degradation of AhR plays an important role in controlling the amount and activity of agonist-activated Ah receptor in the nucleus.

Role of the TA Domain and DNA Binding—The observation that AhR, but not Arnt, is degraded by the proteasome upon TCDD treatment indicates that this TCDD-activated degradation pathway is specific for AhR. Because ubiquitination of a target protein involves a specific structural motif or degron that is central to the specificity of ubiquitin-mediated proteolysis, we examined which regions of AhR are required for TCDD-induced degradation by deletion analysis. As shown in Fig. 7*A*, TCDD induces degradation of AhR in wild type and AhR-defective variant cells (AhR-D), which contains ~10% AhR as compared with wild type cells. Expression of wild type AhR in AhR-D by using retroviral gene transfer restores the AhR protein level and function (as measured in the induction of *CYP1A1* by TCDD) (*lane 5* and data not shown). The expressed AhR is degraded by TCDD (*lane 6*). However, a deletion mutant, AhR$_{1-515}$, that lacks the C-terminal half of AhR (amino acid residues 516–805, consisting of the TA domain) but contains an intact bHLH/PAS domain (residues 1–340) is not degraded by TCDD (*lanes 7* and *8*). The AhR$_{1-515}$ protein retains the ability to dimerize with Arnt and to bind DRE sequences but is not capable of mediating transcription, because of the lack of the TA domain (data not shown and Ref. 27); thus, these results suggest that the TA domain of AhR serves as a degron for degradation. AhR$_{1-421}$, which is similar to AhR$_{1-515}$ in structure and function, is also resistant to degradation by TCDD, further confirming the requirement of the TA domain in AhR degradation. Next, we tested whether Arnt plays a role in AhR degradation, because the TA activity of AhR is regulated and activation of the TA domain occurs in the presence of ligand and Arnt (26, 30). As shown in Fig. 7*B*, Arnt-defective variant cells (Arnt-D) exhibit partial resistance to TCDD-induced AhR degradation (compare *lanes 1–4*). Expression of Arnt or Arnt$_{1-652}$, which lacks the TA domain but retains the bHLH/PAS domain of Arnt (residues 1–515; Ref. 26), fully restores the degradation of AhR by TCDD (*lanes 5–8*), suggesting that there exists Arnt-dependent and Arnt-independent degradation of AhR in Arnt-D cells. Arnt-D variants express a mutant Arnt protein at a low, but detectable level (data not shown and Ref. 41); the mutant Arnt can bind AhR but is less stable than wild type Arnt (41). It is possible that this mutant Arnt contributes to AhR degradation in Arnt-D cells.

AhR mediates transcriptional regulation via binding to the DRE sequences of a target gene. Therefore, we tested whether DNA binding plays a role in AhR degradation. AhRR39A, in which arginine 39 was replaced with alanine, is incapable of DNA binding and induction of transcription (28). As shown in Fig. 8*A*, degradation of AhRR39A expressed in AhR-D cells by TCDD, is reduced. Because AhR forms a dimer with Arnt for

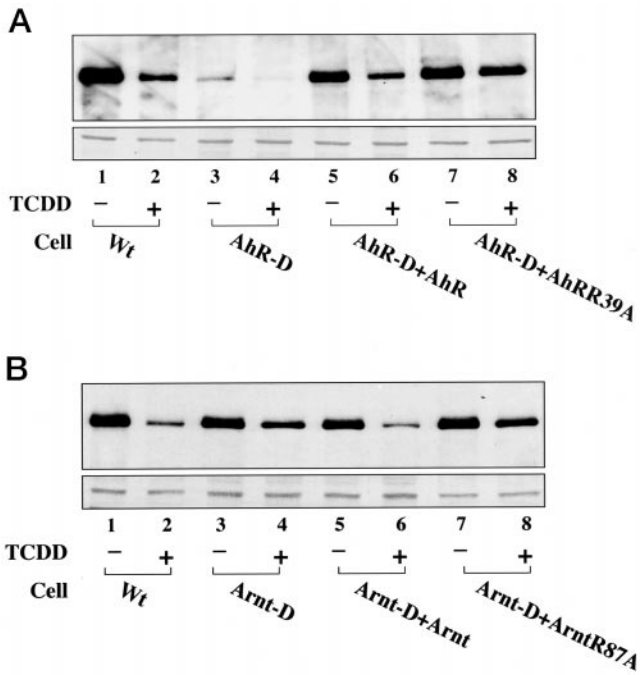

FIG. 8. **Analyses of DNA-binding mutants for AhR degradation.** AhR and AhRR39A were expressed in AhR-D cells (*A*) and Arnt and ArntR87A were expressed in Arnt-D cells (*B*) by using retroviral expression as described under "Experimental Procedures." The cells were treated with TCDD for 5 h, and total cell extract was analyzed by immunoblotting with an anti-AhR antibody. *Lower panel* shows actin blotted with an anti-actin antibody, as a control. *Wt*, wild type.

DNA binding, we examined a DNA-binding mutant of Arnt (R87A) for AhR degradation. Expressing ArntR87A in Arnt-D variant cells (28) inhibits the degradation of AhR by TCDD (Fig. 8*B*). These data suggest that binding of the AhR·Arnt dimer to DNA, at least in part, contributes to AhR degradation.

DISCUSSION

Protein degradation through the ubiquitin-proteasomal pathway has been implicated in the signal transduction of a number of transcription factors; 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 Ref. 19). 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 shortening of the $t_{1/2}$ of AhR by TCDD is blocked by inhibition of the 26 S proteasome, implicating the proteasome pathway in the degradation of AhR. Others have obtained a similar conclusion by analyzing steady state AhR using immunoblotting (25).

Because the proteasomal degradation of proteins often requires ubiquitination, two mechanisms may contribute to the degradation of AhR: (*a*) TCDD induces ubiquitination of the AhR protein or (*b*) ubiquitination occurs in an AhR-associated factor that assists in targeting AhR to the proteasome for degradation. Our results reveal that TCDD induces accumulation of high molecular weight, ubiquitinated AhR. Furthermore, degradation of AhR requires functional ubiquitin-activating enzyme E1. Collectively, these data demonstrate that the TCDD-induced proteasomal degradation of AhR involves ubiquitination of AhR. However, these findings do not exclude the possibility that TCDD induces ubiquitination of other proteins that also contribute to AhR degradation. Blocking the ubiquitination of AhR by mutating amino acid residues required for AhR ubiquitination may distinguish whether ubiquitination of additional proteins is involved in AhR degradation.

Our analyses of AhR degradation in ts20 cells reveal that the turnover of AhR in untreated cells also requires E1, indicating that degradation of unliganded AhR is mediated through a ubiquitin-proteasome pathway. Because the unliganded AhR is in a complex with heat shock protein 90 and (AIP) AhR interacting protein in the cytoplasm, it is conceivable that the mechanism for the ubiquitin-proteasomal degradation of unliganded AhR involves dissociation of AhR from the cytoplasmic complex in the absence of an exogenous AhR ligand and therefore is distinct from the mechanism for the degradation of ligand-activated AhR in the nucleus. Elucidation of the mechanism by which the cytoplasmic AhR is degraded may reveal new aspects of the regulation of unliganded AhR, as well as other receptor/transcription factors, such as the glucocorticoid receptor, the estrogen receptor, and the bHLH/PAS factor Sim, which also form a complex with heat shock protein 90 and immunophillins in the absence of an activation signal.

Although regulation through the ubiquitin-proteasome-mediated proteolysis pathway has been implicated for transcription factors, direct evidence of the functional impact of such regulation on the transcriptional responses mediated by the factors is lacking. Our analyses of the induction of endogenous *CYP1A1* gene expression clearly demonstrate that inhibition of the 26 S proteasome enhances the induction of the gene by TCDD, *i.e.* superinduction. These results indicate that TCDD-induced ubiquitin-proteasomal degradation of AhR regulates the activity of AhR in the nucleus by controlling the amount of ligand-activated AhR, so that transcription of the target genes can be maintained at a certain level. This conclusion is further supported by the observation that inhibition of the 26 S proteasome increases the amount of the functional AhR·Arnt complex, in a dose- and time-dependent manner (Fig. 6*B* and Ref. 25). AhR mediates a broad range of toxic responses to TCDD and has been implicated in embryonic development, liver, and immune functions, as well as cell growth and differentiation. It will be intriguing and challenging to test whether the ubiquitin-proteasomal regulation of AhR has a similar functional impact on these more complex biological responses.

Degradation through the ubiquitin-proteasome pathway is specific, because ubiquitination of the proteins involves a specific structural element (degron). The degron is recognized by a specific ubiquitin-conjugating enzyme E2, either alone or in conjunction with ubiquitin-ligase E3. Because TCDD induces degradation of AhR, but not Arnt, it is possible that the AhR protein contains a structural element that functions as a degron for ubiquitination. Deletion analyses of AhR reveal that the C-terminal half of AhR, which consists of the TA domain of AhR, is required for AhR degradation by TCDD, suggesting that the TA domain functions as a degron. Other transcription factors, such as Myc and E2F-1, also contain TA domains that signal the degradation of these transcription factors (42, 43). Overlapping of the degrons with the TA domains of the transcription factors is intriguing and may reflect a conserved mechanism by which activated-transcription factors are regulated through ubiquitin-mediated proteolysis. Because TA domains interact with other transcription proteins, such as histones and basal transcription factors, the TA domains may also contribute to the ubiquitination and regulation of these proteins through proteasomal degradation.

Previous studies on the TA activities of AhR and Arnt reveal

that the TA activity of AhR is inhibited through an inhibitory domain (30); activation of the TA occurs in the presence of ligand and Arnt. The AhR TA domain mediates the induction of *CYP1A1* by TCDD. On the other hand, Arnt contains a TA domain that is constitutively active but not required for the induction of *CYP1A1*. Thus, regulation of the TA activities of AhR and Arnt after ligand stimulation is an integral part of the transcriptional function of AhR and Arnt (26, 30). Our analyses of Arnt and an Arnt deletion mutant expressed in Arnt-D cells indicate that there exists both Arnt-dependent and -independent degradation of AhR, suggesting that the AhR degron is, in part, regulated through interaction with Arnt. This notion is further supported by the observation that loss of DNA binding of AhR or Arnt reduces AhR degradation (see below). Alternatively, the mutant Arnt in the Arnt-D cells that can bind AhR and is expressed at a low but detectable level because of reduced stability (41) contributes to AhR degradation in Arnt-D cells.

Substitution of residues arginine 39 of AhR or arginine 87 of Arnt with alanine abolishes the DNA binding activity of AhR and Arnt (28). Degradation of the expressed AhRR39A in AhR-D or the wild type AhR in Arnt-D cells expressing ArntR87A is reduced; these results suggest that DNA binding is, at least in part, involved in AhR degradation by TCDD. The simplest explanation of these results is that there are two mechanisms of AhR degradation: (*a*) DNA binding-dependent, in which activated AhR dimerizes with Arnt and binds to the DRE, followed by proteasomal degradation and (*b*) DNA binding-independent, in which activated AhR is degraded before binding to DNA. Identification of the components of the ubiquitin system(s) that mediate AhR degradation in the nucleus and reconstitution of the degradation pathway(s) may provide insights into the two mechanisms.

The bHLH/PAS proteins comprise a large family of transcription factors that have been implicated in a number of biological functions, such as hypoxic response (44, 45), circadian rhythm control (46), embryonic development (47), and transcriptional response to xenobiotics (5). The transcriptional regulation by the bHLH/PAS factors often involves signal-induced activation of a bHLH/PAS factor, followed by dimerization of the factor with another bHLH/PAS protein and binding to specific enhancer sequences of the target genes. Regulation of bHLH/PAS proteins after activation by a signal is largely unclear at present, although proteasomal degradation has been shown for the control of the hypoxia-inducible factor Iα (HIF1α), a bHLH/PAS factor that binds Arnt and mediates the transcriptional response to hypoxia (44, 45). Because of the similarities in the structure, signaling pathway, and function among the bHLH/PAS factors, the AhR-mediated transcriptional gene regulation constitutes a useful model for the signal transduction of other bHLH/PAS proteins. Understanding the molecular steps of the ubiquitin-proteasomal degradation of AhR will provide new insights into the regulation of bHLH/PAS transcription factors in general.

Acknowledgments—We thank Drs. M. Luster and A. Munson for support and guidance during the study, Dr. A. Poland for valuable advice, Drs. F. Chen, L. Oram, and J. M. Matheson for review of the manuscript, and H. Michael for secretarial assistance. We also thank Dr. R. R. Kopito for providing the E36 and ts20 cells and Dr. J. P. Whitlock, Jr., for the hepa1c1c7, AhR-D, Arnt-D and reconstituted variant cells.

REFERENCES

1. Whitlock, J. P., Jr. (1999) *Annu. Rev. Pharmacol. Toxicol.* **39,** 103–125
2. Hankinson, O. (1995) *Annu. Rev. Pharmacol. Toxicol.* **35,** 307–340
3. Poland, A., and Knutson, J. C. (1982) *Annu. Rev. Pharmacol. Toxicol.* **22,** 517–554
4. Poland, A., and Glover, E. (1980) *Mol. Pharmacol.* **17,** 86–94
5. Whitlock, J. P., Jr., Okino, S. T., Dong, L., Ko, H. P., Clarke-Katzenberg, R., Ma, Q., and Li, H. (1996) *FASEB J.* **10,** 809–818
6. Fernandez-Salguero, P. M., Hilbert, D. M., Rudikoff, S., Ward, J. M., and Gonzalez, F. (1996) *Toxicol. Appl. Pharmacol.* **140,** 173–179
7. Safe, S. H. (1986) *Annu. Rev. Pharmacol. Toxicol.* **26,** 371–399
8. Mimura, J., Yamashita, K., Nakamura, K., Morita, M., Takagi, T. N., Nakao, K., Ema, M., Sogawa, K., Yasuda, M., Katsuki, M., and Fujii-Kuriyama, Y. (1997) *Genes Cells* **2,** 645–654
9. Luster, M., Faith, R., and Clark, G. (1979) *Ann. N. Y. Acad. Sci.* **31,** 473–486
10. Kolluri, S., Weiss, C., Koff, A., and Gottlicher, M. (1999) *Genes Dev.* **13,** 1742–1753
11. Fernandez-Salguero, P. M., Pineau, T., Hilbert, D. M., McPhail, T., Lee, S. S. T., Kimura, S., Nebert, D. W., Rudikoff, S., Ward, J. M., and Gonzalez, F. J. (1995) *Science* **268,** 722–726
12. Schmidt, J. V., Su, G. H., Reddy, J. K., Simon, M. C., and Bradfield, C. A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 6731–6736
13. Weis, C., Kolluri, S. K., Kiefer, F., and Gottlicher, M. (1996) *Exp. Cell Res.* **226,** 154–163
14. Ma, Q., and Whitlock, J. P., Jr. (1996) *Mol. Cell. Biol.* **16,** 2144–2150
15. Reiners, J. J., Jr., and Cliff, R. E. (1999) *J. Biol. Chem.* **274,** 2502–2510
16. Ma, Q., and Whitlock, J. P., Jr. (1997) *J. Biol. Chem.* **272,** 8878–8884
17. Carver, L. A., and Bradfield, C. A. (1997) *J. Biol. Chem.* **272,** 11452–11456
18. Meyer, B. K., Pray-Grant, M. G., Vanden Heuvel, J. P., and Perdew, G. H. (1998) *Mol. Cell. Biol.* **18,** 978–988
19. Hershko, A., and Ciechanover, A. (1998) *Annu. Rev. Biochem.* **67,** 425–479
20. Varshavsky, A. (1997) *Trends Biochem. Sci.* **22,** 383–387
21. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) *Annu. Rev. Biochem.* **65,** 801–847
22. Hochstrasser, M. (1996) *Annu. Rev. Genet.* **30,** 405–439
23. Reick, M., Robertson, R. W., Pasco, D. S., and Fagan, J. B. (1994) *Mol. Cell. Biol.* **14,** 5653–5660
24. Pollenz, R. S. (1996) *Mol. Pharmacol.* **49,** 391–398
25. Davarinos, N., and Pollanz, R. (1999) *J. Biol. Chem.* **274,** 28708–28715
26. Ko, H. P., Okino, S. T., Ma, Q., and Whitlock, J. P., Jr. (1996) *Mol. Cell. Biol.* **16,** 430–436
27. Gao, L., Dong, L., and Whitlock, J. P., Jr. (1998) *J. Biol. Chem.* **273,** 15358–15365
28. Dong, L., Ma, Q., and Whitlock, J. P., Jr. (1996) *J. Biol. Chem.* **271,** 7942–7948
29. Miller, A. G., Israel, D. L., and Whitlock, J. P., Jr. (1983) *J. Biol. Chem.* **258,** 3523–3527
30. Ma, Q., Dong, L., and Whitlock, J. P., Jr. (1995) *J. Biol. Chem.* **270,** 12697–12703
31. Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., and Struhl, K. (1998) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York
32. Denison, M. S., Fisher, J. M., and Whitlock, J. P., Jr. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 2528–2532
33. Lusska, A., Shen, E., and Whitlock, J. P., Jr. (1993) *J. Biol. Chem.* **268,** 6575–6580
34. Han, Y., Weinman, S., Boldogh, I., Walker, R. K., and Braisier, A. R. (1998) *J. Biol. Chem.* **274,** 787–794
35. Adams, J., and Stein, R. (1996) *Ann. Rep. Med. Chem.* **31,** 279–283
36. Poland, A., and Glover, E. (1988) *Arch. Biochem. Biophys.* **261,** 103–111
37. Khissiin, A., and Leclercq, G. (1999) *FEBS Lett.* **448,** 160–166
38. Wang, K., Nath, R., Posner, A., K. R., Buroker-Kilgore, M., Hajimohammadreza, I., Probert, A., Jr., Marcoux, F., Ye, Q., Takano, E., Hatanaka, M., Maki, M., Caner, H., Collins, J., Fergus, A., Lee, K., Lunney, E., Hays, S., and Yuen, P. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 6687–6692
39. Ward, C., Omura, S., and Kopito, R. (1995) *Cell* **83,** 121–127
40. Kulka, R., Raboy, B., Schuster, R., Parag, H., Diamond, G., Ciechanover, A., and Marcus, M. (1988) *J. Biol. Chem.* **263,** 15726–15731
41. Numayama-Teuruta, K., Kobayashi, A., Cogawa, K., and Fujii-Kuriyama, Y. (1997) *Eur. J. Biochem.* **246,** 486–495
42. Salghetti, S. E., Kim, S. Y., and Tansey, W. P. (1999) *EMBO J.* **18,** 717–726
43. Campanero, M. R., and Flemington, E. K. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 2221–2226
44. Li, H., Ko, H. P., and Whitlock, J. P., Jr. (1996) *J. Biol. Chem.* **271,** 21262–21267
45. Semenza, G. L. (1998) *Curr. Opin. Genet. Dev.* **8,** 588–594
46. Sassone-Corsi, P. (1997) *Nature* **389,** 443–444
47. Crews, S. (1998) *Genes Dev.* **12,** 607–620

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced Degradation of Aryl Hydrocarbon Receptor (AhR) by the Ubiquitin-Proteasome Pathway: ROLE OF THE TRANSCRIPTION ACTIVATON AND DNA BINDING OF AhR

Qiang Ma and Kimberly T. Baldwin

J. Biol. Chem. 2000, 275:8432-8438.
doi: 10.1074/jbc.275.12.8432