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Biochemical Pharmacology 70 (2005) 1623–1633
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Role of the carboxy-terminal transactivation domain and active transcription in the ligand-induced and ligand-independent degradation of the mouse Ah^{b-1} receptor

Richard S. Pollenz *, Jesal Popat, Edward J. Dougherty

Department of Biology, University of South Florida, BSF 110, 4202 E Fowler Ave, Tampa, FL 33620, USA

Received 23 August 2005; accepted 12 September 2005

Abstract

To assess the importance of transactivation domains (TAD), DNA binding and transcription on the degradation of the AH receptor (AHR), Hepa-1 cells were pre-treated with actinomycin D (AD) or cycloheximide (CHX) and exposed to 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD). AD or CHX did not affect nuclear localization or DNA binding of the AHR but inhibited ligand-induced degradation. In contrast, AD or CHX did not inhibit geldanamycin (GA) induced degradation of the AHR. To assess the role of the COOH-terminal TAD in AHR degradation, stop codons were placed at nucleotide 1501 and 1921 of the Ah^{b-1} AHR coding region to generate AHR_{500} and AHR_{640}. Stable cell lines were generated and exposed to TCDD. Cells expressing AHR_{500} did not induce CYP1A1 protein, but exhibited significant degradation of AHR_{500}. Cells expressing AHR_{640} induced CYP1A1 protein to 50% of the level of cells expressing wild type AHR and exhibited significant degradation of AHR_{640}. Importantly, AD and CHX did not inhibit the TCDD-induced degradation of either AHR_{500} and AHR_{640} and these receptors showed a more rapid profile of ligand-induced degradation compared to cells expressing wild type AHR. TCDD exposure to Hepa-1 cells with reduced aryl hydrocarbon receptor nuclear translocator (ARNT), showed ligand-induced degradation of the AHR that was not blocked by AD. However, AD inhibited TCDD-induced degradation when ARNT expression was restored. These results show that multiple mechanisms exist for the ligand and GA-induced degradation of the AHR and suggest that ligand-induced degradation can switch between two mechanisms depending on the presence of a functional TAD and the binding to DNA.

Keywords: Aryl hydrocarbon receptor; TCDD; Geldanamycin; Protein degradation; Cycloheximide

1. Introduction

The aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor that is a member of the basic-helix-loop-helix Per/ARNT/Sim (bHLH-PAS) family of proteins. The current model of AHR-mediated signal transduction proposes that the AHR is activated by ligand, associates with the aryl hydrocarbon receptor nuclear translocator (ARNT) protein in the nucleus and then binds with core xenobiotic response elements (XRE) to modify gene regulation (reviewed in refs. [1–3]). Since many transcription factors are limited in the duration that they activate or repress genes, the proteolytic degradation of these proteins has become an important aspect of regulating responses (reviewed in refs. [4,5]). Proteolytic degradation has been shown to be involved in signal transduction systems involving NF-κB [6], glucocorticoid receptors [7], p53 [8], the bHLH/PAS protein HIF-1α [9,10] and estrogen receptors [11–13]. The mechanism involved in turning off AHR-mediated signaling and the regulation of this pathway across species are especially critical with respect to halogenated aromatic compounds that are not readily metabolized or cleared from an organism.

The ligand-induced degradation of the AHR has been demonstrated both in vivo and in vitro but the mechanism involved in the degradation process has been illusive (reviewed in ref. [14]). For example, while it has been hypothesized that the ligand-induced degradation of the

Abbreviations: AD, actinomycin D; AHR, aryl hydrocarbon receptor; ARNT, Ah receptor nuclear translocator; CHX, cycloheximide; ER, estrogen receptor; GA, geldanamycin; GAR-HRP, goat anti-rabbit antibodies conjugated to horseradish peroxidase; GAR-RHO, goat anti-rabbit antibodies conjugated to rhodamine; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic response element

* Corresponding author. Tel.: +1 813 974 1596; fax: +1 813 974 3263.
E-mail address: pollenz@chuma1.cas.usf.edu (R.S. Pollenz).

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doi:10.1016/j.bcp.2005.09.006

AHR is mediated via the 26S proteasome pathway following ubiquitylation [15–17], the direct ubiquitylation of the endogenous AHR has not been demonstrated and no E3 ligases have been identified that mediate the degradation process. In addition, there is no consensus regarding the subcellular location of the degradation events. Stable cell lines expressing an AHR with a mutant nuclear localization signal show ligand-induced degradation with similar kinetics to wild type AHR [18]. However, the ligand-induced nuclear accumulation of the AHR was not completely inhibited in these studies and a constitutively nuclear AHR has also been shown to be susceptible to degradation by the 26S proteasome [19]. In addition, the AHR can be degraded in a ligand-independent manner following exposure to geldanamycin (GA) [16,18,20,21]. Interestingly, GA treatment results in nuclear accumulation of the AHR, but the degradation time course is more rapid than TCDD-induced degradation and the AHR does not become transformed to a DNA binding species [16]. These findings suggest that different pathways may mediate the degradation of the AHR following exposure to GA or TCDD. Thus, it is important to determine why there are multiple pathways and when each pathway functions during AHR-mediated signaling.

A connection between active transcription and AHR degradation has been implied in studies showing that Hepa-1 cells treated with the transcription inhibitor actinomycin D (AD), exhibit reduced levels of TCDD-induced degradation [22]. Additional studies show that ligand-induced degradation of the AHR is also inhibited by translation inhibitors typified by cycloheximide (CHX) [23]. It has been hypothesized that the inhibition of ligand-induced degradation by AD or CHX is due to either: (i) reduced transformation or DNA binding by the AHR; (ii) reduced induction of a degradation factor mediated via the AHR complex; (iii) loss of a CHX-sensitive factor that was required for AHR degradation; (iv) a requirement for active transcription by the AHR·ARNT complex. To resolve some of these questions, studies were performed in the presence of AD or CHX to determine the importance of the COOH-terminal transactivation domain of the AHR in ligand and GA-induced degradation.

2. Materials and methods

2.1. Materials

TCDD (98% stated chemical purity) was obtained from Radian Corp. (Austin, TX) and was dissolved in dimethylsulfoxide (Me_2SO). Geldanamycin, actinomycin D and cycloheximide were purchased from Sigma (St. Louis) and dissolved in Me_2SO. G418 sulfate was purchased from Sigma.

2.2. Buffers

PBS is 0.8% NaCl, 0.02% KCl, 0.14% Na_2HPO4, 0.02% KH_2PO_4, pH 7.4. SDS sample buffer is 60 mM Tris, pH 6.8, 2% SDS, 15% glycerol, 2 mM EDTA, 5 mM EGTA 10 mM DTT, 0.005% bromphenol blue. Lysis buffer is 60 mM Tris, pH 6.8, 2% SDS, 15% glycerol, 2 mM EDTA, 5 mM EGTA, 10 mM DTT, 0.5% NP-40, 20 mM sodium molybdate 0.005% bromphenol blue. 5× gel shift buffer is 50 mM Hepes, pH 7.5, 15 mM $MgCl_2$, 50% glycerol. TTBS is 50 mM Tris, 0.2% Tween 20, 150 mM NaCl, pH 7.5. TTBS+ is 50 mM Tris, 0.5% Tween 20, 300 mM NaCl, pH 7.5. BLOTTO is 5% dry milk and 20 mM DL-histidine in TTBS.

2.3. Cells and growth conditions

Wild type Hepa-1c1c7 (Hepa-1) and type I (LA-I) Hepa-1 variants were a generous gift from Dr. James Whitlock Jr. (Department of Pharmacology, Stanford University). Mouse 10T1/2 embryonic fibroblasts, rat A7H smooth muscle cells, C4 Hepa-1 and C4 vT{2} cells were obtained from ATCC (Manassas, VA). Hepa-1 and A7 cells were propagated in DMEM supplemented with 10% fetal bovine serum (FBS). C4 and vT{2} cells were propagated in alpha-MEM supplemented with 10% heat-inactivated FBS. 10T1/2 cells were propagated in BME supplemented with 10% heat-inactivated FBS. All cells were passaged at 1-week intervals and used in experiments during a 3-month period at approximately 70–90% confluence. For treatment regimens, stated concentrations of TCDD, GA or Me_2SO were administered directly into growth media for the indicated incubation times.

2.4. Antibodies

Either of two specific antibodies (termed A-1 and A-1A) was used to detect the AHR and are identical to those described previously [24,25]. All antibodies are affinity-purified IgG fractions. For Western blot analysis goat anti rabbit antibodies conjugated to horseradish peroxidase (GAR-HRP) were utilized. For immunohistochemical studies, goat anti-rabbit IgG conjugated to rhodamine (GAR-RHO) was used. Both of these reagents were purchased from Jackson Immunoresearch (West Grove, PA). Antibodies against rabbit β-actin were purchased from Sigma (St. Louis, MO). Antibodies against rat CYP1A1 were purchased from Chemicon Intl. (Temecula, CA).

2.5. Preparation of total cell lysates

Following treatment, cell monolayers were washed twice with PBS and detached from plates by trypsinization (0.05% trypsin/0.5 mM EDTA). Cell pellets were washed with PBS and sonicated directly in a 1:1 mixture of lysis buffer and SDS sample buffer for 12 s. Lysates were

immediately heated for 3 min at 95 °C and then sonicated for an additional 5 s. Samples were stored at −20 °C until analysis.

2.6. Western blot analysis and quantification of protein

Protein samples were resolved by denaturing electrophoresis on discontinuous polyacrylamide slab gels (SDS-PAGE) and were electrophoretically transferred to nitrocellulose. Immunochemical staining was carried out with varying concentrations of primary antibody (see text and figure legends) in BLOTTO buffer supplemented with DL-histidine (20 mM) for 1–2 h at 22 °C. Blots were washed with three changes of TTBS+ for a total of 45 min. The blot was then incubated in BLOTTO buffer containing a 1:10,000 dilution of GAR-HRP for 1 h at 22 °C and washed in three changes of TTBS+ as above. Prior to detection, the blots were washed in PBS for 5 min. Bands were visualized with the enhanced chemiluminescence (ECL) kit as specified by the manufacturer (Amersham, Arlington Hts., IL). Multiple exposures of each set of samples were produced. The relative concentration of target protein was determined by computer analysis of the autoradiographs as previously described in detail [25–27].

2.7. Preparation of nuclear extracts and EMSA

Nuclear extracts were prepared as previously described in detail [28] and protein quantified by the Coomassie Plus assay (Pierce, Rockford, IL). For EMSA, a double stranded XRE fragment corresponding to the consensus XRE-1 of the *CYP1A1* promoter [29], was labeled with [^{32}P]dCTP by Klenow fill in. Each EMSA reaction contained 10 μg of nuclear extract, 1× gel shift buffer, KCl (80 mM) and polydIdC (0.1 mg/ml). For competition experiments, 250 ng pre-immune IgG to IgG specific to the AHR (A-1A) was also added to the reaction mixture. Samples were incubated at 22 °C for 15 min. Approximately 4 ng of [^{32}P]-labeled XRE was then added to each sample, mixed and the incubation continued for an additional 15 min at 22 °C. The samples were resolved on 5% acrylamide/0.5% TBE gels, dried and exposed to film.

2.8. Immunofluorescence staining and microscopy

All immunocytochemical procedures (cell plating, fixation and staining) were carried out as previously described [24–26]. Cells were observed on an Olympus IX70 microscope. On average, 15–20 fields (5–20 cells each) were evaluated on each coverslip and 3–4 fields were photographed with a digital camera at the same exposure time to generate the raw data. Experiments were repeated at least two times.

2.9. Generation of stable cell lines expressing AHR truncations

Full-length cDNA to the Ah^{b-1} AHR was ligated into the pLNCX2 retroviral expression vector (Clontech, Mountain View, CA) as previously described to generate pMAHR-WT [18,30]. To generate COOH-terminal deletions in the AHR, PCR was used with 3′ oligonucleotides that contained stop codons inserted at either nucleotide 1501 or 1921. The cDNA fragments were then ligated into pLNCX2 to generate pMAHR-500 (expressing an AHR that contains amino acids 1–500) and pMAHR-640 (expressing an AHR that contains amino acids 1–640). Virus particles were used to infect the parental LA-I Hepa-1 variant cell line as described previously [18] and neomycin-resistant colonies selected in 800 μg/ml G418 sulfate (Sigma, St. Louis, MO). For all experiments described in this report, at least three independent stable cell lines were evaluated. All cells were maintained in selective media during propagation, but G418 was not present in the media when cells were plated for experiments. Control LA-I cells used in the studies were infected with naked virus and maintained under ideal selective pressure as the AHR expressing cells.

2.10. Statistical analysis

Target protein bands were normalized to internal standards (actin) and the normalized densitometry units compared by ANOVA and Tukey–Kramer multiple comparison tests using InStat software (GraphPad Software Inc., San Diego, CA). Results are presented as mean ± S.E. A probability of error value of <0.05 was considered significant.

3. Results

3.1. Effect of AD and CHX on ligand and GA-induced degradation of the endogenous AHR

To formally investigate the connection between active transcription and ligand-induced degradation of the AHR, Hepa-1 cells were pre-treated with AD or CHX and then exposed to GA or TCDD. A representative experiment is shown in Fig. 1. In cells pre-treated with vehicle (Fig. 1A), the AHR was reduced by 75% and 80% following exposure to TCDD or GA, respectively. In addition, treatment with TCDD, but not GA results in the induction of the endogenous *CYP1A1* gene as determined by the detection of CYP1A1 protein. In contrast, treatment of cells with AD prior to exposure to TCDD (Fig. 1B), blocks both the TCDD-induced degradation of the AHR as well as the induction of CYP1A1. However, AD treatment did not affect the level of GA-induced degradation of the AHR. Identical results were observed when cells were pre-treated

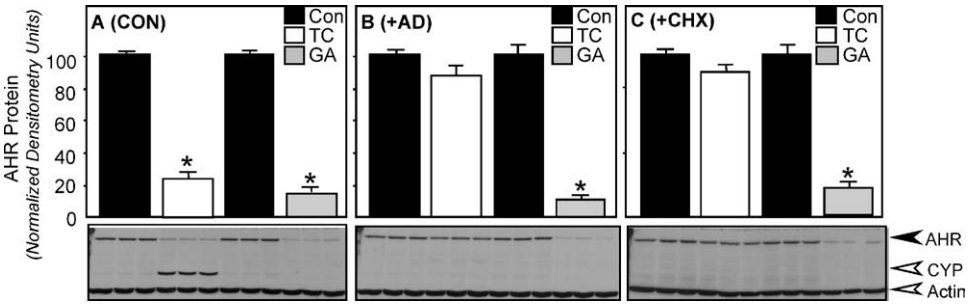


Fig. 1. Effect of AD and CHX on ligand and GA-induced degradation of the endogenous AHR in Hepa-1 cells. Triplicate plates of Hepa-1 cells were treated with 0.05% Me_2SO (Panel A), 0.30 μM AD (Panel B) or 10 μg/ml CHX (Panel C) for 2 h at 37 °C. Cells were then exposed to TCDD (2 nM) or GA (100 nM) for an additional 4 or 2 h, respectively, and total cell lysates prepared. Equal amounts of total cell lysates were resolved by SDS-PAGE, blotted and stained with A-1A anti-AHR IgG (1.0 μg/ml), anti-β-actin IgG (1:1000) or anti-CYP1A1 IgG (1:1000). Reactivity was visualized by ECL with GAR-HRP (1:10,000). The level of AHR protein at each time point was divided by the corresponding level of actin and the average ± S.E. of the three independent samples plotted as a function of the vehicle treated control cells (100%). *Statistically different from vehicle treated control; $p < 0.005$.

with CHX (Fig. 1C). As expected, CHX treatment blocks the induction of CYP1A1 by TCDD and results in the inhibition of AHR degradation. However, as observed in the AD treated cells, CHX had no significant impact on the level of GA-induced degradation of the AHR.

Previous studies suggest that species differences exist in the time course and magnitude of AHR degradation [26,30,31]; thus, it was pertinent to determine whether the affects of AD and CHX were specific to the Ah^{b-1} AHR. Rat smooth muscle cells (A7) and mouse 10T1/2 embryonic fibroblasts (expressing the Ah^{b-2} receptor) were treated with AD or CHX and exposed to TCDD or GA. A representative study is shown in Fig. 2. In both cell lines, treatment with TCDD resulted in complete loss of the endogenous AHR protein and this loss was inhibited in the presence of AD or CHX (Fig. 2A). In contrast, as shown in the Hepa-1 line, AD or CHX failed to block the GA-induced degradation of the AHR in either the A7 or 10T1/2 cells (Fig. 2B). Thus, the ability of translation or transcription inhibitors to block the TCDD-induced degradation of the AHR but not the GA-induced degradation appears to be universal and is not specific to distinct species of AHR. Collectively, the results in Figs. 1 and 2 are noteworthy for two reasons. First, they link active transcription *and* translation to the ligand-induced degradation of the AHR and provide a correlation to results from steroid hormone receptors. Indeed, both AD and CHX result in inhibition of estradiol-induced degradation of the ERα [12,32,33]. Secondly, these results show that TCDD and GA induced degradation utilize distinct mechanisms even though both pathways terminate at the 26S proteasome.

3.2. Effect of AD on nuclear translocation and DNA binding of the endogenous AHR

Transcription is inhibited by AD by intercalating into the DNA and blocking mRNA elongation [34]. Since AD is affecting DNA structure, it is possible that it could impact AHR degradation by inhibiting the binding of the AHR·ARNT heterodimer to DNA or by affecting the localization of the AHR to the nucleus. To evaluate these possibilities, cells were treated with vehicle or AD for 120 min, incubated with TCDD for an additional 60 min and stained for AHR or used to generate nuclear extracts. Fig. 3A shows that AD does not impact the TCDD-induced

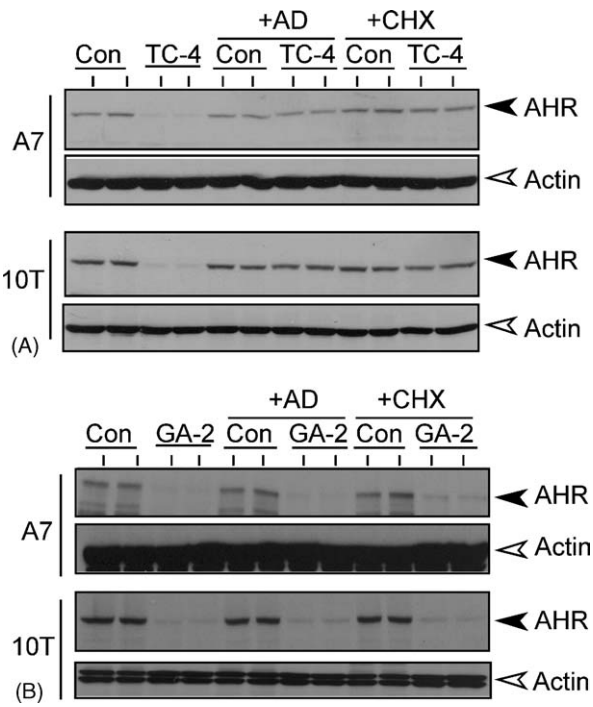
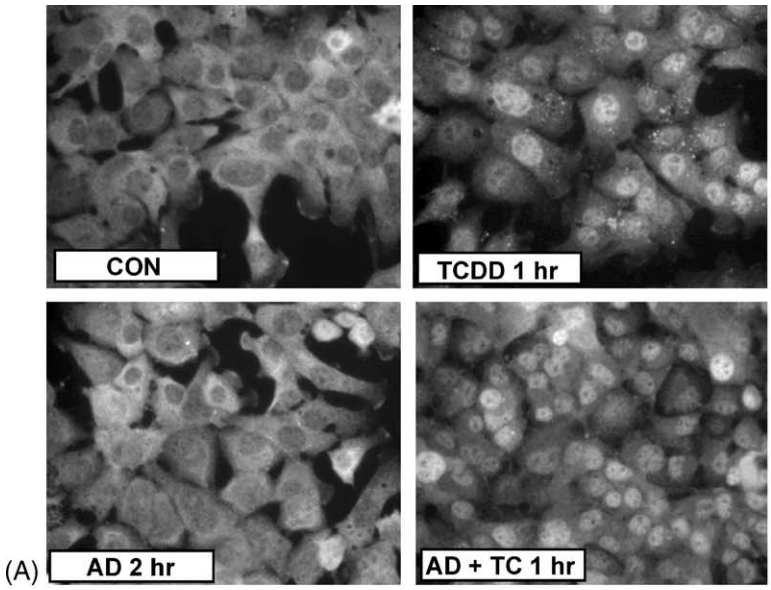


Fig. 2. Effect of AD and CHX on TCDD and GA-induced degradation of the endogenous AHR in A7 and 10T1/2 cells. Duplicate plates of rat A7 or mouse 10T1/2 cells were treated with AD (0.30 μM), CHX (10 μg/ml) or Me_2SO (0.05%) for 2 h at 37 °C and then exposed to TCDD (2 nM) or GA (100 nM) for an additional 4 or 2 h, respectively. Equal amounts of total cell lysates were resolved by SDS-PAGE, blotted and stained with A-1A anti-AHR IgG (1.0 μg/ml) and anti-β-actin IgG (1:1000). Reactivity was visualized by ECL with GAR-HRP (1:10,000). (A) A7 and 10T1/2 cells exposed to the indicated inhibitors and TCDD. (B) A7 and 10T1/2 cells exposed to the indicated inhibitors and GA.



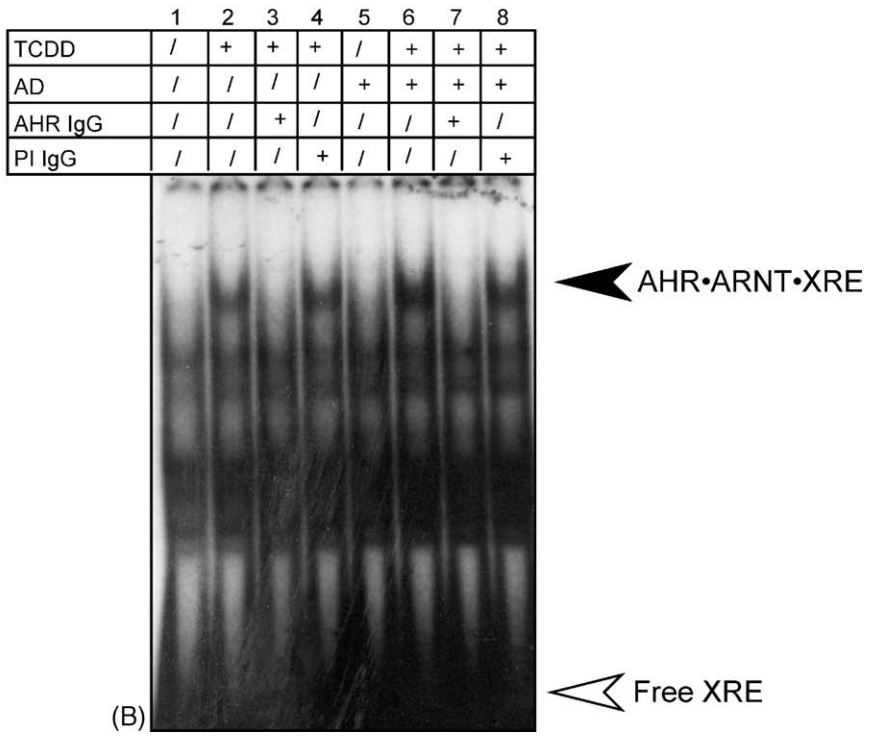


Fig. 3. Effect of AD on nuclear translocation and DNA binding of the endogenous AHR in Hepa-1 cells. (A) Hepa-1 cells were grown on glass coverslips and treated with AD (0.30 μM) or Me_2SO (0.05%) for 2 h and then exposed to TCDD (2 nM) for an additional hour. Cells were then fixed and incubated with A-1 anti-AHR IgG (1.0 μg/ml) and visualized with GAR-RHO IgG (1:400). All panels were photographed with identical exposures. (B) Hepa-1 cells were treated with AD (0.3 μM) or Me_2SO (0.05%) for 2 h at 37 °C and then exposed to TCDD (2 nM) for an additional hour. Nuclear extracts were prepared and equal amounts evaluated by EMSA as detailed in Section 2. Lanes 1 and 5, extracts from vehicle treated cells; lanes 2 and 6, extracts from TCDD treated cells; lanes 3 and 7, extracts from TCDD treated cells incubated with A-1A anti-AHR IgG (250 ng); lanes 4 and 8, extracts from TCDD treated cells incubated with pre-immune IgG (250 ng).

translocation of the AHR to the nucleus. However, AD could still be affecting the ability of the AHR to dimerize with ARNT and bind DNA. To address this issue, the presence of AHR·ARNT heterodimers was evaluated by gel shift. In cells pre-treated with vehicle, a strong shift was detected in the presence of TCDD (Fig. 3B, lane 2) that was competed by the presence of AHR antibodies (Fig. 3B, lane 3). Importantly, in cells treated with AD, specific AHR complexes were also detected (Fig. 3B, lanes 6 and 8) and showed the same level of intensity as complexes isolated from cells pre-treated with vehicle. Thus, these results rule out the possibility that AD is impacting AHR degradation

by blocking nuclear import or DNA binding and suggest that inhibition of degradation is distal to these events.

3.3. Generation and analysis of AHRs containing truncations of the transactivation domain (TAD)

To directly evaluate the role of AHR-mediated transactivation in the mechanism of ligand and GA-induced degradation, the putative TAD of the Ah^{b-1} AHR (mAHR) was deleted. PCR was used to generate a full-length mAHR (AHR_{WT}) and two truncated mAHRs by introducing stop codons into the open reading frame as detailed in Materials and Methods. AHR_{500} and AHR_{640} are missing the COOH-terminal 305 and 165 amino acids, respectively (Fig. 4A). AHR_{500} serves the role of an AHR that is missing all three TAD regions and is transcriptionally inactive [35,36]. Since the COOH-terminal 305 amino acids also contain eight lysine residues (illustrated in Fig. 4A), deletion of this region also serves to test whether these residues could serve as potential sites for ubiquitylation. AHR_{640} contains the acidic domain but is missing both the proline and serine-rich domains and should exhibit partial transcriptional activity [35,36].

The strategy for the physiological analysis of AHR_{500} and AHR_{640}, was to stably express the proteins in the LA-I variant Hepa-1 line using retroviral infection and assess transcriptional activity by measuring the induction of the endogenous CYP1A1 protein. Stable cell lines have been used previously to assess the nuclear localization of mAHR

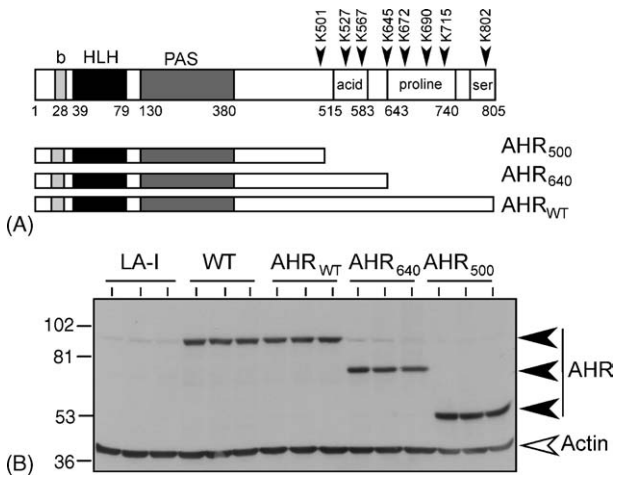


Fig. 4. Generation of stable cell lines. (A) Schematic representation of AHR constructs used to assess TAD activity. b, basic region; HLH, helix-loop-helix; PAS, Per/Arnt/Sim homology domain. Numbers indicate amino acids and the location of lysines are shown by arrowheads. (B) Equal amounts of total cell lysates from the indicated lines were resolved by SDS-PAGE, blotted and stained with A-1A anti-AHR IgG (1.0 μg/ml) and anti-ß-actin IgG (1:1000). Reactivity was visualized by ECL with GAR-HRP (1:10,000). LA-I, parental line used to produce the stable lines; WT, wild type Hepa-1 cells. Each lane of the AHR_{WT}, AHR_{640} and AHR_{500} samples represents an independent stable cell line.

and to express the Ah^{b-2} AHR [18,30]. Using this method, several stable lines were selected and the expression of the mAHR evaluated by Western blotting. Fig. 4B shows the expression of AHR_{500}, AHR_{640} and AHR_{WT} in three independent cell lines in comparison to the WT Hepa-1 cells and the parental LA-I cell line. Importantly, the overall level of expression of AHR_{500}, AHR_{640} and AHR_{WT} were similar to the level of endogenous AHR found in WT Hepa-1 cells. Immunocytochemical analysis of the various cell lines showed the AHR to be expressed in >85% of the population (R.S. Pollenz, unpublished results).

3.4. Analysis of stable cell lines expressing AHR_{500}, AHR_{640} and AHR_{WT}

To determine if TCDD-mediated gene regulation correlated to the degradation of the mAHR, cell lines expressing AHR_{500}, AHR_{640}, and AHR_{WT} were treated with TCDD for 16 h and evaluated for the expression of mAHR, CYP1A1 and actin. The induction of CYP1A1 protein was used to assess the transcriptional activity of the various constructs. A representative experiment is shown in Fig. 5. In WT Hepa-1 cells, CYP1A1 protein was strongly induced following TCDD exposure and mAHR levels were reduced by approximately 85%. Nearly identical levels of CYP1A1 induction were observed in the AHR_{WT} stable cell line (Fig. 5A and B) and the level of AHR_{WT} was reduced by 70% after 16 h of TCDD exposure (Fig. 5C). In contrast, the AHR_{500} cells exhibited no detectable CYP1A1 protein above the level in the parental LA-I cells. However, despite the lack of transcriptional activity, the AHR_{500} protein was reduced by approximately 65% in the presence of TCDD (Fig. 5C). The AHR_{640} protein was also reduced by approximately 70% in the presence of TCDD but resulted in CYP1A1 induction to 50% of the level observed in the WT Hepa-1 and AHR_{WT} cells. Thus, the level of AHR degradation in all stable cell lines was similar even though the transactivation potential of the AHR_{500} and AHR_{640} was 0 and 50% of that observed in the AHR_{WT}. These results suggest that active transcription via the AHR·ARNT complex *is not* a prerequisite for ligand-induced degradation and that the inhibition of degradation of the endogenous AHR by AD is likely related to the reduced expression of a gene product.

3.5. Effect of MG-132 on ligand-induced degradation AHR_{500}, AHR_{640} and AHR_{WT}

To verify that the truncation of the AHR or the use of a stable transfection model did not affect the degradation pathway, cells expressing AHR_{500}, AHR_{640} and AHR_{WT} were treated with the proteasome inhibitor MG-132 and then exposed to TCDD. Fig. 6 illustrates that TCDD-induced degradation of all receptors is blocked by

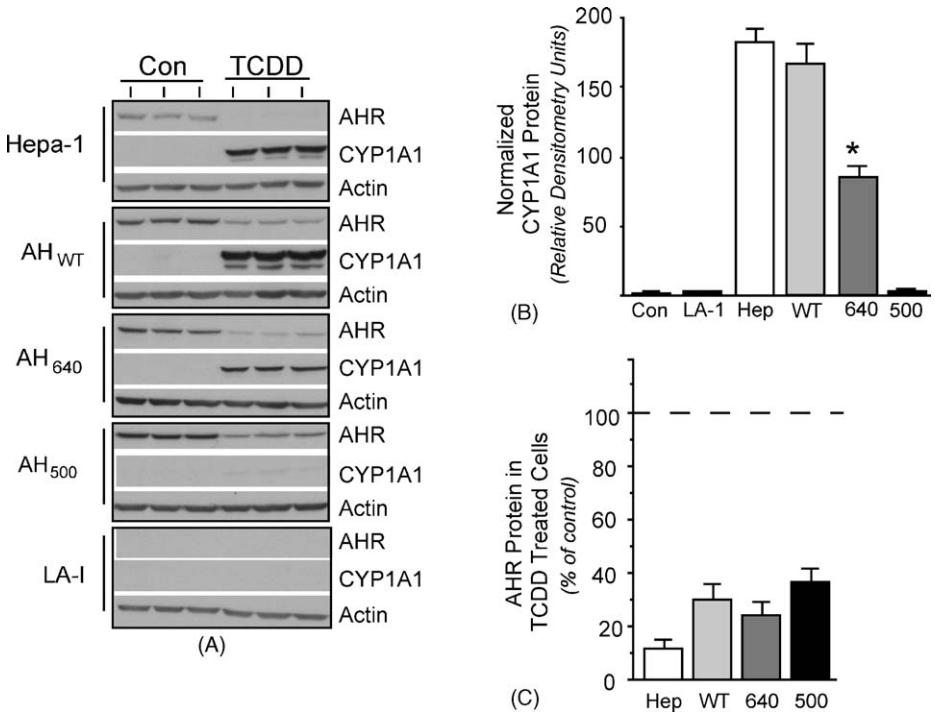


Fig. 5. Analysis of stable cell lines expressing AHR_{500}, AHR_{640} and AHR_{WT}. (A) The indicated cell lines were treated with TCDD (2 nM) or Me_2SO (0.05%) for 16 h at 37 °C and total cell lysates prepared. Equal amounts of total cell lysates were then resolved by SDS-PAGE, blotted and stained with A-1A anti-AHR IgG (1.0 μg/ml), anti-β-actin IgG (1:1000) or anti-CYP1A1 IgG (1:1000). Reactivity was visualized by ECL with GAR-HRP (1:10,000). (B) The level of CYP1A1 protein in the TCDD treated samples was divided by the corresponding level of actin and the average ± S.E. of the three independent samples plotted. *Statistically different from TCDD treated AHR_{WT} cells; $p < 0.005$. (C) The level of AHR protein in the TCDD treated samples was divided by the corresponding level of actin and the average ± S.E. of the three independent samples plotted as percent of vehicle treated samples (100%, dashed line).

MG-132. Additional studies using calpain or lysosomal inhibitors failed to block the TCDD-mediated degradation (R.S. Pollenz, unpublished results). These findings are consistent with those analyzing the endogenous AHR in numerous cell culture lines [15–17]. Thus, deletion of the COOH-terminal TAD or stable expression does not appear to impact degradation via the 26S proteasome.

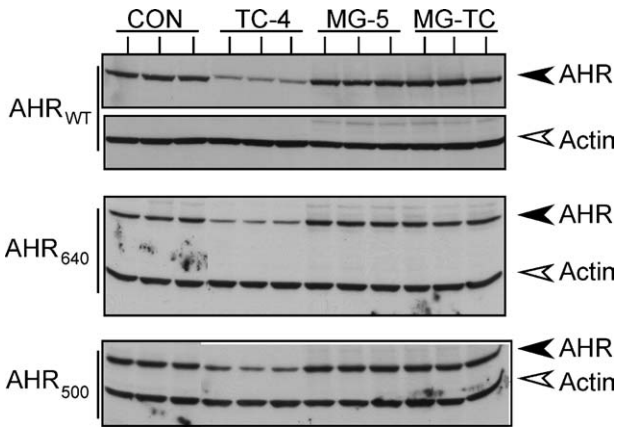


Fig. 6. Effect of MG-132 on ligand-induced degradation AHR_{500}, AHR_{640} and AHR_{WT}. Triplicate samples of the indicated cell lines were treated with Me_2SO (0.05%) for 5 h (CON); Me_2SO (0.05%) for 1 h followed by exposure to TCDD (2 nM) for an additional 4 h (TC-4); MG-132 (10 μM) for 5 h (MG-5) or MG-132 (10 μM) for 1 h followed by exposure to TCDD (2 nM) for an additional 4 h (MG-TC). All samples were harvested at the same time and total cell lysates prepared. Equal amounts of total cell lysates were resolved by SDS-PAGE, blotted and stained with A-1A anti-AHR IgG (1.0 μg/ml) and anti-β-actin rabbit IgG. Reactivity was visualized by ECL with GAR-HRP (1:10,000).

3.6. Effect of AD and CHX on ligand-induced degradation of AHR_{500}, AHR_{640} and AHR_{WT}

Since the AHR_{WT}, AHR_{500} and AHR_{640} were degraded following ligand exposure, it was pertinent to investigate whether the degradation mechanism could be blocked by AD or CHX. Thus, stable cells expressing AHR_{500}, AHR_{640} or AHR_{WT} were pre-treated with AD or CHX and then exposed to TCDD. A representative Western blot of whole cell lysates is shown in Fig. 7. Consistent with the results for the endogenous AHR in WT cells, the TCDD-induced degradation of AHR_{WT} was effectively inhibited by both CHX and AD. Surprisingly though, neither AD nor CHX inhibited the TCDD-induced degradation of AHR_{500} or AHR_{640}. Since this result was unexpected, experiments were repeated on at least four additional stable lines and in all cases the degradation of AHR_{WT} was always inhibited by AD and CHX while no affect was observed on lines carrying AHR_{500} and AHR_{640}. These results suggest that the ligand-induced mechanism of degradation can switch between two distinct pathways depending on the presence of the COOH-terminal domain. Since the degradation of the AHR_{WT} can be blocked by AD/CHX, the results

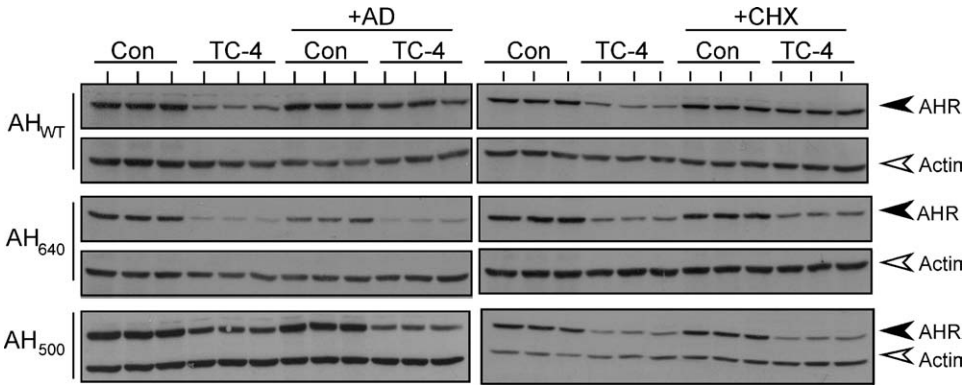


Fig. 7. Effect of AD and CHX on ligand-induced degradation of AHR_{500}, AHR_{640} and AHR_{WT}. Triplicate plates of the indicated cell lines were treated with Me_2SO (0.05%), AD (0.30 μM), or CHX (10 μg/ml) for 2 h at 37 °C. Cells were then exposed to TCDD (2 nM) or GA (100 nM) for an additional 4 or 2 h, respectively, and total cell lysates prepared. Equal amounts of total cell lysates were resolved by SDS-PAGE, blotted and stained with A-1A anti-AHR IgG (1.0 μg/ml), anti-β-actin IgG (1:1000) or anti-CYP1A1 IgG (1:1000). Reactivity was visualized by ECL with GAR-HRP (1:10,000). CON = Me_2SO treated cells. T-4 = TCDD treated cells.

implicate the COOH-terminal 165 amino acids of the AHR as a possible site for the initiation of the AD/CHX-sensitive mechanism of degradation.

3.7. Time course of ligand-induced degradation of AHR_{500}, AHR_{640} and AHR_{WT}

Since both the AHR_{500} and AHR_{640} were susceptible to TCDD-induced degradation, a time course experiment was completed to determine whether the kinetics of degradation were similar to those of AHR_{WT}. Two different time course experiments were completed. In the first, cells were treated with TCDD for 0, 1, 2, 4 and 6 h and total cell lysates evaluated for the level of AHR and actin. Results from a representative experiment are presented in Fig. 8. The results show that the AHR_{500} and AHR_{640} proteins are degraded along a more rapid time course and to a much greater magnitude over the first 2 h of TCDD exposure as compared to the AHR_{WT}. Indeed, the levels of AHR_{500} and AHR_{640} were approximately 30–38% of control levels at the 120-min time point as compared to 70–75% for the AHR_{WT}. Analysis of the slopes indicated that the degradation of AHR_{500} and AHR_{640} was approximately 2.5 times more rapid than the AHR_{WT} during the first 2 h of TCDD exposure. To verify this finding, a second experiment was carried out in which cells were treated with TCDD for 0, 30, 60, 90 and 120 min. The results are presented in the inset in Fig. 8. As observed in the first study, the time course of degradation was significantly increased. The change in kinetics and loss of sensitivity to AD/CHX is consistent with a change in the mechanism of degradation. Interestingly, GA-induced degradation is also not blocked by AD/CHX and results in a more rapid level of degradation of the AHR than that induced by ligand [16,18]. Therefore, in the absence of a functional COOH-terminal region the results support a hypothesis that ligand-induced degradation of the truncated AHRs can proceed via an alternative pathway than that used for a full length AHR.

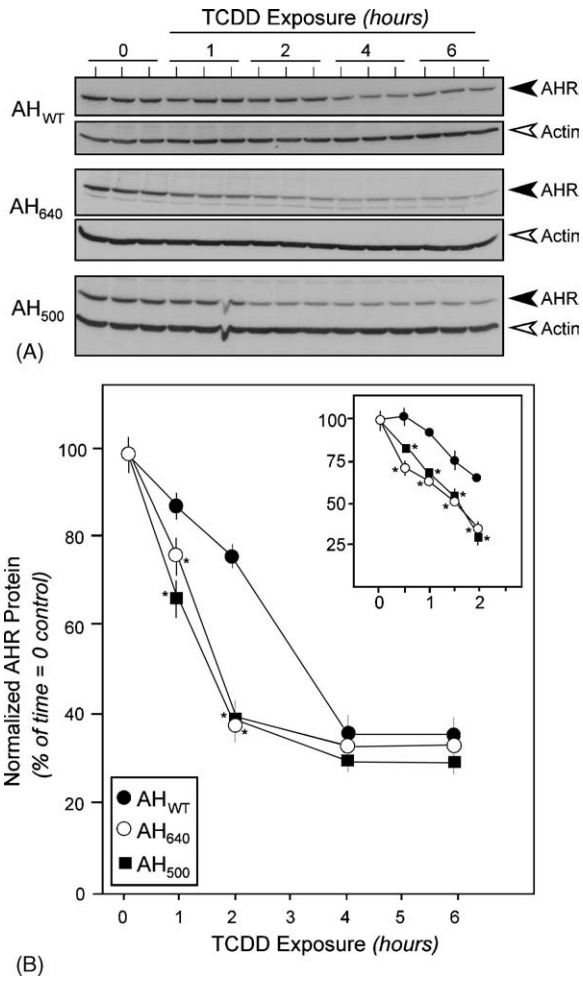


Fig. 8. Time course of ligand-induced degradation of AHR_{500}, AHR_{640} and AHR_{WT}. The indicated cell lines were treated with 0.05% Me_2SO (time 0 control), or TCDD (2 nM) for the indicated times (h) at 37 °C. Dosing was staggered so that all cells were harvested at the same time. Equal amounts of total cell lysates were resolved by SDS-PAGE, blotted and stained with A-1A anti-AHR IgG (1.0 μg/ml) or anti-β-actin IgG (1:1000). Reactivity was visualized by ECL with GAR-HRP (1:10,000). The level of AHR protein at each time point was divided by the corresponding level of actin and the average ± S.E. of the three independent samples plotted as a function of the vehicle treated control cells (100%). *Statistically different from time = 0 control; $p < 0.01$. The inset shows the plot of cells treated with TCDD for 0, 30, 60, 90 and 120 min.

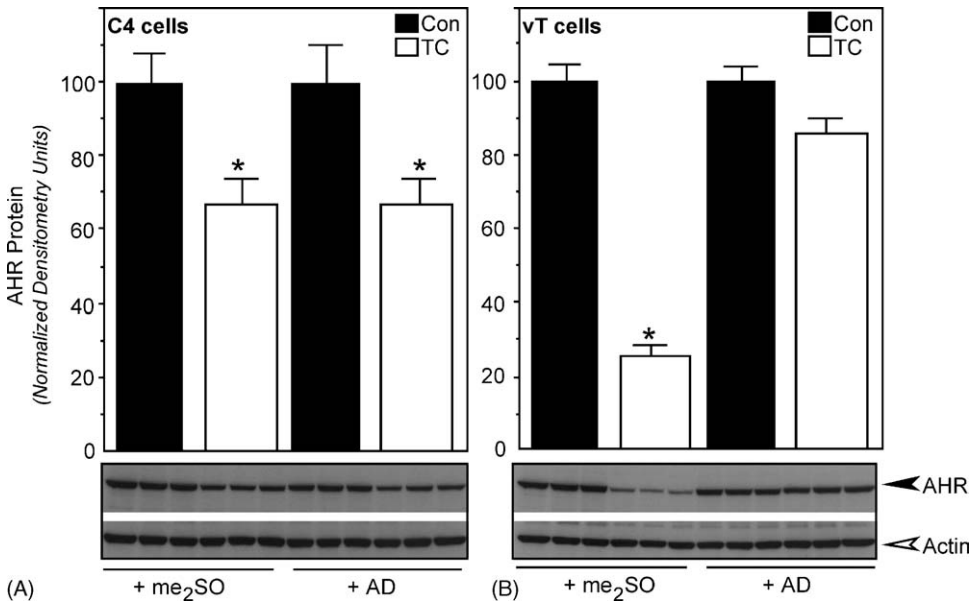


Fig. 9. Role of ARNT in ligand-induced degradation. Triplicate plates of ARNT-deficient cells (C4) or ARNT-proficient cells (vT) were treated with Me_2SO (0.05%) or AD (0.30 μM) for 2 h at 37 °C. Cells were then exposed to TCDD (2 nM) for additional 4 h and total cell lysates prepared. Equal amounts of total cell lysates were resolved by SDS-PAGE, blotted and stained with A-1A anti-AHR IgG (1.0 μg/ml) and anti-β-actin IgG (1:1000). Reactivity was visualized by ECL with GAR-HRP (1:10,000). The level of AHR protein at each time point was divided by the corresponding level of actin and the average ± S.E. of the three independent samples plotted as a function of the vehicle treated control cells (100%). *Statistically different from vehicle treated control; $p < 0.005$.

3.8. Role of ARNT in ligand-induced degradation

Since the previous results suggest that there are multiple mechanisms involved in the ligand-induced degradation of the AHR, it was pertinent to assess the degradation of the full length AHR in cells lacking ARNT. For these studies, C4 Hepa-1 cells with reduced ARNT, and a C4 line expressing human ARNT (vT cells) were used [37]. In the C4 cells, the endogenous AHR can bind ligand and translocate to the nucleus but it does not form a complex with ARNT or bind consensus XRE motifs [24,37]. Thus, it has been used as a model in which the AHR does not bind DNA. Cells were pretreated with Me_2SO or AD and then incubated with TCDD. The level of endogenous AHR determined by quantitative Western blotting and a representative experiment is shown in Fig. 9. TCDD resulted in a modest reduction of AHR in the C4 cells (35%) that was not inhibited by treatment with AD (Fig. 9A). In contrast, TCDD treatment of the recombinant vT cells, resulted in an enhanced level of degradation that was blocked by exposure to AD (Fig. 9B). Thus, these results suggest that the inhibition of degradation by AD requires a full length AHR and functional ARNT protein as well as DNA binding. However, the results also imply that in the absence of DNA binding, the mechanism of degradation of the endogenous AHR in C4 cells is occurring via the pathway observed for AHR_{500} and AHR_{640}.

4. Discussion

It is clear that the degradation of many nuclear receptors is an important physiological response to agonist stimulation and that degradation can be linked to binding at enhancer regions and transcriptional activity (reviewed in refs. [4,5]). Previous studies that have shown that AD [22] and CHX [23] can block ligand-induced degradation of the mouse Ah^{b-1} AHR. It has been hypothesized that the inhibition of ligand-induced degradation by AD or CHX may be due to either: (i) reduced transformation or DNA binding by the AHR; (ii) reduced induction of a degradation factor mediated via the AHR complex; (iii) loss of a CHX-sensitive factor that was required for AHR degradation; (iv) a requirement for active transcription by the AHR·ARNT complex. However, the validity of each of these hypotheses and a connection between transcription and translation in AHR degradation has not been explored. The results in the current report resolve some of these issues by showing that AD does not impact nuclear translocation or DNA binding of the AHR complex. This implies that the affects of AD and CHX must be distal to these processes. What then is the connection between degradation, DNA binding and the TAD?

The key finding of this report, is that the degradation mechanism is more complex than originally thought and appears to involve two distinct pathways that function under different situations. The identification of two mechanisms highlights the importance of clearing the receptor from the cell. Degradation of the full length AHR in a physiological context appears to require a factor that is sensitive to AD and CHX. In this context, dimerization with ARNT, a functional TAD and DNA binding are also required for efficient degradation, and it appears that the mechanism of degradation that is insensitive to AD/CHX is not functioning. Interestingly, AD and CHX

also inhibit ligand-induced degradation of the ER [32,33]. The similarity of the two signaling pathways is intriguing since studies show that the AHR and ER share common co-activators involved in gene regulation (reviewed in refs. [38]). The sensitivity of both pathways to AD/CHX also suggests that it is likely that the AHR·ARNT complex is not inducing the factor required for AHR or ER degradation. This hypothesis is supported by the finding that ubiquitin ligases or conjugating enzymes are not among the genes induced by the AHR following ligand exposure [39–44]. However, recent studies showed that ligand-activated AHR could be detected in association with the ER in MCF-7 nuclear extracts and appeared to promote the degradation of the ER [45]. Thus, these studies suggest that degradation of the AHR may be linked with DNA binding and transcription and it may participate in the recruitment of factors that degrade the ER under certain conditions. Indeed, studies have shown that there is a connection between the potency of a TAD and the degradation of the corresponding protein [46]. It has also been shown that proteasomal enzymes can be recruited to the promoters of activated genes [11–13,32,47] and that the AHR has a cyclical pattern of binding at the *CYP1A1* enhancer region in human MCF-7 cells that is consistent with periods of degradation [48]. Therefore, like other nuclear receptors, it appears that a key point in the physiological degradation of the AHR is during DNA binding and recruitment of the transcriptional machinery. What then is the physiological relevance of the degradation mechanism that is not sensitive to AD/CHX?

First, this pathway must involve a distinct set of proteins since it does not requite active translation. In addition, the finding that GA-induced degradation of the AHR is also insensitive to AD or CHX, indicates that this degradation pathway does not require the dimerization or DNA binding of the AHR. Indeed, previous studies have shown that GA treatment results in the nuclear accumulation of the AHR but does not lead to association with ARNT or DNA [16]. This conclusion is also supported by the studies in the C4 cells that indicate ligand-induced degradation is not sensitive to AD in the absence of ARNT [17]. In addition, the degradation of the truncated AHR_{500} and AHR_{640} is also not sensitive to AD or CHX. In all these instances, the common theme is that the AHR is not functioning in a physiological context. Thus, it is possible that the AD/CHX insensitive degradation pathway may exist to clear a protein from the cell that is perceived to be misfolded or not localized properly. In the case of the AHR_{500} and AHR_{640} proteins, it may be that the proteins are missing critical domains that are substrates for important factors that are reduced in the presence of AD/CHX. Within the last 165 amino acids of the Ah^{b-1} AHR are 4 lysines that are conserved in position between mouse Ah^{b-2}, rat and human AHRs, as well as conserved serine residues that are predicted to be phosphorylation sites. Therefore, GA-induced degradation as well as the AHR_{500} and AHR_{640} proteins will provide important models to assess this alternative degradation pathway and determine the importance of AHR degradation in signal transduction.

Acknowledgement

This work was supported by a grant from the National Institutes of Health to R.S.P. (ES 10991).

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