0026-895X/02/6204-806–816$7.00
MOLECULAR PHARMACOLOGY
Copyright © 2002 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 62:806–816, 2002

Vol. 62, No. 4
1722/1012390
Printed in U.S.A.

Ligand-Dependent and Independent Modulation of Aryl Hydrocarbon Receptor Localization, Degradation, and Gene Regulation

ZHIJUAN SONG and RICHARD S. POLLENZ

Department of Biology, University of South Florida, Tampa, Florida

Received March 7, 2002; accepted June 20, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Changes in the concentration or subcellular location of the key proteins involved in signal transduction pathways have been shown to impact gene regulation. Studies were designed to evaluate the relationship between aryl hydrocarbon receptor (AHR) localization, stability, and gene regulation in a defined system where the endogenous AHR protein could be evaluated. The findings indicate that treatment of cells with geldanamycin (GA) or MG-132 (an inhibitor of the 26S proteasome) results in nuclear translocation of the endogenous AHR in both human HepG2 and murine Hepa-1 cells without induction of endogenous CYP1A1 protein. Exposure to GA resulted in the degradation of AHR by >90% in the nucleus via the 26S proteasome. Importantly, the reduced level of AHR resulted in a 50% reduction in the maximal level of CYP1A1 induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In all treatments the concentration of the AHR nuclear translocator (ARNT) protein was unchanged and had no impact on the localization of the AHR. Thus, ligand-independent translocation of the AHR to the nucleus was not sufficient to induce CYP1A1 in the absence of ligand, but reductions in the level of the endogenous AHR protein pool shifted the dose-response curve for TCDD to the right.

The aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor that is a member of the basic-helix-loop-helix periodicity/ARNT/single-minded (Per/ARNT/Sim) family of proteins (reviewed in Hahn, 1998; Whitlock, 1999; Gu et al., 2000). The AHR binds ligands typified by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and modulates gene regulation after dimerization with the nuclear ARNT protein. Although these two aspects of the AHR signaling pathway are well established, recent studies continue to add complexity to AHR-mediated gene regulation with respect to the importance of AHR stability, concentration, subcellular location, and the association of the AHR with chaperone proteins. It has been shown, for example, that the AHR is rapidly degraded after ligand binding in vivo and in vitro (Pollenz, 1996, Pollenz et al., 1998; Roman et al., 1998; Davarinos and Pollenz, 1999; Sommer et al., 1999; Ma and Baldwin, 2000) and that inhibition of AHR degradation results in increased levels of ligand mediated gene induction (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000; Ma et al., 2000). Thus, these studies indicate that the concentration of the AHR protein has a profound impact on the magnitude and duration of gene regulation and raises the question of how the process is regulated. Because the AHR contains both nuclear export signals and nuclear localization signals, it has been hypothesized that degradation via the 26S proteasome may be influenced by the subcellular localization of the AHR (Davarinos and Pollenz, 1999; Pollenz and Barbour, 2000). Indeed, studies have shown that the ligand-mediated degradation of the AHR can be inhibited in HepG2 cells that have been exposed to the nuclear export blocker leptomycin B (Pollenz and Barbour, 2000). Therefore, it becomes critical to understand how the subcellular location of the AHR is modulated in both the liganded and unliganded states.

The unliganded AHR exists in a multiprotein complex containing one molecule of AHR, two molecules of hsp90, a protein termed p23, and an immunophilin-like protein named XAP, AIP, or ARA9 (Meyer et al., 1998; Meyer and

This work was supported by National Institute of Environmental Health Sciences grant ES10401.

ABBREVIATIONS: AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; Per-ARNT-Sim, periodicity/ARNT/single-minded; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; hsp90, 90-kDa heat-shock protein; GA, geldanamycin; MG-132, *Z*-Leu-Leu-Leu-aldehyde; DMSO, dimethyl sulfoxide; LMB, leptomycin B; PBS, phosphate-buffered saline; TTBS, Tris-buffered saline with Tween 20; DMEM, Dulbecco's modified Eagle's medium; BLOTTO, bovine lacto transfer optimizer; MENG, MOPS/EDTA/NaN3/glycerol; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; XRE, xenobiotic response element; GAR-HRP, goat anti-rabbit horseradish peroxidase; GAM-RHO, goat anti-rabbit rhodamine; PAGE, polyacrylamide electrophoresis; ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; MEF, mouse embryo fibroblast.

Perdew, 1999; LaPres et al., 2000; Kazlauskas et al., 2001). The relationship of these proteins to the degradation of AHR represents an important area of research because their association with the AHR may mask key domains for nuclear import, nuclear export, ubiquitination, or phosphorylation. Recent studies suggest that the protein responsible for maintaining a stable AHR-hsp90 complex is the immunophilin-like protein XAP2 (Meyer et al., 1998; Meyer and Perdew, 1999; Kazlauskas et al., 2000; LaPres et al., 2000). Results show that when XAP2 is expressed in COS-1 cells, it enhances the level of cytosolic AHR (Meyer and Perdew, 1999), can prevent the ubiquitination of the AHR (Kazlauskas et al., 2001), and also results in enhanced AHR-mediated gene regulation (Meyer et al., 1998; LaPres et al., 2000). In addition, the relationship between the association of hsp90 and immunophilins with the AHR and its degradation has been investigated. Several studies have evaluated the affect of geldanamycin (GA) exposure on AHR-mediated signaling. GA is a benzoquinone ansamycin that directly associates with ATP/ADP binding site of hsp90 (Grenert et al., 1997) and can disrupt the formation of heterocomplexes such as hsp90-pp60v-src (Whitesell et al., 1994) and Raf-1/hsp90 (Schulte et al., 1995). Treatment of both human HeLa and mouse Hepa cells with GA results in dramatic reductions in the level of endogenous AHR protein within 2 h of exposure in a dose- and time-dependent manner without affecting the level of XAP2 (Chen et al., 1997; Meyer et al., 2000). Thus, these studies are consistent with a hypothesis that loss of chaperone proteins or changes in the conformation of the AHR-hsp90 complex plays a key role in the stability and subcellular localization of the AHR and its recognition by the 26S proteasome.

To gain additional insight into these issues, studies were designed to evaluate the relationship between AHR localization, stability, and gene regulation in a defined system in which the endogenous AHR protein could be evaluated. The strategy of these studies was to manipulate the location of the endogenous AHR in a ligand-independent manner and then assess TCDD-mediated gene regulation and AHR protein concentration. The findings indicate that treatment of cells with GA or MG-132 (an inhibitor of the 26S proteasome) results in nuclear translocation of the endogenous AHR in both human HepG2 and murine Hepa-1 cells without induction of the *CYP1A1* gene. Importantly, treatment of these cell lines with GA leads to degradation of AHR in the nucleus via the 26S proteasome, resulting in an overall reduction in the maximal level of CYP1A1 that is induced by TCDD. Thus, ligand independent translocation of the AHR to the nucleus was not sufficient to induce CYP1A1 but reductions in the level of the endogenous AHR protein pool by GA shifted the dose-response curve for TCDD to the right.

Materials and Methods

Materials. TCDD (98% stated chemical purity) was obtained from Radian Corp. (Austin, TX) and was solubilized in dimethyl sulfoxide (DMSO). Leptomycin B (LMB) and GA were purchased from Sigma (St. Louis, MO). MG-132 was purchased from Calbiochem (San Diego, CA). The pGudLUC1.1 vector was a generous gift from Dr. Michael Denison (University of California Davis, Davis, CA)

Buffers. PBS is 0.8% NaCl, 0.02% KCl, 0.14% Na_2HPO_4, and 0.02% KH_2PO_4, pH 7.4; 2× gel sample buffer is 125 mM Tris, pH 6.8, 4% SDS, 25% glycerol, 4 mM EDTA, 20 mM dithiothreitol, and 0.005% bromphenol blue. Tris-buffered saline is 50 mM Tris, 150 mM NaCl, pH 7.5. TTBS is 50 mM Tris, 0.2% Tween 20, and 150 mM NaCl, pH 7.5. TTBS+ is 50 mM Tris, 0.5% Tween 20, and 300 mM NaCl, pH 7.5. BLOTTO is 5% dry milk in TTBS; 2× lysis buffer is 50 mM HEPES, pH 7.4, 40 mM sodium molybdate, 10 mM EGTA, 6 mM $MgCl_2$, and 20% glycerol; 5× gel shift buffer is 50 mM HEPES, pH 7.5, 15 mM $MgCl_2$, 50% glycerol; and 0.5× Tris/borate/EDTA is 45 mM Tris-borate, 1 mM EDTA. MENG is 25 mM MOPS, 10 mM EDTA, 0.02% NaN_3, and 10% glycerol.

Cells and Growth Conditions. Wild-type Hepa-1c1c7(Hepa-1) and type II Hepa-1 variants were a generous gift from Dr. James Whitlock, Jr. (Department of Pharmacology, Stanford University, Stanford, CA). These cells were propagated in DMEM supplemented with 5% fetal bovine serum. HepG2 cells were obtained from American Type Culture Collection (Manassas, VA). HepG2 cells were propagated in DMEM supplemented with 10% fetal bovine serum. Hepa-1 cells stably integrated with an XRE-driven reporter vector (H1L1.1c2; Garrison et al., 1996) were a generous gift from Dr. Michael Denison. H1L1.1c2 cells were propagated in DMEM supplemented with 5% fetal bovine serum. All cells were passaged at 1-week intervals and used in experiments during a 2-month period at approximately 70% confluence. For treatment regimens, TCDD, MG-132, and LMB were administered directly into growth media for the indicated incubation times. The vehicle used for TCDD and MG-132 was DMSO and the final concentration of DMSO ranged from 0.2 to 0.8%. The vehicle used for LMB was 70% methanol and the final concentration in the media was <0.02%.

Antibodies. Specific antibodies against either the AHR (A-1, A-1A) or ARNT protein (R-1) are identical to those described previously (Pollenz et al., 1994; Holmes and Pollenz, 1997). All antibodies are affinity-purified IgG fractions. For Western blot analysis, goat anti-rabbit antibodies conjugated to horseradish peroxidase (GAR-HRP) were used. For immunohistochemical studies, goat anti-rabbit IgG conjugated to rhodamine (GAR-RHO) were used. Both of these reagents were purchased from Jackson Immunoresearch (West Grove, PA). Polyclonal rabbit β-actin antibodies were purchased from Sigma.

In Vitro Expression of Protein. Recombinant protein was produced from expression constructs using the TNT Coupled Rabbit Reticulocyte Lysate Kit essentially as detailed by the manufacturer (Promega, Madison, WI). Upon completion of the 90-min reaction, samples were either combined with an equal volume of 2× gel sample buffer and boiled for 5 min, or stored at −80°C for use in functional studies

Preparation of Total Cell Lysates. After treatment, cell monolayers were washed twice with PBS and detached from plates by trypsinization (0.05% trypsin/0.5 mM EDTA). Cell pellets were then washed with PBS and suspended in 50 to 100 μl of ice-cold 2× lysis buffer supplemented with Nonidet P-40 (0.5%), leupeptin (10 ug/ml), and aprotinin (20 μg/ml). Cell suspensions were immediately sonicated for 10 s, supplemented with phenylmethylsulfonyl fluoride (100 μM, final concentration), and sonicated for an additional 10 s. A small portion of the lysate was then removed for protein determination and the remainder was combined with an equal volume of 2× gel sample buffer, vortexed, and immediately heated for 5 min at 100°C. Samples were stored at −20°C. Protein concentrations were determined by the Coomassie Brilliant Blue plus assay (Pierce, Rockford, IL.) with bovine serum albumin as the standard

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 to 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-HP for 1 h at 22°C and washed in three changes of TTBS+ as above. Before

detection, the blots were washed in Tris-buffered saline for 5 min. Bands were visualized with the enhanced chemiluminescence (ECL) kit as specified by the manufacturer (Amersham Biosciences, Piscataway, NJ). Multiple exposures of each set of samples were produced. The relative concentration of target protein was determined by computer analysis of the autoradiographs as detailed previously (Pollenz, 1996; Holmes and Pollenz, 1997; Pollenz et al., 1998).

Immunofluorescence Staining and Microscopy. All immunocytochemical procedures (cell plating, fixation, and staining) were carried out as described previously (Pollenz et al., 1994; Pollenz, 1996; Holmes and Pollenz, 1997). Cells were observed on an Olympus IX70 microscope. On average, 15 to 20 fields (5 to 20 cells each) were evaluated on each coverslip and three to four fields were photographed with a digital camera at the same exposure time to generate the raw data. Experiments were repeated at least two times.

In Vitro Activation of AHR-ARNT Complexes and Electrophoretic Mobility Shift Assay. For EMSA, a double-stranded fragment corresponding to the consensus XRE-1 of the murine *CYP1A1* promoter (mXRE) has been described previously (Shen and Whitlock, 1992). For in vitro activation, approximately 25 ng of recombinant AHR and ARNT protein (2–6 μl of the TNT reaction) were combined with MENG buffer in a 60-μl reaction. Each sample was then supplemented with TCDD (16 nM) or DMSO (0.5%) and incubated at 30°C for 2 h. Activated samples (15 μl) were then incubated at 22°C for 15 min in 1× gel shift buffer supplemented with KCl (80 mM) and polydIdC (0.1 mg/ml). Approximately 4 ng of ^{32}P-labeled XRE was added to each sample and the incubation continued for an additional 15 min at 22°C. The samples were resolved on 5% acrylamide/0.5% Tris/borate/EDTA gels, dried, and exposed to film. In some instances, activated samples were analyzed by Western blotting to assess expression of AHR and ARNT.

Transfection and Reporter Gene Assay. All transfections were carried out using LipofectAMINE (Invitrogen, Carlsbad, CA) as detailed by the manufacturer. The reporter gene construct used in these studies was pGudLuc 1.1, which contains a 484-base pair fragment from the murine CYP1A1 promoter (Garrison et al., 1996). Cells were transfected with the indicated plasmids and a constitutive β-galactosidase expression vector (pSV-β-galactosidase), and treated with TCDD or vehicle for the times indicated in the text. Cells were then scraped from plates in 1× Reporter Gene Buffer (Promega) and luciferase and β-galactosidase activity was quantified as detailed by the manufacturer. The raw luciferase activity was then divided by the β-galactosidase activity to control for transfection efficiency. In all experiments, the overall trend of the data was never changed by the normalization procedure.

Generation of ARNT-GFP Expression Construct. The entire coding region of the mRANT cDNA was isolated by polymerase chain reaction and cloned in frame into the pcDNA3.1/CT-GFP-TOPO vector (Invitrogen), to generate an ARNT protein with a GFP tag at the C terminus (pmARNT-GFP). Functional expression was evaluated by Western blotting of in vitro-expressed protein with anti-ARNT (R-1) antibodies. The ARNT-GFP was also shown to dimerize with mAHR and bind XRE sequences in vitro (R. S. Pollenz, unpublished observations).

Statistical Analysis. The EC_{50} values representing the concentration of ligand at which luciferase activity was 50% of maximal were obtained by nonlinear regression using InStat software (GraphPad Software, San Diego, CA). EC_{50} values represent the mean ± S.E. of three independent experiments.

Results and Discussion

Exposure of Hepa-1 cells to GA or MG-132 Results in Accumulation of Endogenous AHR in the Nucleus without Induction of Endogenous CYP1A1 Protein. In the past few years, numerous studies have shown that the ligand bound AHR is rapidly degraded after ubiquination via the 26S proteasome (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000). Similarly, previous reports have shown that treatment of various cell lines with GA also results in rapid degradation of the AHR (Chen and Perdew, 1997; Meyer et al., 2000). Therefore, it was of interest to determine whether the mechanism of GA-mediated degradation was similar to that initiated after ligand binding. For these studies, Hepa-1 cells were treated with MG-132 for 2 h and then exposed to GA for 0 to 3 h. Total cell lysates were then evaluated for the level of AHR and ARNT protein by quantitative Western blotting. The results show that basal AHR protein levels (90.1 ± 7.2 relative densitometry units) are reduced by 45 (50.2 ± 5.0), 62 (32.2 ± 1.3), and 92% (7.2 ± 1.0) after 0.5, 1.0, and 3.0 h of GA treatment, respectively (Fig. 1A). Importantly, the GA-mediated degradation of the AHR was inhibited when cells were treated with the proteasome inhibitor MG-132 before GA exposure. Similar results were observed when lactacystein was used to inhibit proteasome activity but not when calpain or lysosomal protease inhibitors were used (data not shown). Thus, the results suggest that the degradation of AHR after GA treatment is mediated by the 26S proteasome. To further assess the effect of GA on the AHR signaling pathway, the level of ARNT protein was also evaluated. In contrast to the degradation of the AHR, GA treatment had a minimal effect on the level of ARNT protein at any time points investigated (Fig. 1B). Thus, the mechanism of AHR degradation mediated by GA shows similarity to TCDD-mediated degradation with regard to the proteolytic enzyme (26S proteasome), time course (maximal loss of AHR after 3 h exposure), level of degradation (>90% compared with controls), and lack of effect on ARNT.

Because of the similarities between GA and TCDD-mediated degradation, it was pertinent to evaluate the subcellular location of the AHR in the presence of GA. Hepa-1 cells were treated with MG-132, GA, or TCDD for the time frames indicated in Fig. 2 and then fixed and stained for the endogenous AHR. As reported previously (Pollenz et al., 1994; Pollenz, 1996), the AHR showed a predominant cytoplasmic distribution in control treated Hepa-1 cells that became nuclear after 3 h of TCDD exposure (Fig. 2, parts 1, 2, 5, and 6). In addition, TCDD-mediated degradation of the AHR was inhibited by pretreatment of cells with MG-132 and the AHR remained predominantly nuclear for the duration of the experiment (Fig. 2, parts 7 and 8). Treatment with MG-132 alone also resulted in the nuclear accumulation of the AHR in the absence of TCDD (Fig. 2, parts 3 and 4). Importantly, the AHR exhibited a predominantly nuclear location after 30 to 60 min of GA treatment (Fig. 2, parts 9, 10, 13, and 14), and remained nuclear but was significantly reduced after the 2- and 3-h time points (Fig. 2, parts 17, 18, 21, and 22). In addition, the GA-mediated degradation of AHR (loss of staining), was inhibited after pretreatment of cells with MG-132 and the AHR remained predominantly nuclear at all time points evaluated (Fig. 2, parts 11, 12, 15, 16, 19, 20, 23, and 24). Thus, these results show that the treatment of cells with either GA or MG-132 results in translocation of the endogenous AHR to the nucleus in the absence of TCDD. Therefore, the GA-mediated degradation of the AHR may require nuclear localization.

Recent studies suggest that the AHR translocated into the nucleus after treatment of primary embryo fibroblasts with MG-132 can form functional complexes with the ARNT pro-

tein and induce CYP1A2 transcription (Santiago-Josefat et al., 2001). Because both MG132 and GA induced the nuclear translocation of the AHR in the Hepa-1 cell line, several studies were initiated to assess the induction of endogenous CYP1A1 protein by these compounds. In the first study, Hepa-1 cells were treated with MG-132, TCDD, GA, or pretreated with MG-132 for 2 h followed by exposure to GA or TCDD. At the end of each treatment, total cell lysates were prepared and the levels of AHR and CPY1A1 protein were evaluated by Western blotting. A representative experiment is shown in Fig. 3A. Consistent with the data in Fig. 1, treatment with GA or TCDD resulted in greatly reduced levels of endogenous AHR that was inhibited by treatment with MG-132. However, despite the fact that GA or MG-132 induced nuclear localization of the AHR, the only treatment regimens that resulted in the induction of CYP1A1 were those containing TCDD. Thus, both GA and MG-132 failed to induce endogenous CYP1A1 protein in Hepa-1 along a time frame similar to that of TCDD.

To confirm these findings and account for the possibility that GA or MG-132 was affecting the endogenous CYP1A1 protein, the effects of GA and MG-132 were evaluated in a Hepa-1 cell line (H1L1.1c2) containing a stably integrated luciferase reporter under control of a 434-base pair fragment of the murine CYP1A1 promoter (Garrison et al., 1996). H1L1.1c2 cells were incubated with GA, TCDD, or MG-132 for 1 to 8 h and the level of luciferase activity measured in cell lysates. A representative experiment is shown in Fig. 3B. The results show that treatment with TCDD results in a 10-fold induction of luciferase activity at the 6-h time point, whereas treatment with GA or MG-132 fails to significantly induce luciferase activity above background levels at any of the time

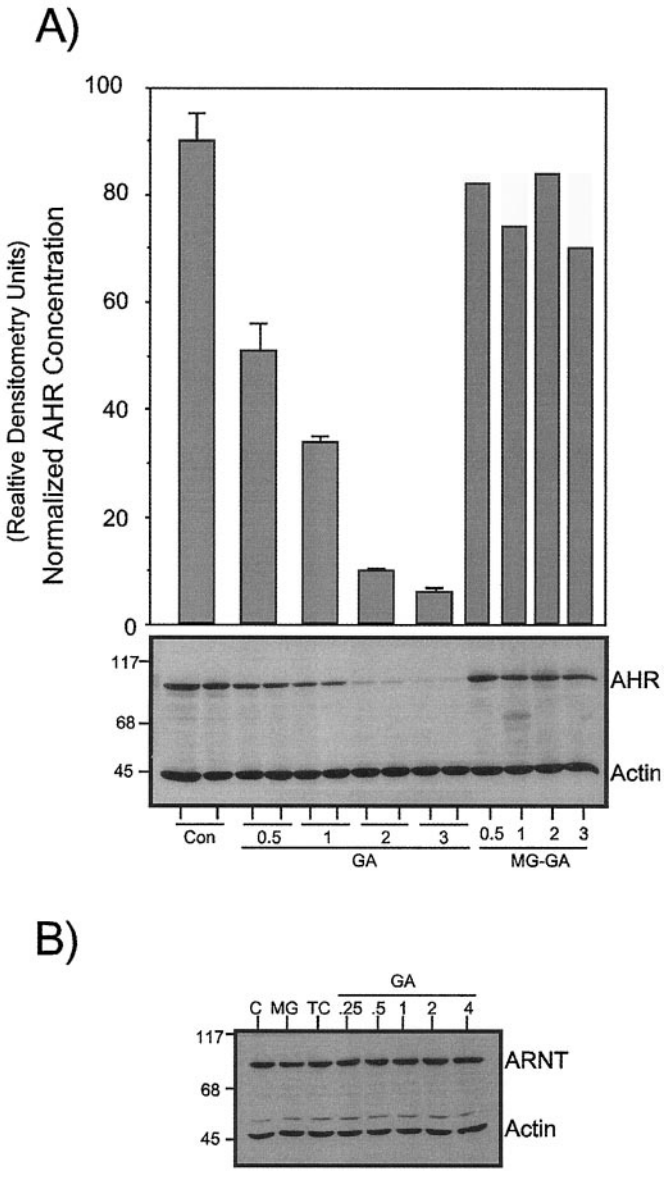

Fig. 1. Western blot analysis of AHR and ARNT protein in Hepa-1 cells exposed to GA. Duplicate plates of Hepa-1 cells were exposed to 0.1% DMSO (Con) or MG-132 (10 μM) for 2 h at 37°C and then exposed to GA (100 nM) for 0.5, 1, 2, or 3 h. Cell pellets were then sonicated in lysis buffer total cell lysates resolved by SDS-PAGE. Gels were then blotted and stained with A-1A IgG (1.0 μg/ml) or R-1 IgG (1.0 μg/ml) and actin IgG (1:1,000) and visualized by ECL with GAR-HRP IgG (1:12,000). A, analysis of AHR. Numbers below blot correspond to house of GA exposure. The relative expression of AHR was quantified by densitometry and normalized to the level of actin. The bars represent the average ± S.E. of two independent samples. GA, cells exposed to GA alone. MG-GA cells exposed to MG-132 for 2 h before GA. B, analysis of ARNT. Numbers above blot correspond to hours of GA exposure. C, cells exposed to DMSO (0.1%) for 3 h; MG, cells exposed to MG-132 (10 μM) for 3 h; TC, cells exposed to TCDD (2 nM) for 3 h.

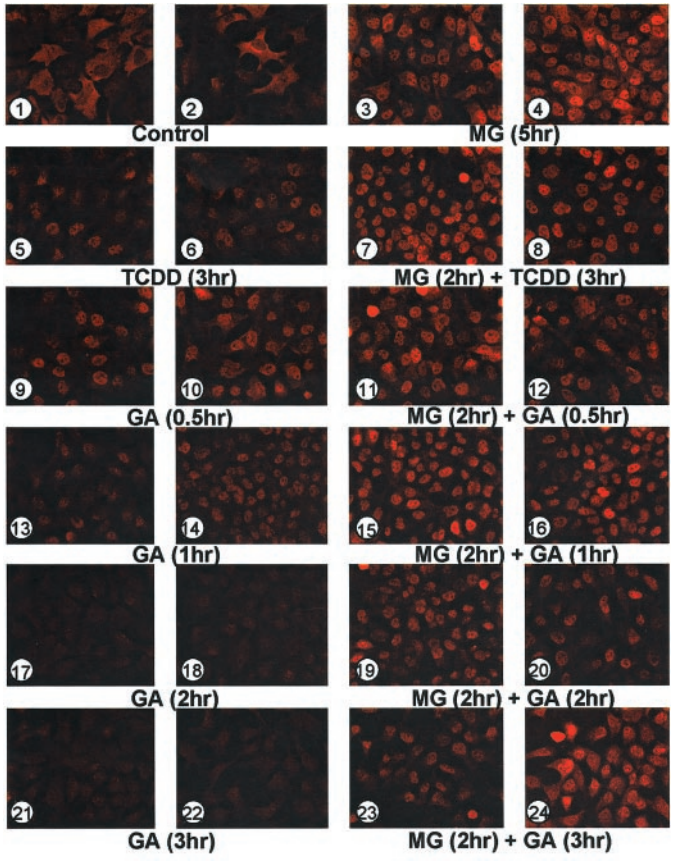

Fig. 2. Immunofluorescence microscopy of mAHR protein expression in Hepa-1 cells. Hepa-1 cells were grown on glass coverslips and exposed to DMSO (0.1%), MG-132 (10 μM), TCDD (2 nM), GA (100 nM) or combinations of these compounds for the indicated times. After exposure, cells were fixed and stained with 2.0 μg/ml A-1 IgG followed by GAR-RHO (1:400). All cells were photographed with a digital camera with the identical exposure. Each pair of represents two fields from the same coverslip. The specificity of the A-1 antibody is demonstrated by the lack of staining in cells treated with GA for 3 h (21–22) and has been shown previously (Pollenz et al., 1994; Pollenz, 1996; Holmes and Pollenz, 1997).

points. Thus, these results are consistent with the data presented in Fig. 3A but are in contrast to studies that have observed induction of CYP1A2 by MG-132 in mouse embryo fibroblast (MEF) primary cell cultures (Santiago-Josefat et al., 2001). The differences in the studies could result from the use of MEF primary cells, the analysis of CYP1A2, or the duration of incubation with MG-132. This is a key point because previous studies have noted that long-term incubation with MG-132 (>8 h) results in rounding of cells from the culture dish (Davarinos and Pollenz, 1999). Because changes in *CYP1A1* gene induction have been observed in cells that are detached from plates (Sadek and Allen-Hoffmann, 1994), it is possible that some of the changes in CYP1A2 induction may be related to changes in cell morphology as well. Nevertheless, the results presented here clearly show that GA or MG-132 does not significantly induce endogenous CYP1A1 in either Hepa-1 or HepG2 cells.

Exposure of Hepa-1 cells to GA and TCDD Results in Induction of Endogenous CYP1A1 Protein That Is Reduced in Magnitude. Because GA binds to hsp90 and can disrupt protein-protein interactions, it has been used to assess numerous receptor systems that are associated with hsp90 (Whitesell et al., 1994; Schulte et al., 1995; Grenert et al., 1997). For the AHR signal transduction pathway, recent studies suggest that GA treatment results in disruption of the AHR-hsp90-p23 complex and inhibits TCDD-mediated nuclear localization of the AHR, the formation of AHR-ARNT complexes, and the subsequent AHR-mediated gene induction (Kazlauskas et al., 2001). Because the data presented in Fig. 2 show that treatment of cells with GA does not inhibit the ability of the AHR to be translocated to the nucleus, it was pertinent to assess whether GA-treated cells were responsive to TCDD. For these studies, Hepa-1 cells were exposed to GA or DMSO for 1 h. After this initial exposure, the culture media were removed from all plates and replaced with fresh media that had been equilibrated with CO_2. Cells were then treated with TCDD for an additional 6 h and the level of CYP1A1 protein evaluated by Western blotting. A representative experiment is shown in Fig. 4A. Consistent with Fig. 3, cells treated with GA alone did not induce CYP1A1 to detectable levels. In contrast, cells pretreated with GA before TCDD exposure induced endogenous CYP1A1 protein to levels that were approximately 60% of levels induced in cells treated with TCDD alone. Thus, treatment of cells with GA did not block the AHR-mediated signaling pathway, but resulted in a reduction in the maximal level of CYP1A1 protein that was induced.

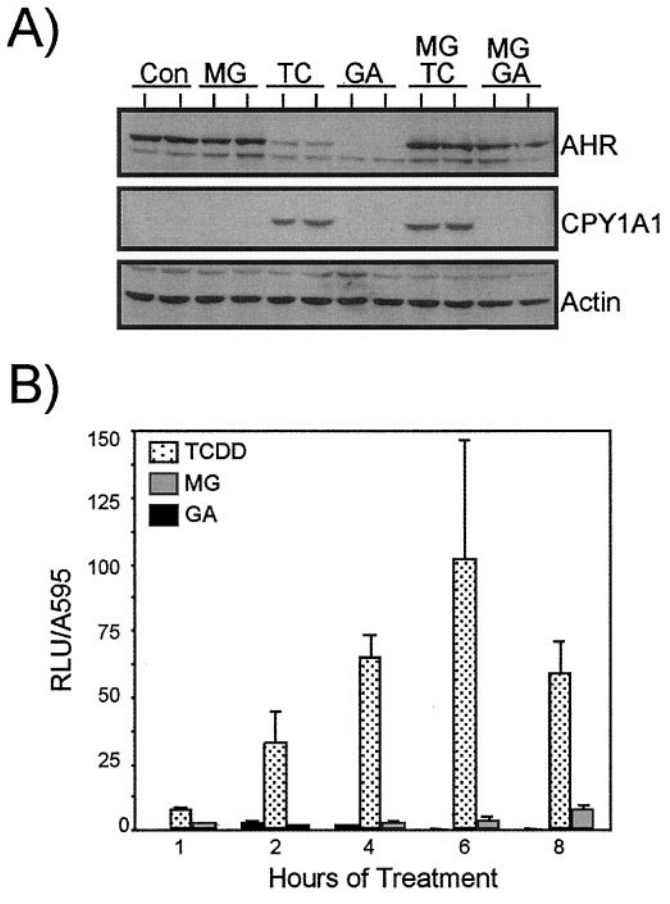

Fig. 3. Western blot analysis of gene induction in Hepa-1 cells exposed to GA and MG-132. A, duplicate plates of Hepa-1 cells were exposed to DMSO (0.1%) for 8 h (Con); MG-132 (10 μM) for 8 h (MG); TCDD (2 nM) for 6 h (TC); GA (100 nM) for 6 h (GA); MG-132 for 2 h followed by TCDD (2 nM) for 6 h (MG/TC); or MG-132 for 2 h followed by GA (100 nM) for 6 h. Cell pellets were then sonicated in lysis buffer and the total cell lysates resolved by SDS-PAGE. Gels were then blotted and stained with A-1A IgG (1.0 μg/ml), anti-P4501A1 (1:1,000), or actin IgG (1:1,000) and visualized by ECL with GAR-HRP IgG (1:12,000). B, H1L1.1c2 cells were incubated with GA (100 nM), TCDD (2 nM), or MG-132 (10 μM) for 1 to 8 h and the level of luciferase activity measured in cell lysates. Luciferase activity was normalized to total cellular protein. The bars represent the average ± S.E. of six independent samples.

To gain insight into the induction of CYP1A1 in the presence of GA and TCDD, studies next focused on the formation of AHR-ARNT dimers. For these studies, AHR and ARNT proteins were produced in vitro, mixed together, and incubated with increasing amounts of GA (100–400 nM) for 1 h at 30°C. The mixture was then incubated with TCDD for an additional 2 h at 30°C and subjected to EMSA as detailed. The results show that specifically shifted AHR-ARNT complexes are detected in all samples activated in the presence of TCDD (Fig. 4B). Importantly, the presence of GA did not inhibit the formation of the AHR-ARNT complex after TCDD binding, even when the concentration of GA was 4-fold higher than that used in the cell culture studies. Thus, these results are consistent with the ability of TCDD to induce endogenous CYP1A1 in Hepa-1 cells exposed to GA. These results are in sharp contrast to recent studies of Kazlauskas et al. (2001). However, it is important to note that in these studies the investigators carried out the in vitro transcription and translation reactions *in the presence* of GA. Thus, this type of experiment represents the analysis of *newly* synthesized AHR and is distinct from the analysis of the mature AHR-hsp90 complexes being evaluated here (see further discussion under *Conclusions*).

Exposure of HepG2 Cells to GA Results in Degradation and Nuclear Accumulation of the Endogenous AHR. Because the mouse and human AHR differ in size and stability, it was pertinent to assess whether the effects of GA observed on Hepa-1 cells were also observed in the human HepG2 cell line. For these studies, HepG-2 cells were treated with MG-132 or DMSO for 2 h followed by GA for 0.5 to 3 h. Total cell lysates were then evaluated for AHR expression by Western blotting. A representative experiment is shown in Fig. 5. The results show that AHR protein levels are reduced by greater than 95% after 3 h of GA exposure and the loss of AHR protein is inhibited by treatment of cells with MG-132

(Fig. 5A). Thus, these results are consistent with those observed in the Hepa-1 cell line (Fig. 1A). To determine whether GA caused the AHR to localize to the nucleus, HepG2 cells were treated with GA or TCDD and then fixed and stained for the endogenous AHR. A representative experiment is shown in Fig. 5B. The distribution of the AHR in untreated HepG2 cells was generally cytoplasmic, although the nuclear compartment exhibited some staining above background levels (Fig. 5B, 1). However, after 1 h of TCDD exposure, the AHR localized predominantly to the nuclear compartment and cytoplasmic staining was reduced. Importantly, the AHR also exhibited a predominantly nuclear distribution after 30 min of GA treatment (Fig. 5, parts 4 and 5), and remained nuclear but was significantly reduced after 2 h of GA exposure (Fig. 5, part 6). In addition, the loss of AHR staining (AHR degradation) was inhibited after pretreatment of cells with MG-132 and remained nuclear (data not shown). Thus, these results are consistent with those observed in Hepa-1 cells and show that GA exposure results in translocation of the endogenous AHR to the nucleus in 2 different cell lines from distinct species.

Exposure of HepG2 Cells to GA and TCDD Results in Induction of Endogenous CYP1A1 Protein That Is Reduced in Magnitude. It was next important to assess whether GA affected TCDD-mediated gene regulation in the HepG2 cell line. For these studies, HepG2 cells were exposed to GA or DMSO for 1 h. After this initial exposure, the culture media were removed from all plates and replaced with fresh media that had been equilibrated with CO_2. Cells were then treated with TCDD for an additional 6 h and the level of CYP1A1 protein evaluated by Western blotting. A representative experiment is shown in Fig. 6. As demonstrated in the Hepa-1 cell line, GA or TCDD treatment resulted in the induction of CYP1A1 protein, although the overall level of induction in GA-treated cells was reduced by approximately 30% compared with cells treated with TCDD alone. Thus, these results are consistent with those observed in the Hepa-1 cell line.

Because the treatment of both Hepa-1 and HepG2 cells with GA seemed to affect the ability of TCDD to induce CYP1A1 at saturating levels of TCDD (2 nM), it was pertinent to carry out a dose-response experiment to observe

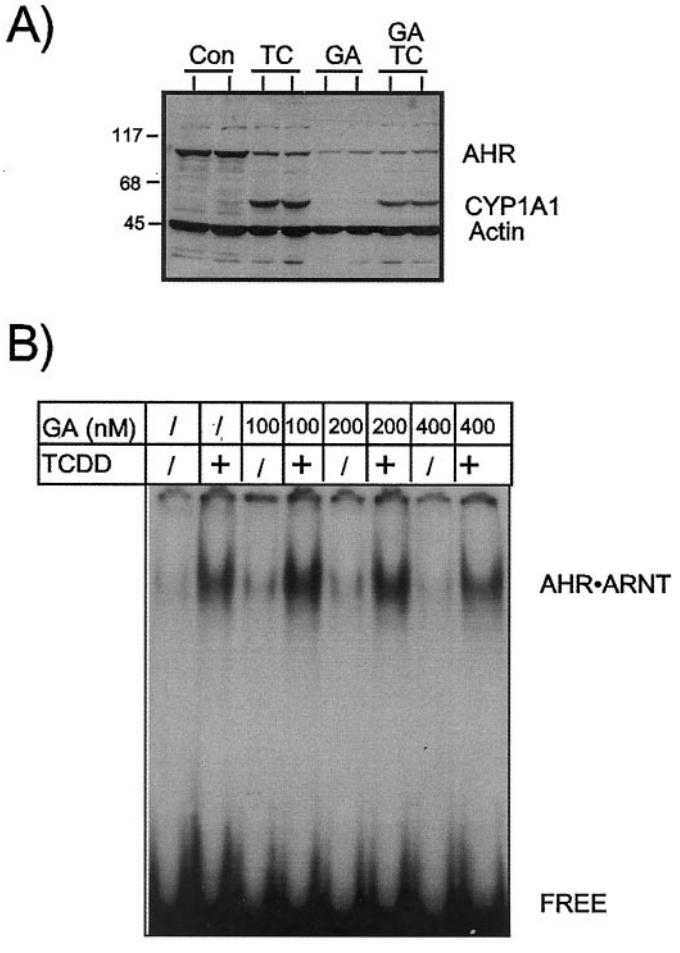

Fig. 4. Affect of GA on TCDD-mediated gene induction in Hepa-1 cells. A, duplicate plates of Hepa-1 cells were exposed to GA (100 nM) or DMSO (0.1%) for 1 h. After the initial exposure, the culture media was removed from all plates and replaced with fresh media. Cultures were then incubated for an additional 6 h (Con and GA) or treated with 2 nM TCDD for 6 h (TC or GA/TC). Cell pellets were then sonicated in lysis buffer and the total cell lysates resolved by SDS-PAGE. Gels were then blotted and stained with A-1A IgG (1.0 μg/ml), anti-P4501A1 (1:1,000), or actin IgG (1:1,000) and visualized by ECL with GAR-HRP IgG (1:12,000). B, EMSA. Approximately 25 ng of in vitro-translated AHR and ARNT protein were combined in 60 μl of MENG buffer and incubated in the presence of DMSO (0.5%) or GA (100, 200, or 400 nM) for 1 h at 30°C. The samples were then incubated in the presence of TCDD (10 nM) for and additional 2 h at 30°C and analyzed by EMSA as detailed under *Materials and Methods*. The specifically shifted AHR-ARNT complex is indicated and could be inhibited by incubation with A-1A or R-1 antibodies (data not shown).

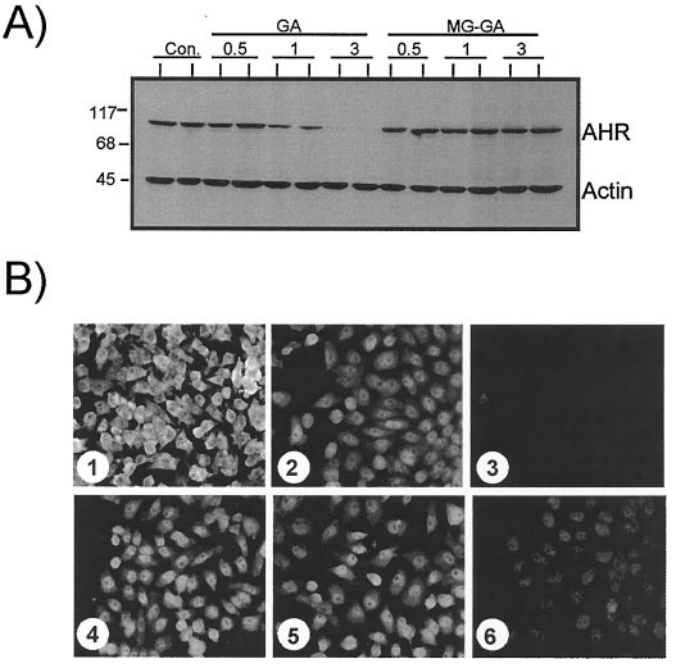

Fig. 5. Affect of GA on AHR protein in HepG2 cells. A, duplicate plates of HepG2 cells were exposed to 0.1% DMSO (Con) or MG-132 (10 μM) for 2 h at 37°C and then exposed to GA (100 nM) for 0.5, 1, or 3 h. Cell pellets were then sonicated in lysis buffer and total cell lysates were resolved by SDS-PAGE. Gels were blotted and stained with A-1A IgG (1.0 μg/ml) and actin IgG (1:1,000) and visualized by ECL with GAR-HRP IgG (1:12,000). GA, cells exposed to GA alone. MG-GA cells exposed to MG-132 for 2 h before GA. B, HepG2 cells were grown on glass coverslips and exposed to DMSO (0.1%) for 3 h (1); TCDD (2 nM) for 1 h (2); or GA (100 nM) for 30 min (4,5) or 3 h (6). After exposure, cells were fixed and stained with A-1 IgG (2.0 μg/ml) followed by GAR-RHO (1:400). 3 represents staining of control treated cells with preimmune IgG (2.0 μg/ml) and GAR-RHO (1:400). All cells were photographed with a digital camera with the identical exposure.

induction at limiting concentrations of TCDD. For these studies, HepG2 cells were exposed to GA or DMSO for 1 h. After this initial exposure, the culture media were removed from all plates and replaced with fresh media that had been equilibrated with CO_2. Cells were then treated with TCDD (10, 40, or 200 pM) for an additional 6 h and the level of CYP1A1 protein evaluated by Western blotting. A representative experiment is shown in Fig. 6, B and C. The results show that cells treated with TCDD alone induce endogenous CYP1A1 in a dose-dependent manner. In contrast, cells treated with GA for 1 h before treatment with TCDD do not induce significant levels of CYP1A1 at the 10 or 40 pM concentrations and show minimal levels of CYP1A1 after exposure to 200 nM TCDD. It is also important to note that the cells treated with GA show an 80 to 90% reduction in the level of AHR protein compared with cells that were not exposed to GA. Thus, exposure of cells to GA seems to reduce the sensitivity of the cells to limiting concentrations of TCDD.

To gain further insight into this finding, a complete dose-response experiment for TCDD was carried out in the presence or absence of GA. To facilitate the analysis of AHR-mediated gene induction, HepG2 cells were transfected with a TCDD-responsive reporter construct (pGudLuc 1.1) and allowed to recover for 16 h. Cells were then treated with GA for 1 h, the media replaced as described above and the cells treated with graded concentrations of TCDD for 6 h. Luciferase activity was measured and normalized to β-galactosidase as detailed and a representative experiment is shown in Fig. 7. In cells treated with TCDD alone, luciferase activity increased in a dose-dependent manner with an EC_{50} of 100 ± 30 pM from three independent experiments. In contrast, the dose-response curve for TCDD in GA-treated cells seemed to be shifted to the right (EC_{50}, 250 ± 80 pM) and was reduced in magnitude by approximately 50% compared with cells exposed to TCDD alone. Importantly, cells treated with GA alone did not induce luciferase activity compared with control treated cells. Because previous studies have shown that GA does not affect the TCDD-mediated formation of AHR-ARNT

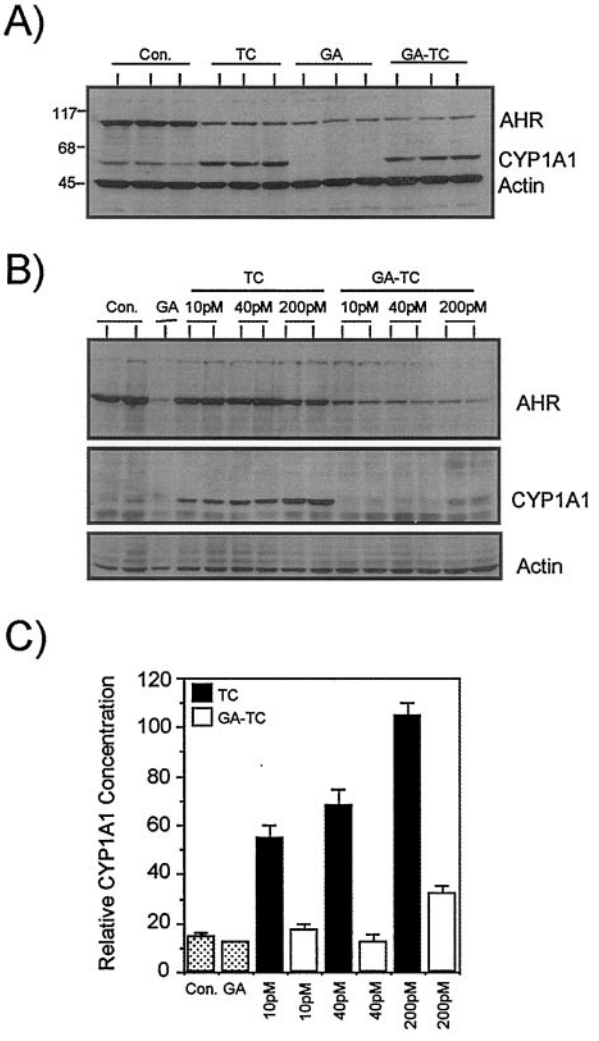

Fig. 6. Affect of GA on TCDD-mediated gene regulation of HepG2 cells. A, triplicate plates of HepG2 cells were exposed to GA (100 nM) or DMSO (0.1%) for 1 h. After the initial exposure, the culture media were removed from all plates and replaced with fresh media. Cultures were then incubated for an additional 6 h (Con and GA) or treated with 2 nM TCDD for 6 h (TC or GA/TC). Cell pellets were then sonicated in lysis buffer and the total cell lysates were resolved by SDS-PAGE. Gels were then blotted and stained with A-1A IgG (1.0 μg/ml), anti-P4501A1 (1:1000), or actin IgG (1:1,000) and visualized by ECL with GAR-HRP IgG (1:12,000). B, triplicate plates of HepG2 cells were exposed to GA (100 nM) or DMSO (0.1%) for 1 h. After the initial exposure, the culture media was removed from all plates and replaced with fresh media. Cultures were then incubated an additional 6 h (Con and GA) or treated with TCDD (10, 40, 200 pM) for 6 h (TC or GA-TC). Cell pellets were then sonicated in lysis buffer and the total cell lysates resolved and stained as indicated in A. C, the relative expression of the CYP1A1 in blot in B was quantified as detailed under *Materials and Methods*. The bars represents the average ± S.E. of two independent samples, except for GA.

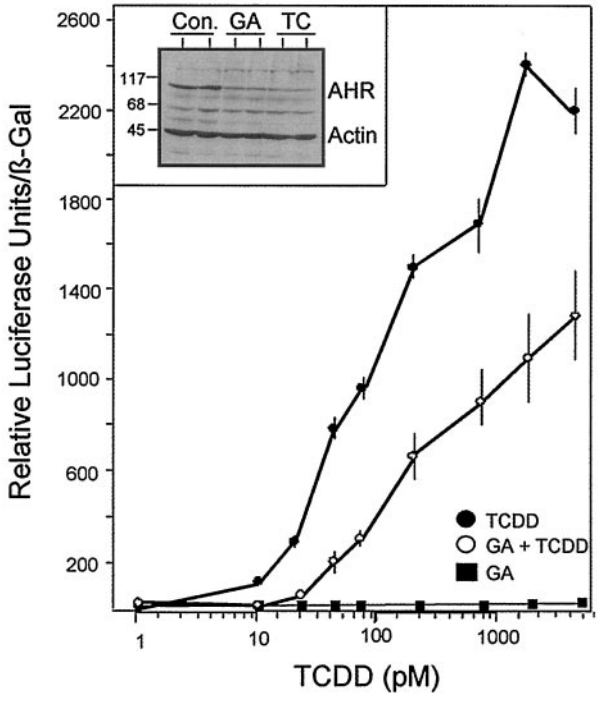

Fig. 7. Dose-response of TCDD in the presence of GA. HepG2 cells were cotransfected with pGudLuc 1.1 and pSV-β-galactosidase. 24 h after transfection, triplicate plates of cells were exposed to GA (100 nM) or DMSO (0.1%) for 1 h, the culture media were removed, and cells were provided fresh media. The cells were then exposed to TCDD (1, 10, 30, 80, 300, 800, 2500, 6000 pM) for an additional 7 h. Cells were harvested into reporter lysis buffer and assayed for luciferase and β-galactosidase activity. Data are expressed as the mean ± S.E. of three independent samples. EC_{50} values were calculated by nonlinear regression as detailed under *Materials and Methods*. Inset, Western blot of AHR protein in transfected cells exposed to GA (100 nM) or TCDD (2 nM) for 7 h.

complexes, or the DNA binding of these complexes at saturating levels of TCDD (Fig. 4), these studies support the hypothesis that the reduced magnitude of gene induction in the presence of GA is the result of the loss of AHR protein caused by proteolytic degradation and a reduced affinity of the AHR for TCDD. This hypothesis is consistent with studies that have observed reduced levels of CYP1A1 induction in cells after down-regulation of the AHR after long-term exposure to TCDD (Pollenz et al., 1998) and the observation that Hepa-1 cell lines expressing low levels of AHR have reduced levels of TCDD-inducible CYP1A1 (Legraverend et al., 1982; Israel and Whitlock, 1984). However, although GA does not bind to the AHR or ARNT, it is also possible that GA is affecting the *trans*-activation potential of the remaining AHR-ARNT complexes by an indirect mechanism.

AHR Is Degraded in the Nuclear Compartment of HepG2 Cells after Exposure to GA. Because treatment of both Hepa-1 and HepG2 cells with GA resulted in dramatic translocation of the AHR to the nucleus that seems to precede the degradation event (Figs. 1, 2, and 5), studies next focused on the subcellular location of the GA-mediated degradation of the AHR. Previous studies have shown that the TCDD-mediated degradation of the AHR can be inhibited by LMB in HepG2 cells (Davarinos and Pollenz, 1999). To determine whether blocking nuclear export could inhibit GA-mediated degradation, HepG2 cells were incubated with LMB for 2 h and then exposed to GA or TCDD for an additional 1 to 4 h. Total cell lysates were then evaluated for AHR and ARNT protein by Western blotting and a representative experiment is shown in Fig. 8.

The results show that LMB treatment alone does not affect the concentration of AHR or ARNT protein (Fig. 8). The results also show that LMB can block the TCDD-mediated degradation of the AHR in the HepG2 cell line. These results are consistent with previous reports in HepG2 cells (Davarinos and Pollenz, 1999; Pollenz and Barbour, 2000) and serve as a positive control for the integrity of the LMB and its ability to block the export of the nuclear AHR. When identical studies were done in the presence of GA however, AHR levels were significantly reduced after 1 and 2 h of GA treatment whether cells were treated with LMB or not. Similar results were observed when the concentration of LMB was

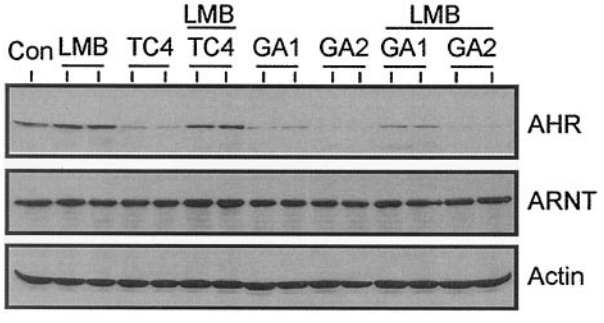

Fig. 8. Effect of LMB on GA-mediated degradation of the AHR. Duplicate plates of HepG2 cells were exposed to LMB (10 nM) or DMSO (0.1%) for 2 h. After the initial exposure, the control and LMB-treated cells were incubated for an additional 4 h (Con, LMB), treated with 2 nM TCDD for 4 h (TC4, LMB/TC4) or treated with GA (100 nM) for 1 h (GA1, LMB/GA-1) or 2 h (GA2, LMB/GA-1). Gels were then blotted and stained with A-1A IgG (1.0 μg/ml) or R-1 IgG (1.0 μg/ml) and actin IgG (1:1,000) and visualized by ECL with GAR-HRP IgG (1:12,000). Note that LMB blocks the TCDD-mediated degradation of the AHR but not the GA-mediated degradation of the AHR.

increased to 50 nM (data not shown). Thus, treatment of HepG2 cells with LMB did not block the GA-mediated degradation of the AHR. Because the AHR would be nuclear after 30 min of GA treatment (Fig. 5), these results imply that the GA-mediated degradation of the AHR is occurring in the nucleus of HepG2 cell line. Because these results are in sharp contrast to those observed with TCDD-treated cells (Davarinos and Pollenz, 1999; Pollenz and Barbour, 2000), the data suggests that although the AHR 26S proteasome seems to degrade the AHR after either TCDD or GA exposure, the subcellular location of the degradation events may be distinct.

Role of ARNT Protein Concentration in the Subcellular Localization of the Unliganded AHR. It is curious that the AHR becomes nuclear after exposure to GA, TCDD, and MG-132. Each of these compounds affects a different aspect of the AHR signaling pathway and each treatment results in a distinct endpoint such as gene induction, degradation or stability. The mechanism behind the MG-132–mediated nuclear translocation of the AHR is currently unclear. However, recent studies carried out in primary MEFs suggest that treatment of cells with MG-132 results in a 2- to 3-fold increase in the level of ARNT protein because of increased transcription of the *ARNT* gene (Santiago-Josefat et al., 2001). Therefore, it has been proposed that increased levels of nuclear ARNT protein affect the cellular location of the AHR. Although this is a viable hypothesis, it is important to note that for ARNT protein concentration to have a direct effect on AHR localization, the timing of the nuclear translocation of the AHR and the increase in ARNT protein levels must correlate. To begin to address this issue, Hepa-1 or HepG2 cells were treated with MG-132 for 0 to 8 h and the level of ARNT protein determined by Western blotting. A representative experiment is shown in Fig. 9A. The data show that the level of ARNT protein remains essentially constant at all the time points evaluated (Fig. 9A). Identical results were observed in the HepG2 cell line (data not shown). Therefore, MG-132 did not affect the level of ARNT protein during the time that the AHR became localized to the nucleus (2–4 h). It should also be noted that analysis of ARNT protein expression by immunohistochemical techniques showed that ARNT remained nuclear under all treatment conditions (data not shown).

To directly assess the relationship between the level of ARNT protein and AHR localization, a construct expressing an ARNT-GFP fusion protein was generated. Analysis of the ARNT-GFP protein in in vitro assays indicated that the protein was capable of binding to AHR and associating with DNA (data not shown). To evaluate the effect of ARNT-GFP expression on the localization of endogenous AHR, Hepa-1 or HepG2 cells were transfected with the ARNT-GFP expression vector, allowed to express the protein for 24 h and then fixed and stained for the endogenous AHR with or without exposure to MG-132. A representative experiment in the Hepa-1 cell line is shown in Fig. 9B.

Several points are important. First, if changes in ARNT protein are causing the AHR to localize to the nucleus to the same degree as treatment with MG-132, then there should be a strong shift in endogenous AHR location in cells expressing ARNT-GFP. Second, if such a shift in AHR location is observed, merging of the ARNT-GFP (green) and AHR staining (red) would show a yellow/orange color in the compartment

where the AHR and ARNT-GFP colocalize. In Fig. 9B, parts 1 and 4, the location of the endogenous AHR is consistent with previous studies in Hepa-1 cells, in that the staining is predominantly cytoplasmic. Cells expressing ARNT-GFP were observed throughout the culture and GFP was localized exclusively within the nucleus (Fig. 9B, parts 2, 5, and 8). The nuclear localization of the ARNT-GFP protein is consistent with the location of endogenous ARNT in cells and tissues (Sojka et al., 2000; Sojka and Pollenz, 2001). Importantly, there is no change in the distribution of the AHR in

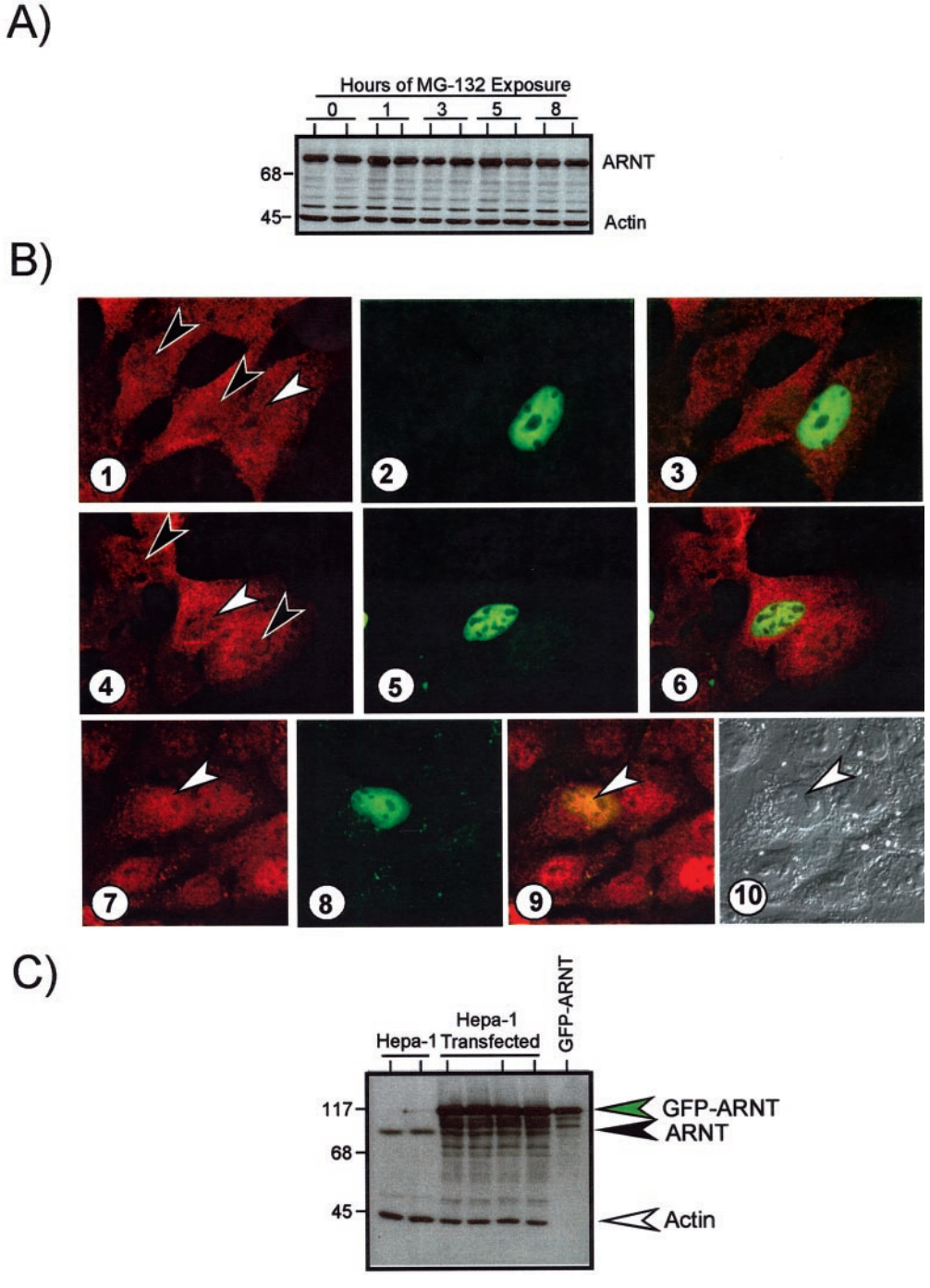

Fig. 9. Affect of ARNT protein concentration on AHR localization and stability. A, Hepa-1 cells were incubated with MG-132 (10 μM) for 1 to 8 h. Cell pellets were then sonicated in lysis buffer and total cell lysates resolved by SDS-PAGE. Gels were blotted and stained with R-1 IgG (1.0 μg/ml) and actin IgG (1:1,000) and visualized by ECL with GAR-HRP IgG (1:12,000). B, Hepa-1 cells were transfected with pmARNT-GFP and allowed to recover for 24 h. Cells were treated with Mg-132 (10 μM) or DMSO for 4 h and fixed and stained with 2.0 μg/ml A-1 IgG followed by GAR-RHO (1:400). 1, 3 and 7, staining for AHR. Black arrow, nucleus of cells that are not expressing ARNT-GFP; white arrow, nucleus of cell that is expressing mARNT-GFP. 2, 5 and 8, expression of ARNT-GFP. 3, 6 and 9, merge of AHR staining and ARNT-GFP. 10, Normaski image of cells shown in 7 to 9 to visualize the nucleus. C, duplicates of the cells described in B. Hepa-1 cells were transfected with pmARNT-GFP and allowed to recover for 24 h. Cell pellets were then sonicated in lysis buffer and total cell lysates resolved by SDS-PAGE. Gels were blotted and stained with R-1 IgG (1.0 μg/ml) and actin IgG (1:1,000) and visualized by ECL with GAR-HRP IgG (1:12,000). Hepa-1, nontransfected Hepa-1 cells; ARNT-GFP, sample of ARNT-GFP produced by in vitro transcription and translation.

the cells expressing ARNT-GFP and the merged image shows only red and green, indicating very little colocalization of these proteins. As a positive control for AHR and ARNT colocalization in the nucleus, ARNT-GFP transfected cells were also treated with MG-132 for 4 h before fixation (Fig. 9B, parts 7–10). The dramatic redistribution of the AHR to the nucleus can be clearly observed (compare parts 1, 4, and 7; also see Fig. 2), and when the AHR and ARNT-GFP images are merged the colocalization is represnted by a yellow/orange nucleus.

To obtain an estimate of the level of GFP-ARNT protein in the transfected cells, plates of cells were harvested and evaluated by Western blotting. A representative blot is shown in Fig. 9C. The results show that the GFP-ARNT could be detected in the transfected cells and was in excess of the endogenous ARNT by approximately 10-fold. Therefore, because the transfection efficiency of the population was approximately 20% (determined by counting the cells expressing GFP in numerous fields of view), then the overall level of GFP-ARNT protein in each transfected cell was approximately 50-fold higher than the endogenous ARNT. Thus, these results, which suggest that ARNT does not play a significant role in the localization of the AHR in the absence of ligand stimulation, make it unlikely that MG-132 affects AHR localization by such a mechanism.

Conclusions and Implications

Receptor-mediated signal transduction pathways involve numerous protein-protein interactions that seem to be dependent on the affinity of the various proteins for each other and their level of expression (reviewed in Pahl and Baeuerle, 1996). Therefore, changes in protein concentrations or subcellular locations can impact a given signaling pathway in a very significant manner. For the AHR-mediated signaling pathway, the issues of subcellular location and ligand-mediated degradation of the AHR have been a growing area of interest, especially as they relate to changes in gene regulation. Thus, exposure of cells to GA or MG-132 provides a model to evaluate the correlation between AHR location, concentration and response.

One of the key findings of the current study is that GA or MG-132 treatment of Hepa-1 and HepG2 cells results in the nuclear accumulation of the endogenous AHR without induction of reporter genes or endogenous CYP1A1. Because the AHR is not actively shuttling between the cytoplasm and nucleus in these cell lines (Davarinos and Pollenz, 1999; Pollenz and Barbour, 2000), the nuclear accumulation cannot be related to a block in nuclear export. Thus, these results show that nuclear localization alone is not sufficient to induce reporter genes or the endogenous CYP1A1 without the addition of TCDD. However, treatment of both Hepa-1 and HepG2 cells with GA before exposure to TCDD reduced the magnitude of CYP1A1 induction and shifted the dose-response curve to the right. There are several possibilities to explain these results. First, exposure to GA could affect ligand binding to the AHR. However, two independent studies have shown that GA does not affect ligand binding to the AHR in vitro and in vivo (Chen and Perdew, 1997; Kazlauskas et al., 2001). Second, GA could affect the dimerization of AHR and ARNT or the DNA binding of the AHR-ARNT complex. Results presented in Fig. 4B show that GA does not affect the ability of saturating levels of TCDD to induce AHR-ARNT complexes capable of binding DNA at concentrations of GA as high as 400 nM. Because activation of the AHR in this assay requires binding of TCDD, these results also show that GA is not impacting ligand binding to the AHR. Third, GA could affect the induction of CYP1A1 by inhibiting translocation of the liganded AHR to the nucleus, as suggested previously (Kazlauskas et al., 2001). However, the results presented in Figs. 2 and 5 clearly show that GA induces the translocation of the endogenous AHR to the nucleus in both human and mouse cells. Thus, it is likely that the reduced response to TCDD in the presence of GA is the result of degradation of the AHR and a resulting decrease in the number of AHRs that can mount a response. This is an important finding in that it highlights the importance of AHR protein concentration in the response of the cells to ligands and is consistent with the hypothesis that cells modulate AHR-mediated signaling in part via degradation of the AHR (Davarinos and Pollenz, 1999; Ma et al., 2000; Pollenz and Barbour, 2000). In addition, the fact that the AHR may be degraded in the nucleus after GA exposure suggests that the conformation of the AHR and its association with additional proteins (XAP, hsp90, etc.) may determine the subcellular compartment in which it is destroyed and might also affect the affinity of the ligand to the active site.

It is important to note that GA-mediated nuclear localization of the AHR observed in the current studies is in sharp contrast to the report of Kazlauskas et al. (2001), who hypothesized that GA blocks the nuclear translocation of the AHR by blocking the maturation of the AHR-hsp90-XAP2-p23 complex and subsequent binding to nuclear import receptors. Although some of their data are consistent with such a conclusion, it is important to consider the state of the AHR at the time of the analysis in their studies and whether they were studying a mature AHR multiprotein complex or an AHR in the process of forming the multiprotein complex. This is a critical point in the interpretation of the results because their studies dealing with AHR binding to nuclear import proteins were carried out in vitro on AHR protein that had been newly translated in the presence of GA. This means that as the AHR was being synthesized, it was being complexed with a modified hsp90 already bound to GA (Grenert et al., 1997). This would result in a modified conformation of the AHR-hsp90 complex that would probably affect the binding of accessory proteins such as XAP2 or p23 and affect subsequent interactions to import proteins. This is a much different scenario compared with the studies reported here, in which the endogenous AHR-hsp90-XAP2-p23 complexes are being evaluated in a culture cell over a 4- to 6-h period after GA exposure. Thus, because GA seems to cause the dissociation of the XAP2 from the mature AHR-hsp90-XAP2-p23 complex (Kazlauskas et al., 2001), and because it has been shown that XAP2 is critical in maintaining a stabilized AHR in a mulitprotein complex in the cytoplasm (Meyer et al., 1998; Meyer and Perdew, 1999; LaPres et al., 2000), it would be expected that dissociation of XAP2 from the core AHR-hsp90 complex would result in nuclear localization of the AHR in a ligand-independent manner. This is clearly the result observed in Figs. 2 and 5. Interestingly, the report of Kazlauskas et al. (2001) is also consistent with this view because the results indicate that a significant percentage of the AHR-GFP fusion protein translocated from the cytoplasm

to nucleus when cell cultures containing mature AHR complexes were treated with GA. Thus, GA seems to affect both the maturation and nuclear translocation of newly synthesized AHR by blocking the binding of XAP2 and p23 (Kazlauskas et al., 2001) but also results in the nuclear localization of the mature AHR complex and its degradation due to loss of the XAP2 protein. Future studies are aimed at determining the mechanism whereby the liganded and unliganded AHR are recognized by the 26S proteasome and the importance of AHR concentration in AHR-mediated signal transduction.

Acknowledgments

We thank Dr. Gary Perdew for helpful discussions of this work and Annette Hendrix for technical assistance on several experiments.

References

Chen H-S, Singh SS, and Perdew GH (1997) The Ah receptor is a sensitive target of geldanamycin-induced protein turnover *Arch. Biochem. Biophys.* **348:**190–198.

Davarinos NA and Pollenz RS (1999) Aryl hydrocarbon receptor imported into the nucleus following ligand binding is rapidly degraded via the cytoplasmic proteasome following nuclear export *J. Biol. Chem.* **274:**28707–28715.

Garrison PM, Tullis K, Aarts JM, Brouwer A, Giesy JP, and Denison MS (1996) Species specific recombinant cell lines as bioassay systems for the detection of TCDD-like chemicals. *Fund. Appl. Toxicol.* **30:**194–203.

Grenert JP, Sullivan WP, and Fadden P (1997) The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation. *J. Biol. Chem.* **272:**23843–23850.

Gu YZ, Hogenesch JB, and Bradfield CA (2000) The PAS superfamily: sensors of environmental and developmental signals, *Annu Rev Pharmacol Toxicol;* **40:**519–561.

Hahn ME (1998) The Ah receptor: a comparative perspective. *Comp. Biochem. Phys.* **121:**23–53.

Holmes J and Pollenz RS (1997) Determination of ARNT protein concentration and subcellular localization in hepatic and non-hepatic cell culture line. *Mol Pharmacol.* **52:**202–211.

Israel DI and Whitlock JP Jr (1984) Regulation of cytochrome P1–450 gene transcription by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin in wild type and variant mouse hepatoma cells. *J. Biol. Chem.* **259:**5400–5402.

Kazlauskas A, Poellinger L, and Pongratz I (2000) The immunophilin-like protein XAP2 regulates ubiquitination and subcellular localization of the dioxin receptor. *J. Biol. Chem.* **275:**41317–41324.

Kazlauskas A, Sundstrom S, Poellinger L, and Pongratz I (2001) The hsp90 chaperone complex regulates intracellular localization of the dioxin receptor. *Mol. Cell. Biol.* **21:**2594–2607.

LaPres JJ, E. Glover E, Dunham EE, Bunger MK and, Bradfield CA (2000) ARA9 modifies agonist signaling through an increase in cytosolic aryl hydrocarbon receptor, *J. Biol. Chem.* **275:**6153–6159.

Legraverend C, Hannah RR, Eisen HJ, Owens IS, Nebert DW, and Hankinson O (1982) Regulatory gene product of the Ah locus. *J. Biol. Chem.* **257:**6402–6407.

Ma Q and Baldwin KT (2000) 2, 3, 7, 8-tetrachloro-dibenzo-p-dioxin induced degradation of aryl hydrocarbon receptor (Ahr) by the ubiquitin-proteasome pathway *J. Biol. Chem.* **275:**8432–8438.

Ma Q, Renzelli AJ, Baldwin KT, and Antonini JM (2000) Superinduction of CYP1A1 gene expression, *J. Biol. Chem.* **275:**2676–12683.

Meyer BK and Perdew GH (1999) Characterization of the AHR-hsp90-XAP2 ore complex and the role of the immunophilin-related protein XAP2 in AhR stabilization, *Biochemistry* **38:**8907–8917.

Meyer BK, Petrulis JR, and Perdew GH (2000) Aryl hydrocarbon receptor levels are selectively modulated by the hsp90-associated immunophilin homolog XAP2 *Cell Stress & Chaperones* **5:**243–254.

Meyer BK, Pray-Grant MG, Vanden Heuval JP, and Perdew GH (1998) Hepatitis B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity *Mol Cell. Biol.* **18:**978–988.

Pahl HL and Baeuerle PA (1996) Control of gene expression by proteolysis *Cur. Opin. Cell Biol.* **8:**340–347.

Pollenz RS (1996) The Ah-receptor but not the Arnt protein is rapidly depleted in hepatic and non-hepatic culture cells exposed to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin. *Mol. Pharmacol.* **49:**391–398.

Pollenz RS and Barbour ER (2000) Analysis of the complex relationship between nuclear export and aryl hydrocarbon-mediated gene regulation *Mol. Cell Biol.* **20:**6095–6104.

Pollenz RS, Santostefano MJ, Klett E, Richardson VM, Necela B, and Birnbaum LS (1998), A single oral dose of TCDD results in sustained depletion of AHR protein in female Sprague-Dawley rats. *Toxicol. Sci.* **42:**117–128.

Pollenz RS, Sattler CA, and Poland A (1994) The aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein show distinct subcellular localizations in Hepa 1c1c7 cells by immunofluorescence microscopy. *Mol Pharmacol.* **45:**428–438.

Roman BL, Pollenz RS, and Peterson RE (1998) AHR and ARNT expression and CYP1A1 induction in the adult male rat reproductive tract. *Toxicol. Applied Phramacol.* **150:**228–239.

Sadek CM and Allen-Hoffmann BL (1994) Suspension-mediated induction of Hepa 1c1c7 Cyp1a-1 expression is dependent on the Ah receptor signal transduction pathway *J. Biol. Chem.* **269:**31505–31509.

Santiago-Josefat B, Pozo-guisada E, Mulero-navarro S and Fernandez-Salguero PM (2001) Proteasome inhibition induces nuclear translocation and transcriptional activation of the dioxin receptor in mouse primary fibroblasts in the absence of xenobiotics *Mol. Cell Biol.* **21:**1700–1709.

Schulte TW, Blagosklonny MV, Ingui C, and Neckers LM (1995) Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association, *J. Biol. Chem.* **261:**24585–24588.

Shen ES and Whitlock JP Jr (1992) Protein-DNA interactions at the dioxin responsive enhancer. *J. Biol. Chem.* **267:**6815–6819.

Sojka K, Kern C, and Pollenz RS (2000) Subcellular localization expression of ARNT protein in developing mouse and chicken. *The Anatomical Record* **260:**327–334.

Sojka K and Pollenz RS (2001) Expression and subcellular localization of aryl hydrocarbon receptor nuclear translocator (ARNT) isoforms in developing rainbow trout. *Marine Biotechnology* **3:**416–427.

Sommer RJ, Sojka K, Pollenz RS, Cooke P, and Peterson RE (1999) AHR and ARNT protein and mRNA concentrations in rat prostate: Effects of stage of development and TCDD. *Toxicol. Appl. Pharmacol.* **155:**77–189.

Whitesell L, Minmaugh EG, Costa BD, Meyers CE, and Neckers LM (1994) Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc Natl Acad Sci USA* **91:**8324–8328.

Whitlock JP (1999) Induction of cytochrome P4501A1, *Annu Rev. Pharmacol. Toxicol.* **39:**103–125.

Address correspondence to: Dr. Richard S. Pollenz, Department of Biology, SCA110, 4202 E. Fowler Ave, University of South Florida, Tampa, FL 33620. E-mail: pollenz@chuma1.cas.usf.edu