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Functional Analysis of Murine Aryl Hydrocarbon (AH) Receptors Defective in Nuclear Import: Impact on AH Receptor Degradation and Gene Regulation

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ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that is also a substrate for the 26S proteasome. However, the subcellular location of the degradation events or the requirement for nuclear transport has not been resolved. To gain insight into both ligand-dependent and independent degradation of the AHR, studies were designed to evaluate the relationship between AHR localization, stability, and gene regulation in a defined cell culture model system. The strategy of these studies was to generate stable cell lines expressing murine AHR proteins that were defective in nuclear import and then to assess the location of the AHR, the time course of AHR degradation, and the level of induction of endogenous CYP1A1 protein after exposure to 2,3,7,8-tetrachlorodibezo-*p*-dioxin (TCDD), geldanamycin (GA), or the protease inhibitor carbobenzoxy-L-leucyl-L-leucyl-leucinal (MG-132). Mutation within the putative nuclear localization sequence (NLS) resulted in AHR mutants that were severely defective in nuclear import as evaluated by immunocytochemical staining after exposure to TCDD, GA, or MG-132. Importantly, the NLS mutants exhibited identical levels of degradation along a similar time course as wild-type AHR after exposure to TCDD or GA when stably expressed in either murine hepatoma cells (Hepa-1) or hamster lung cells (E36). In contrast, the NLS mutants were severely defective in ligand-mediated induction of CYP1A1 expression. These findings imply that the proteolytic machinery present in the cytoplasmic compartment is sufficient to degrade the AHR and that nuclear translocation, binding with ARNT, or DNA binding are not necessary for efficient degradation of the AHR.

The aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor that is a member of the basic-helix-loop-helix (bHLH) periodicity/aryl hydrocarbon nuclear translocator (ARNT)/single-minded family of proteins. The current model of AHR-mediated signal transduction proposes that the AHR is activated by ligand, associates with the ARNT protein in the nucleus to modify gene regulation, and then becomes degraded (reviewed in Hahn, 1998; Whitlock, 1999; Gu et al., 2000). The mechanism whereby the AHR is degraded has been studied in a number of laboratories, and the consensus is that the liganded AHR is ubiquinated and then degraded by the 26S proteasome pathway (reviewed in Pollenz, 2002). The importance of controlling the level of activated AHR is underscored by the finding that blockage of degradation results in potentiation of gene induction (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000), and the finding that a constitutively active AHR results in reduced life span and stomach tumors in transgenic mice (Andersson et al., 2002). Thus, it is critical to determine the subcellular location of the degradation events; this will impact the mechanism by which the level of the AHR is regulated, will influence the types of protein-protein interactions that can occur in an active or latent state, and will define the types of ubiquitin ligases involved in the degradation process. From initial studies of the AHR signal transduction pathway, it was shown that ligand-mediated nuclear import preceded AHR degradation and thus it was proposed that degradation occurred within the nuclear compartment (Pollenz et al., 1994; Pollenz, 1996). However, it was later shown that ligand-mediated degradation of the AHR was completely inhibited by leptomycin B (LMB) and that the AHR remained predominately nuclear in the presence of LMB and TCDD (Davarinos and Pollenz, 1999). Importantly, the AHR·ARNT complexes detected in the nucleus of cells treated with LMB

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ABBREVIATIONS: AHR, aryl hydrocarbon receptor; bHLH, basic helix-loop-helix; ARNT, aryl hydrocarbon receptor nuclear translocator; LMB, leptomycin B; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; GA, geldanamycin; MG-132 carbobenzoxy-L-leucyl-L-leucyl-leucinal; hsp90, 90-kDa heat shock protein; Me_2SO, dimethyl sulfoxide; PBS, phosphate-buffered saline; TTBS, Tris-buffered saline with Tween 20; BLOTTO, bovine lacto transfer optimizer; GAR, goat anti-rabbit; HRP, horseradish peroxidase; RHO, rhodamine; NLS, nuclear localization signal; PAGE, polyacrylamide electrophoresis; ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility shift assay; XRE, xenobiotic response element; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's media; PCR, polymerase chain reaction; WT, wild-type.

and TCDD were capable of binding to XRE sequences in gel-shift assays. However, despite the high levels of AHR·ARNT dimers, LMB-treated cells expressed greatly reduced levels of CYP1A1 (Davarinos and Pollenz, 1999; Pollenz and Barbour, 2000). Because the AHR contains at least two putative nuclear export sequences (Ikuta et al., 1998; Davarinos and Pollenz, 1999; Pollenz and Barbour, 2000; Berg and Pongratz, 2001), these results suggest that either the AHR is exported from the nucleus to be degraded or that an inhibitor of AHR degradation is retained within the nuclear compartment in cells treated with LMB. To further assess this question, the degradation of the AHR was evaluated in cells transiently expressing AHR proteins defective in nuclear export. In these studies, it was shown that mutation of leucine 69 to alanine produced an AHR (termed AHR_{A69}) that dimerized with ARNT, bound DNA, and was functional in the induction of TCDD-responsive reporter genes (Pollenz and Barbour, 2000). However, the AHR_{A69} seemed to accumulate in the nucleus after ligand exposure and exhibited reduced levels of degradation. Thus, these studies suggest that either mutation of L69 directly impacts degradation of the AHR in the nucleus or a model in which the AHR is degraded within the cytoplasmic compartment after nuclear export. The later mechanism would allow proteolysis to occur within a cellular compartment distinct from the site of gene activation, as has been described for p53 (Haupt et al., 1997; Roth et al., 1998), $p27^{Kip}$ (Tomoda et al., 1999), and $p27^{Xic1}$ (Chuang and Yew, 2001).

Although the studies on the nuclear export of the AHR provide compelling results, there is growing evidence that the AHR may also be targeted for degradation within the nucleus or may require nuclear translocation for degradation to occur. First, treatment of numerous cell lines with geldanamycin (GA) results in rapid accumulation of the AHR in the nucleus in a ligand-independent manner and subsequent degradation (Chen et al., 1997; Meyer et al., 2000; Song and Pollenz, 2002). However, although the proteasome inhibitor MG-132 can block GA-induced degradation to the same degree as TCDD-mediated degradation, the GA-mediated degradation event is not inhibited by LMB (Song and Pollenz, 2002). Second, a constitutively nuclear AHR (termed DRNLS) has been shown to have a half-life of less than 2 h in the presence or absence of ligand even though degradation can be blocked by MG-132 or mutant ubiquitins (Roberts and Whitelaw, 1999). The interpretation of these results was that the DRNLS was being degraded by the 26S proteasome in the nuclear compartment in a ligand-independent manner, although the affect of LMB on the degradation was not evaluated. Finally, it has also been suggested that the AHR may require DNA binding before being targeted for degradation (Ma and Baldwin, 2000). Collectively, these studies suggest that the degradation of the AHR may occur in the nucleus or require shuttling through the nucleus before the degradation events. In addition, it is possible that nuclear degradation of the AHR may be caused by recognition of an AHR·hsp90·immunophlin core complex that is not in the correct conformation (such as the DRNLS or an AHR associated with hsp90 bound with GA). Importantly, degradation of the bHLH transcription factor MyoD has recently been shown to occur in the nucleus via the 26S proteasome pathway (Floyd et al., 2001). To gain additional insight into both ligand-dependent and independent degradation of the AHR, studies were designed to evaluate the relationship between AHR localization, stability, and gene regulation in a defined cell culture model system. The strategy of these studies was to generate stable cell lines expressing murine AHR proteins that were defective in nuclear import and then to assess the location of the AHR, the time course of AHR degradation and the level of induction of endogenous CYP1A1 after exposure to TCDD, GA, and MG-132.

Materials and Methods

Materials. TCDD (98% stated chemical purity) was obtained from Radian Corp. (Austin, TX) and was solubilized in dimethyl sulfoxide (Me_2SO). LMB and GA were purchased from Sigma (St. Louis). MG-132 was purchased from Calbiochem (San Diego, CA).

Buffers. PBS is 0.8% NaCl, 0.02% KCl, 0.14% Na_2HPO_4, 0.02% KH_2PO_4, pH 7.4. Gel sample buffer (2×) is 125 mM Tris, pH 6.8, 4% SDS, 25% glycerol, 4 mM EDTA, 20 mM dithiothreitol, 0.005% bromphenol blue. Tris-buffered saline is 50 mM Tris and 150 mM NaCl, pH 7.5. 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 in TTBS. Lysis buffer (2×) is 50 mM HEPES, pH 7.4, 40 mM sodium molybdate, 10 mM EGTA, 6 mM $MgCl_2$, and 20% glycerol. Gel shift buffer (5×) is 50 mM HEPES, pH 7.5, 15 mM $MgCl_2$ and 50% glycerol.

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, Stanford, CA). A7 rat smooth muscle cells were purchased from the American Type Culture Collection (Manassas, VA). All 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 GA were administered directly into growth media for the indicated incubation times. The vehicle used for TCDD, GA, and MG-132 was Me_2SO, the final concentration of which ranged from 0.05 to 0.5%.

Antibodies. Specific antibodies against either the AHR (A-1, A-1A) 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 (St. Louis, MO). Polyclonal antibodies specific to rat CYP1A1 were purchased from Chemicon (Temecula, CA).

Generation of Stable Cell Lines Expressing AHR. Wild-type murine AHR cDNA was amplified by PCR and ligated into the retroviral expression vector pLNCX2 to generate pMAHRretro as detailed by the manufacture (BD Clontech, Palo Alto, CA). AHR mutated in the nuclear localization signal (NLS) were generated in pMAHRretro by the QuikChange in vitro mutagenesis system as detailed by the manufacturer (Stratagene, La Jolla, CA) to generate pNLS2retro and pNLS1retro. The retroviral vectors were then transfected into packaging cell lines and viral containing media harvested as detailed by the manufacture (BD Clontech). Viral media was used to infect LA-I Hepa-1 or E36 cells and stable cell lines selected in 800 μg/ml of G-418. Surviving colonies (20–50) were isolated for each AHR and subjected to an additional round of selection. Stable cell lines that survived the second round of selection were analyzed for AHR expression and frozen in liquid nitrogen for future studies. For all experiments described in this report, at least three independent cell lines were evaluated for each AHR studied. All cells were maintained in selective media during propagation, but G-418 was re-

moved from the media during treatment with TCDD, GA, or MG-132. All experiments were carried out during 2-month intervals, and stable lines were checked weekly for changes in the level of AHR expression. Control LA-I or E36 cells used in the studies were infected with naked virus and maintained under identical selective pressure as the AHR-expressing cells.

In Vitro Expression of Protein. Recombinant protein was produced from expression constructs using the TNT Coupled Reticulocyte Lysate System 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 μg/ml), and aprotinin (20 μg/ml). Cell suspensions were immediately sonicated for 10 s, supplemented with phenylmethylsulfonyl fluoride (final concentration, 100 μM), 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 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 (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 1:12,000 dilution of GAR-HRP 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 (5–20 cells each) were evaluated on each coverslip, and 3 to 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.

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) was combined with 25 mM MOPS, 10 mM EDTA, and 10% glycerol 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. The activated samples (15 μl) were then incubated at 22°C for 15 min in 1× gel-shift buffer supplemented with KCl (80 mM) and poly(dI/dC) (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% 45 mM Tris-borate and 1 mM EDTA gels, dried, and exposed to film. In some instances, activated samples were analyzed by Western blotting to assess expression of AHR and ARNT.

Statistical Analysis Statistical analysis was carried out using InStat software (GraphPad Software Inc. San Diego, CA).

Results and Discussion

Generation and Analysis of AHRs Containing Mutations within the NLS. The putative NLS of the AHR is a bipartite sequence that is rich in basic amino acids (Pollenz et al., 1994; Ikuta et al., 1998). It spans amino acids 12 to 41 of the murine AHR and seems to overlap with the basic domain involved in DNA binding (Fukunaga and Hankinson, 1996). At present, the NLS of the AHR has not been evaluated in the context of the complete AHR amino acid sequence but has been characterized through analysis of GFP chimeras containing peptides with the NLS sequence derived from the human AHR (Ikuta et al., 1998). The results of these studies showed that alanine substitutions at either R13, K14, R15, R16, K37, or R38 (the corresponding amino acids in the mouse are R12, K13, R14, R15, K26, or R41; Fig. 1A) resulted in NLS-GFP chimeras that remained predominantly cytoplasmic compared with wild-type NLS-GFP, which was exclusively nuclear (Ikuta et al., 1998). Thus, with these data as a framework, in vitro mutagenesis was used to generate full-length murine AHRs containing alanine substitution at K13 (termed AHR_{NLS1}) or at both R12 and K13 (termed AHR_{NLS2}). Because the NLS domain seems to share basic residues that have been implicated in DNA binding (Fukunaga and Hankinson, 1996), it was important to functionally evaluate AHR_{NLS1} and AHR_{NLS2} proteins before proceeding in the analysis of their degradation.

To determine whether the AHR_{NLS} mutants were defective in nuclear localization, expression plasmids were transfected into a rat smooth muscle cell line, A7, and the subcellular

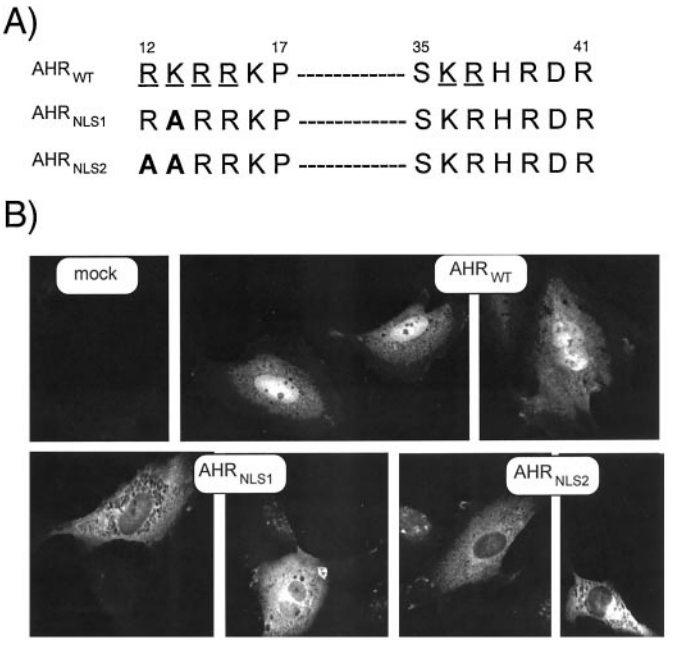

Fig. 1. Mutation of the putative NLS and transient expression in A7 cells. A, amino acid sequences of the NLS domain of the mouse AHR. Numbers indicate amino acid. Underline indicate residues shown to affect localization in GFP-chimeras (Ikuta et al., 1999). Amino acid mutations are shown in bold. B, AHR_{WT}, AHR_{NLS1},and AHR_{NLS2} retroviral vectors were transiently expressed in A7 cells for 24 h. Cells were fixed and stained for AHR as detailed under *Materials and Methods*. Note the lack of nuclear accumulation of the AHRNLS proteins.

location of the AHR was evaluated by indirect immunofluorescence microscopy. The AHR actively shuttles between the cytoplasm and nucleus in the A7 cells and is predominantly nuclear when transiently expressed from plasmid constructs (R. S. Pollenz, unpublished observations). Thus, we predicted that an AHR defective in nuclear import would remain cytoplasmic in the A7 cell compared with wild-type AHR (AHR_{WT}). A representative experiment is shown in Fig. 1B. As expected, AHR_{WT} showed a predominantly nuclear distribution, whereas the AHR_{NLS1} and AHR_{NLS2} remained cytoplasmic. Thus, both NLS mutations dramatically affected the ligand-independent movement of the AHR in the A7 cells. To assess ligand binding, association with ARNT, and DNA binding of the various NLS mutants, AHR_{WT} , AHR_{NLS1}, and AHR_{NLS2} were expressed in vitro and quantified by Western blotting. Equal amounts of each AHR were then mixed with an equal amount of murine ARNT protein, and the samples were incubated in the presence of TCDD or Me_2SO for 2 h at 30°C. The formation of AHR·ARNT complexes were then evaluated by EMSA. Figure 2 shows that both NLS mutants were capable of binding to the consensus XRE sequences in a TCDD-dependent manner. Interestingly, the AHR_{NLS2} produced a band of reduced intensity, suggesting a possible reduction in DNA binding. This result was not unexpected, because studies have indicated that alanine substitution in this region impacts DNA binding (Fukunaga and Hankinson, 1996). However, quantification of the shifted AHR_{NLS2}·ARNT·XRE complexes normalized to the level of AHR protein used in the assay showed that DNA binding of the AHR_{NLS2} was reduced by only 30 ± 8% compared with samples containing AHR_{WT}. To further assess these results, immunoprecipitation studies were carried out to assess ligand-mediated association between the various AHRs and ARNT. The results showed that AHR·ARNT binding was not affected in either of the NLS mutants (data not shown). Thus, these studies indicate that the NLS mutants are 1) defective in nuclear localization, 2) capable of binding ligand, 3) bind with ARNT, and 4) can be transformed to a DNA-binding species.

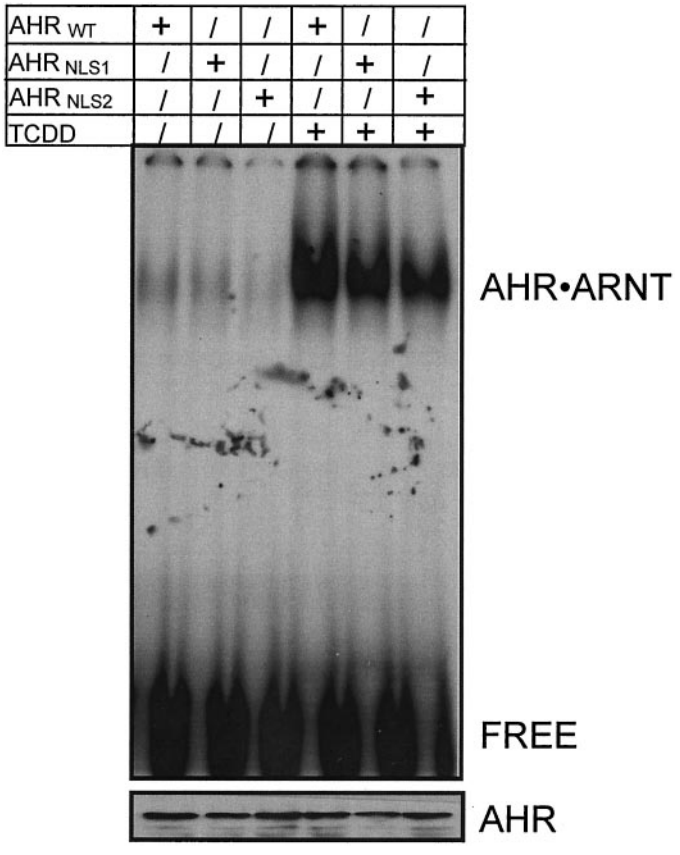

Fig. 2. Functional analysis of mutations within the NLS of the AHR. The indicated AHR protein and mouse ARNT were expressed in vitro, and equal concentrations (approximately 10–15 ng) were mixed, incubated in the presence of TCDD (10 nM) or Me_2SO (0.5%) for 2 h at 30°C, and analyzed immediately by EMSA, as detailed under *Materials and Methods*. The Western blot at the bottom of the EMSA represents an aliquot of the exact sample used for the EMSA that was stained for AHR with A-1A IgG (1.0 μg/ml) and visualized by ECL with GAR-HRP IgG (1:12,000). Note that the concentration of AHR in each sample is similar. The location of the specific AHR·ARNT·XRE complex and free XRE are indicated.

AHR_{NLS2} Is Defective in Nuclear Translocation but Not Degradation after TCDD Exposure. To develop a model system that would allow the analysis of nuclear translocation, degradation and gene regulation of the two AHR-NLS mutants, the cDNAs were ligated into retroviral expression vectors and used to generate cell lines that were stably expressing the AHR_{NLS} and AHR_{WT} proteins. Two different cell lines were used to generate the stable cells. The first was the type I Hepa-1 cell line (LA-I) that has reduced levels of endogenous AHR protein and low levels of ligand-inducible CYP1A1 expression (Israel and Whitlock, 1984). The second was the Chinese hamster lung cell line, E36, that has low levels of AHR protein as determined by Western blotting and has been previously used to evaluate AHR function (Davarinos and Pollenz, 1999; Pollenz and Barbour, 2000; Pollenz et al., 2002). Control cell lines were generated by infecting cells with virus that exressed AHR_{WT} or virus that did not contain an insert. At least 25 independent stable cell lines were isolated for each construct in each cell line. Once selected, each clone was analyzed by Western blotting and immunofluorescence microscopy to assess the level of AHR expression and its subcellular location. Although three independent lines were taken through the battery of studies detailed in this report, the results from single AHR_{NLS1}, AHR_{NLS2}, and AHR_{WT} clones are shown for ease of data presentation.

It was first important to evaluate the level of AHR protein in the various stable cell lines to confirm that it was consistent with the level of endogenous AHR expressed in Hepa-1 cells. Figure 3 shows a representative Western blot of the various stable cell lines compared with wild-type Hepa-1 cells and the parental LA-I line. The results show that LA-I cells express very low levels of endogenous AHR protein, whereas the level of AHR protein in the various stable cell lines approximates the level found in the wild-type Hepa-1 cells. Similar levels of AHR expression were observed in the E36 clones that were selected (data not shown). Studies next focused on the time course of ligand-mediated degradation of the AHR. Cells were treated with TCDD for 0 to 8 h, and the level of AHR protein was quantified by Western blot analysis. Figure 3B shows a representative experiment, and the normalized data are presented in Fig. 3C. The results show that the AHR is degraded in all the stable cell lines along a time course that is similar to that observed in the Hepa-1 cell line. Importantly, the magnitude of AHR degradation in the three stable cell lines was essentially the same at 8 h regardless of the NLS mutation (maximal degradation was approximately 60% of the basal level). In addition, longer times of TCDD

exposure did not result in an increased level of degradation in any of the stable cell lines (data not shown). Thus, the NLS mutations did not seem to affect the time course or magnitude of ligand-mediated degradation. However, it is important to note that although the time course of AHR degradation in the stable cells was similar to that observed in the Hepa-1 cell line, the overall level of degradation in the stable cell lines did not reach the level observed for the endogenous AHR in the Hepa-1 cells. This result mostly likely reflects the difference in the promoter that is driving the expression of the AHR in the stable cell lines (cytomegalovirus promoter) compared with the Hepa-1 cells (endogenous AHR promoter).

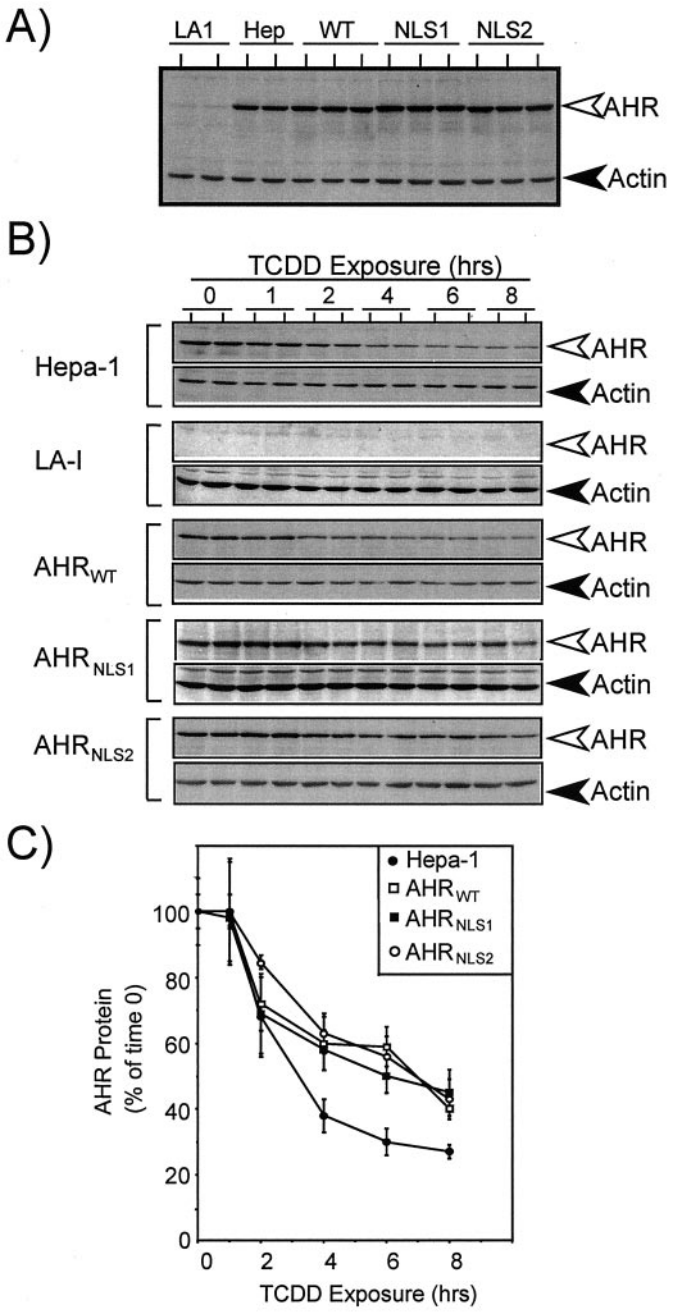

Fig. 3. Western blot analysis of AHR_{NLS} cell lines after exposure to TCDD. A, total cell lysates were generated from duplicate plates of the indicated cell lines and subject to SDS-PAGE. Gels were then blotted and stained with A-1A IgG (1.0 μg/ml) and actin IgG (1:1,000) and visualized by ECL with GAR-HRP IgG (1:12,000). LA1, parental LA-I cells stably expressing retroviral vector; Hep, Hepa-1 cells; WT, AHR_{WT} stable cell line; NLS1, AHR_{NLS1} stable cell line; NLS2, AHR_{NLS2} stable cell line. Note the similar level of AHR expression in all cells. B, duplicate plates of the cell lines shown in A were treated with TCDD (1 nM) or Me_2SO (0.05%) for the indicated times, and total cell lysates were prepared and subjected to SDS-PAGE. Gels were blotted and stained as above. C, AHR and actin bands were quantified by densitometry and normalized as described under *Materials and Methods*. Results are plotted as the percentage of AHR protein relative to the control (time 0) data point. Each point is the mean ± range of the two samples shown in B.

Having established that the NLS mutations did not significantly affect the time course or magnitude of ligand-mediated degradation, we next evaluated the subcellular location of the AHR_{NLS} and AHR_{WT} proteins during TCDD stimulation. Cells were grown on glass coverslips, treated with TCDD or Me_2SO for 0 to 8 h, and then stained for the AHR. Figure 4 shows the results of the AHR_{WT} and AHR_{NLS2} clones in the LA-I cell line. It can be observed that the parental LA-I cells do not exhibit significant staining for the AHR that is consistent with the reduced level of AHR in these cells (Fig. 4, top). However, the AHR_{WT} and AHR_{NLS2} cell lines show intense staining for the AHR that exhibits a predominantly cytoplasmic localization that is consistent with the distribution of the AHR in Hepa-1 cells (see Pollenz et al., 1994; Pollenz, 1996; Davarinos and Pollenz, 1999). Importantly, treatment of the AHR_{WT} cells with TCDD resulted in a dramatic translocation of the receptor to the nucleus at 1 h and a subsequent reduction in the cytoplasmic staining (Fig. 4, right). In addition, the overall level of nuclear AHR staining was reduced by 6 h. The reduced level of staining is consistent with the degradation of the AHR_{WT} shown in Fig. 3. In contrast, TCDD treatment failed to induce a similar level of AHR translocation in the AHR_{NLS2} cells, and the cells clearly exhibited cytoplasmic staining throughout the time course (Fig. 4, left). Similar results were observed in three other AHR_{NLS2} stable cell lines (R. S. Pollenz, unpublished observations). Interestingly, there seemed to be a slight but detectable increase in the nuclear staining for the at 2 and 4 h even though the nuclear staining never exceeded the level observed in the cytoplasm or approached the level of the AHR_{WT}. In addition, the intensity of the cytoplasmic staining of the AHR_{NLS2} became reduced throughout the time course, consistent with the data presented in Fig. 3. The reduced level of ligand-mediated nuclear translocation was not specific to the LA-I cell line. Figure 5 shows that the AHR_{NLS2} also remained cytoplasmic in the presence of TCDD in the stable E36 cell line, whereas the AHR_{WT} showed a change in distribution over the time course. Thus, these results support the hypothesis that mutation of R12 and K13 to alanine results in an AHR that is severely defective in ligand-mediated nuclear import but is not defective in ligand-mediated degradation. Because the stable cell lines both show similar levels of AHR protein expression and are generally clonal with respect to the population of cells that are expressing the AHR (Fig. 4), the difference in function between the AHR_{WT} and AHR_{NLS2} cannot be related to differences in AHR expression. These results imply that ligand-mediated degradation can occur within the cytoplasmic compartment and that shuttling through the nucleus or DNA binding does seem to be required for degradation.

Cell Lines Expressing AHR_{NLS2} Exhibit Reduced Induction of Endogenous CYP1A1 Protein after Exposure to TCDD. Because the AHR_{NLS2} protein seemed to be severely defective in nuclear localization, but still exhibited a

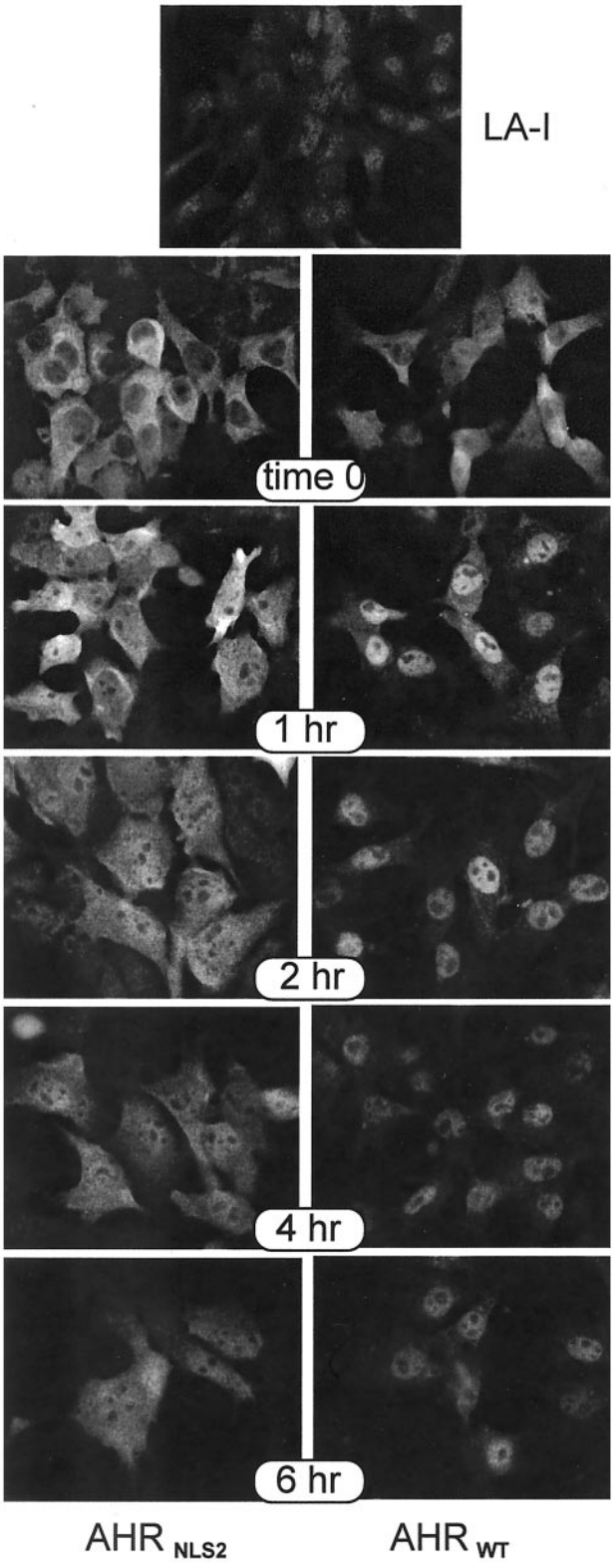

Fig. 4. Immunofluorescence microscopy of AHR_{NLS2} and AHR_{WT} protein expression in stable LA-1 cells after TCDD exposure. The indicated cells were grown on glass coverslips and exposed to TCDD (1 nM) for the indicated times. The time 0 sample was exposed to Me_2SO (0.05%) for 6 h. After exposure, cells were fixed and stained with 2.0 μg/ml A-1A IgG followed by GAR-RHO (1:400). All cells were photographed with a digital camera for identical exposure times. The specificity of the A-1 antibody is demonstrated by the lack of staining in the parental LA-I cells (top). Also note the homogeneity of the stable population and greatly reduced level of nuclear translocation of the AHR_{NLS2}.

slight nuclear accumulation in a ligand-dependent manner, it was pertinent to determine whether cells expressing the AHRNLS2 protein were capable of inducing endogenous CYP1A1. Triplicate plates of LA-I, Hepa-1, AHR_{WT}, AHR_{NLS1}, or AHR_{NLS2} cells were treated with Me_2SO or TCDD for 8 h, and the level of CYP1A1 protein was quantified by Western blot analysis. Figure 6A shows a representative experiment of the TCDD-treated samples (the control samples showed no level of CYP1A1 protein; see time 0 in Fig. 6B). The results show that only the Hepa-1 and AHR_{WT} induced high levels of CYP1A1 protein after 8 h of TCDD exposure, whereas the level of CYP1A1 induced in the AHR_{NLS2} cells was 11% of the AHR_{WT} cells. Because an AHR that was completely defective in nuclear import would be

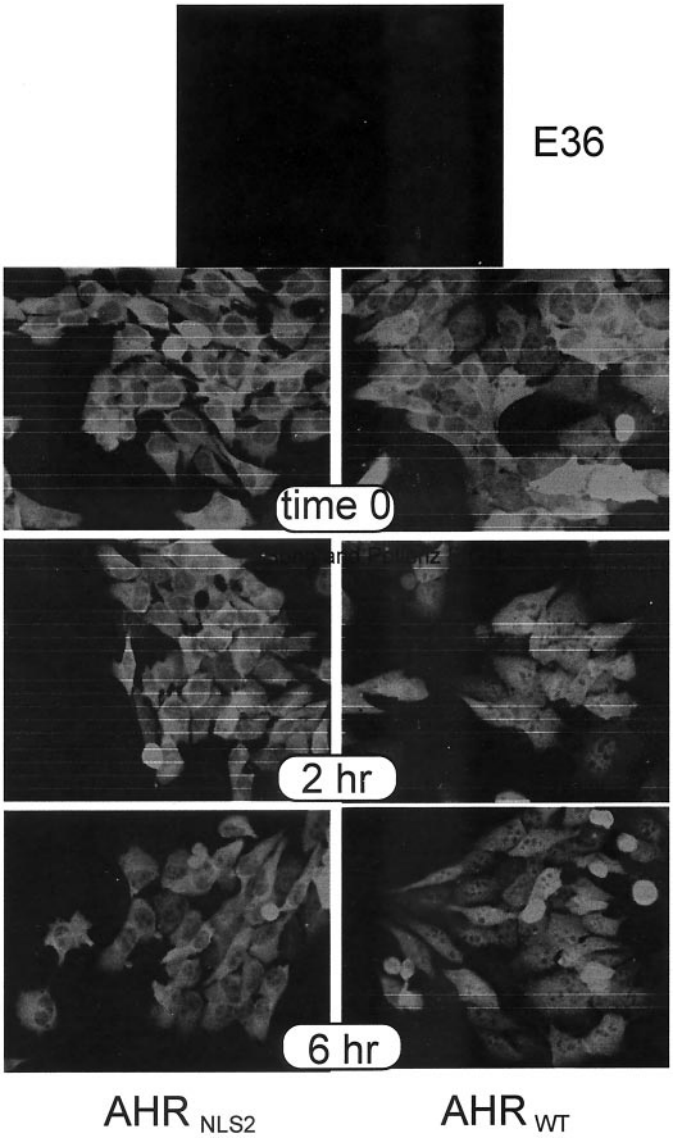

Fig. 5. Immunofluorescence microscopy of AHR_{NLS2} and AHR_{WT} protein expression in stable E36 cells after TCDD exposure. The indicated cells were grown on glass coverslips and exposed to TCDD (1 nM) for the indicated times. The time 0 sample was exposed to Me_2SO (0.05%) for 6 h. After exposure, cells were fixed and stained with 2.0 μg/ml A-1A IgG followed by GAR-RHO (1:400). All cells were photographed with a digital camera for identical exposure times. The specificity of the A-1 antibody is demonstrated by the lack of staining in the parental E36 cells (top). Also note the homogeneity of the stable population and lack of nuclear translocation of the AHR_{NLS2}.

expected to be unable to induce any CYP1A1, the time course of induction was also evaluated. In these studies, AHR_{WT} and AHR_{NLS2} cells were treated with TCDD for 0 to 16 h, and the level of CYP1A1 protein was evaluated by Western blot analysis. The results show that CYP1A1 can be detected as early as 4 h in the AHR_{WT} cells but is not detectable in the AHR_{NLS2} cells for 6 to 8 h. These results are consistent with a protein that is defective in nuclear import, because gene induction is delayed and has a greatly reduced magnitude. Thus, these results add further support that the ANR_{NLS2} is severely defective in nuclear import but confirm that some fraction of the receptor is still able to reach the nucleus and function in TCDD-mediated gene induction. However, these results clearly separate the events of gene regulation from AHR degradation because of the dramatic differences observed between the AHR_{WT} and AHR_{NLS2} cell lines.

AHR_{NLS2} Is Degraded after Exposure to Geldanamycin without Localization to the Nucleus. Previous reports have shown that treatment of various cell lines with GA results in the rapid degradation of the AHR in a ligand-independent manner (Chen and Perdew, 1997; Meyer et al., 2000; Song and Pollenz, 2002). GA is a benzoquinone ansamycin that directly associates with the ATP/ADP binding site of hsp90 and can alter the conformation of hsp90 and its target binding proteins (Grenert et al., 1997). Interestingly, the magnitude of GA-mediated degradation is similar to that observed with TCDD, can be inhibited by MG-132, and also seems to involve nuclear localization of the AHR (Song and Pollenz, 2002). Therefore it was of interest to evaluate whether the GA-mediated degradation would be affected in cells expressing AHR_{NLS} proteins. In the first study, Hepa-1, AHR_{NLS2}, AHR_{NLS1}, and AHR_{WT} cell lines were treated with GA for 0 to 120 min and the level of AHR protein was evaluated by Western blotting.

Figure 7A shows a representative experiment and the normalized data are shown in Figure 7B. The results show that the AHR is degraded in all the stable cell lines along a time course that is similar to that of the Hepa-1 cell line but is more rapid than that observed in cells treated with TCDD (Fig. 3). This result is consistent with previous studies (Song and Pollenz, 2002). Importantly, the magnitude of GA-induced AHR degradation in the three stable cell lines was essentially the same at 120 min regardless of the NLS mutation (maximal degradation was approximately 70 to 80% of

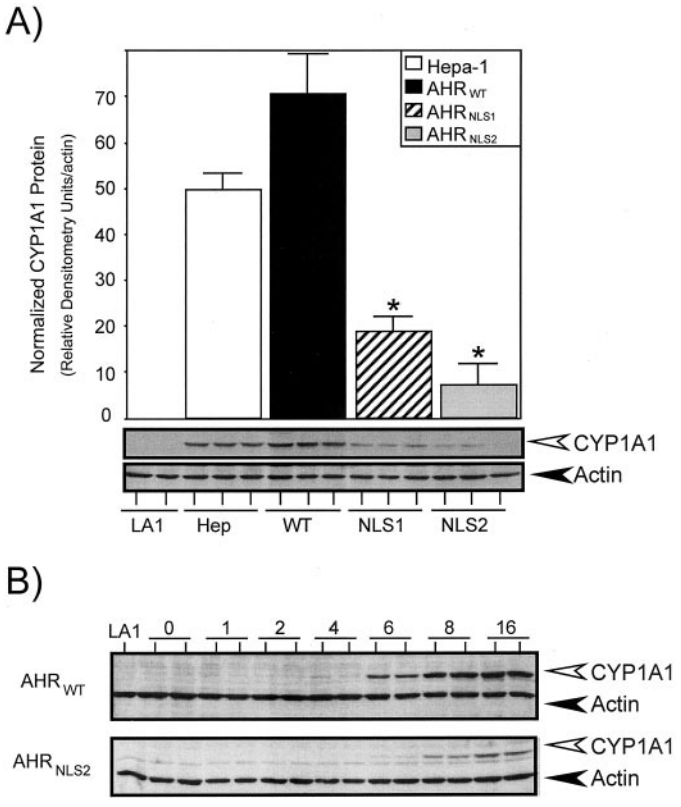

Fig. 6. Analysis of CYP1A1 expression in stable LA- 1 cell lines expressing NLS mutants. A, total cell lysates were generated from triplicate plates of the indicated cell lines and subject to SDS-PAGE. Gels were then blotted and stained with anti-CYP1A1 IgG (1:1,000) and actin IgG (1:1,000) and visualized by ECL with GAR-HRP IgG (1:12,000). CYP1A1 and actin bands were quantified by densitometry and normalized as described under *Materials and Methods*. Results are plotted as the relative densitometry units of the CYP1A1 divided by the relative densitometry units of actin. Each bar is the mean ± S.E. of three samples. *, $p < 0.05$, statistically different from the AHR_{WT} sample. LA1, parental LA-I cells stably expressing retroviral vector; Hep, Hepa-1 cells; WT, AHR_{WT} stable cell line; NLS1, AHR_{NLS1} stable cell line; NLS2, AHR_{NLS2} stable cell line.

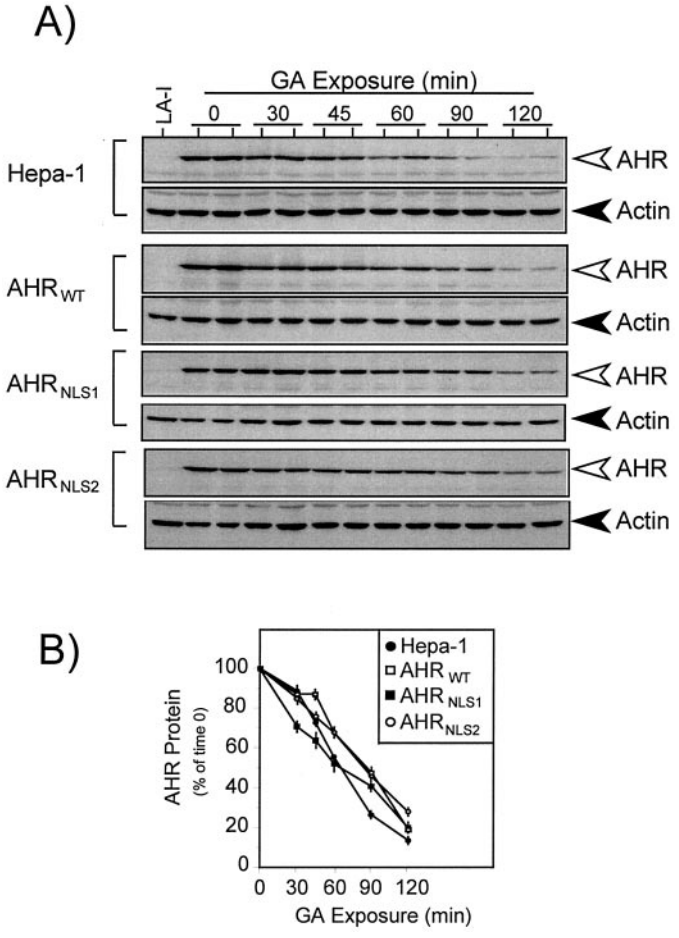

Fig. 7. Western blot analysis of AHR_{NLS} cell lines after exposure to GA. A, duplicate plates of the indicated cell lines were treated with GA (75 nM) or Me_2SO (0.1%) for the indicated times and total cell lysates prepared and subject to SDS-PAGE. Gels were then blotted and stained with A-1A IgG (1.0 μg/ml) and actin IgG (1:1,000) and visualized by ECL with GAR-HRP IgG (1:12,000). B, AHR and actin bands were quantified by densitometry and normalized as described under *Materials and Methods*. Results are plotted as the percentage of AHR protein relative to the control (time 0) data point. Each point is the mean ± range of two samples shown in A.

the basal level) and approximated the level of degradation in the Hepa-1 cell line. Thus, the NLS mutations did not seem to affect the time course or magnitude of GA-mediated degradation. Figure 8 shows the subcellular localization of the AHR_{WT} and AHR_{NLS2} after GA exposure. It can be observed that the AHR_{WT} becomes predominately nuclear after 30 min of GA exposure and that the staining is dramatically reduced after 120 min. The GA-mediated nuclear localization of the AHR_{WT} is consistent with previous studies (Song and Pollenz, 2002). In contrast, GA exposure results in a dramatic reduction in cytoplasmic staining in the AHR_{NLS2} cells with no apparent increase in nuclear staining at either 30 or 120 min. Thus, these results indicate that, like TCDD-mediated degradation, GA-mediated degradation can occur within the cytoplasmic compartment and does not require nuclear import. It is also interesting to note that the AHR_{NLS2} did not exhibit nuclear staining after GA treatment, compared with the slight level of change observed when cells were treated with TCDD (compare Figs. 4 and 8). Thus, because GA does not seem to cause the loss of hsp90 from the AHR complex (Chen et al., 1997; Meyer et al., 2000; Kazlauskas et al., 2001), these results suggest that the AHR·hsp90·GA complex has a much different conformation than the liganded AHR complex even though both undergo nuclear import.

It has also been demonstrated that treatment of various cell lines with MG-132 not only inhibits TCDD and GA-mediated degradation of the AHR but also causes the AHR to translocate to the nucleus (Santiago-Josefat et al., 2001; Song and Pollenz, 2002). To evaluate the affect of MG-132 in the various NLS cell lines, cells were treated with GA, TCDD, or MG-132 for 4 h or pretreated with MG-132 for 2 h followed by exposure to GA or TCDD for an additional 4 h. At the end of each treatment, total cell lysates were prepared and the level of AHR protein was evaluated by Western blotting. A representative experiment is shown in Fig. 9A. In all cell lines evaluated, GA or TCDD exposure resulted in significant reduction in the level of the AHR protein as observed in the previous experiments (Figs. 3 and 6), but degradation was inhibited by pretreatment with MG-132. To determine whether MG-132 resulted in nuclear localization of the AHR, AHR_{WT} and AHR_{NLS2} cells were exposed to MG-132 for 6 h

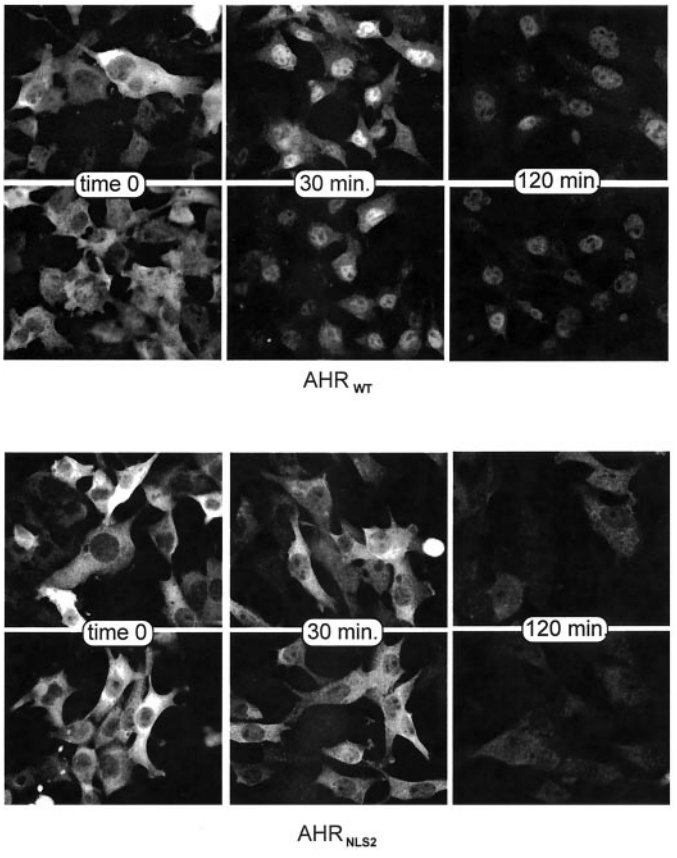

Fig. 8. Immunofluorescence microscopy of AHR_{NLS2} and AHR_{WT} protein expression after GA exposure. The indicated cells were grown on glass coverslips and exposed to GA (75 nM) for 30 or 120 min. The time 0 sample was exposed to Me_2SO (0.2%) for 120 min. After exposure, cells were fixed and stained with A-1A IgG (2.0 μg/ml) followed by GAR-RHO (1:400). All cells were photographed with a digital camera for identical exposure times. Note the loss of staining over time and absence of nuclear translocation of the AHR_{NLS2}.

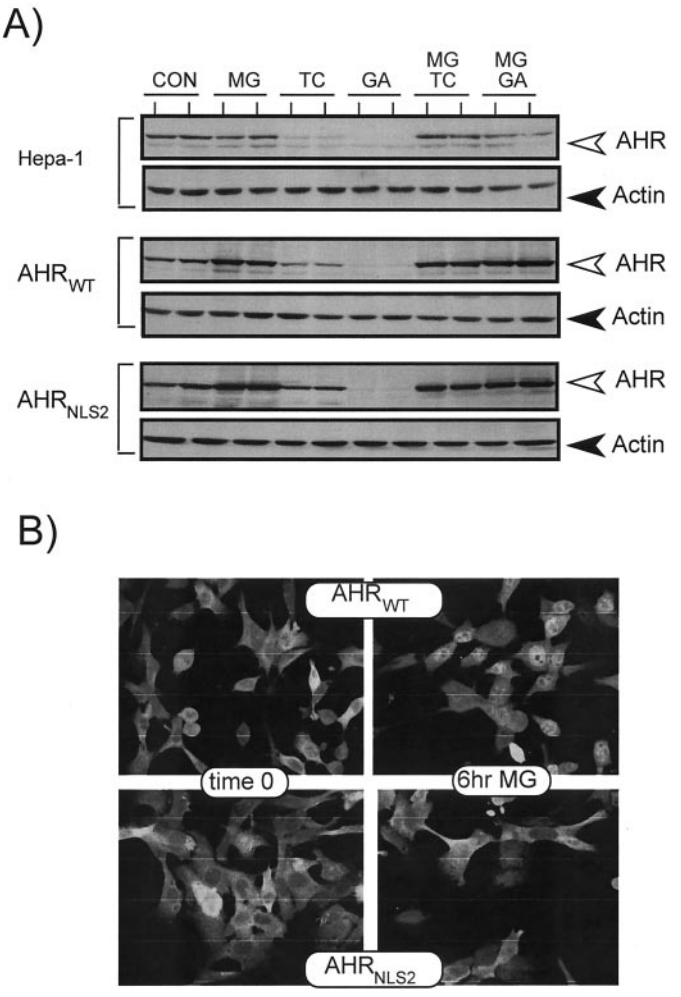

Fig. 9. Analysis of MG-132 exposure on degradation and location of AHR_{NLS}. A, duplicate plates of the indicated cells were exposed to Me_2SO (0.1%) for 6 h (CON); MG-132 (10 μM) for 6 h (MG); TCDD (1 nM) for 4 h (TC); GA (75 nM) for 4 h (GA); MG-132 for 2 h followed by TCDD (1 nM) for 4 h (MG/TC); or MG-132 for 2 h followed by GA (75 nM) for 4 h (MG/GA). Total cell lysates were prepared at the end of each incubation and were subjected to SDS-PAGE. Gels were then 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). B, the indicated cells were grown on glass coverslips and exposed to MG-132 (10 μM) or Me_2SO (0.1%) for 6 h. After exposure, cells were fixed and stained with A-1 IgG (2.0 μg/ml) followed by GAR-RHO (1:400). All cells were photographed with a digital camera for identical exposure times. Note the absence of nuclear translocation of the AHR_{NLS2}.

and then fixed and stained. The results show that AHR_{WT} becomes nuclear after exposure to MG-132, whereas the AHR_{NLS2} remains cytoplasmic at the end of the exposure. Thus, these results add further support that the AHR_{NLS1} and AHR_{NLS2} proteins do not show defects in the degradation pathway compared with AHR_{WT}.

Conclusions and Implications

The proteolytic degradation of transcription factors is an established mechanism for regulating signal transduction pathways and the complexity of the system is slowly emerging (reviewed by Pahl and Baeuerle, 1996; Conaway et al., 2002). Proteolysis has been shown to be involved in such divergent signaling systems as nuclear factor-κB (Palombella et al., 1994), glucocorticoid-mediated signaling (Hoeck et al., 1989), p53 (Haupt et al., 1997; Roth et al., 1998), estrogen receptor (Nawaz et al., 1999), and the bHLH proteins HIF-1a and MyoD (Salceda and Caro, 1997; Huang et al., 1998; Floyd et al., 2001). Recently, the role that degradation of the AHR plays in the regulation of the AHR signal transduction pathway has become a specific area of research, and numerous studies have established that ligand binding results in the rapid degradation of the AHR through the ubiquitin proteasome system in vitro and in vivo (reviewed in Pollenz, 2002). Unfortunately, although AHR degradation itself is well established, there are conflicting results regarding the subcellular location of the ubiquitination and degradation events. Determining the location of AHR degradation is especially pertinent because the types of proteins associated with the AHR complex in the nucleus and cytoplasm are different and could influence the interactions and regulation that occur (Whitlock, 1999; Gu et al., 2000). In addition, there are multiple ubiquitin ligase enzymes that can function in either the nucleus or cytoplasm; these enzymes may even function in cellular compartments distinct from the ultimate site of degradation (reviewed in Glickman and Ciechanover, 2001; Pickart, 2001; Conaway et al., 2002). Such a complex system leaves open the possibility for a myriad of protein-protein interactions. Thus, the key finding of the studies presented in the current report is that inhibition of nuclear import of the AHR does not impact the time course or magnitude of either ligand-dependent or -independent degradation. The implication of these results is that the entire complement of proteolytic machinery needed for AHR degradation is present in the cytoplasmic compartment and that nuclear translocation, binding with ARNT, or binding to DNA is not required for efficient degradation. The ability to observe degradation of the various AHR_{NLS} mutants in both a ligand-dependent and -independent manner is also important because it now provides a model in which to begin to identify the domains of the AHR that are required for the degradation events and determine whether there are distinct sequences required for ligand-dependent and -independent degradation. Because recent studies suggest that AHR-mediated gene regulation is significantly impacted if the AHR is not degraded or is constitutively activated (Davarinos and Pollenz, 1999; Ma et al., 2000; Andersson et al., 2002), an understanding of how AHR degradation is regulated will provide important insight into this signal transduction pathway.

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